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Identification of foot and mouth disease virus carrier and subclinically infected animals and differentiation from vaccinated animals

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Summary

Countries that are free of foot and mouth disease (FMD) are reluctant to use vaccine in the event of an outbreak because of the difficulties this can cause in re-establishing freedom from FMD status to the satisfaction of trading partners. The problem does not lie in distinguishing between vaccinated and recovered animals as vaccinated animals can be tagged or otherwise marked to show that they have been vaccinated; the difficulty is in identifying vaccinated animals that have had contact with live virus and become carriers. The traditional probang test is not sufficiently sensitive and is labour- and laboratory-intensive, but alternative serological tests such as those for antibodies to non-structural proteins (NSPs), or specific immunoglobulin A (IgA) are also not 100% sensitive. However, these newer tests do provide increased security by reducing the likelihood of trading carrier animals and can be used to help define the limits of an outbreak; the use of vaccine to help control an outbreak of FMD in a previously free country still has significant consequences on trade in FMD susceptible animals and their products.

Keywords

Carriers – Diagnosis – Foot and mouth disease – Subclinical infection – Trade – Vaccination.

The host

Diagnosis of foot and mouth disease (FMD) relies heavily on the recognition of clinical signs in affected animals. In high-producing cattle and pigs that have not been vaccinated against FMD, the clinical signs can be almost pathognomonic, but in small ruminants and some breeds of cattle, the clinical signs may be less obvious and easily confused with other conditions. In FMD endemic situations, the indigenous animals that come into frequent contact with the disease often fail to exhibit signs, although this is not always true. The pan-Asian topotype which recently caused the outbreaks in the United Kingdom (UK), was identified when it first entered Taipei China, following isolation from probang samples from local Chinese yellow cattle which were being routinely tested prior to movement (10). In this situation, FMD had not been present in the cattle since 1928, but they had reportedly shown no evidence of disease. Similarly, the large outbreak of FMD in Zimbabwe in 1991 was triggered by the movement of infected Brahman cattle into the Bulawayo showground during a national agricultural show; these particular animals were highly bred and had had no previous contact with FMD and yet showed only a mild lameness.

In sheep and goats, subclinical disease is often the rule rather than the exception. Lameness and mild mouth lesions are frequently seen in sheep, unassociated with FMD infection. In experiments reported by Hughes *et al.* (11), 21% of closely inspected sheep failed to show any clinical signs during infection and 20% had only a single lesion. These lesions heal rapidly and can be easily missed. When FMD spread into Tunisia in 1989, it was mistaken for bluetongue because of the lameness and apparent coronitis; not until the virus affected

cattle was the lameness imputed to FMD, by which time the disease had already spread into Algeria, from where it moved into Morocco.

The virus

The occurrence of subclinical disease is not only dependent on the natural immunity of susceptible species, but also on the strain of FMD virus (FMDV). The Hong Kong topotype of type O FMDV is adapted well to pigs, to the extent that it has been isolated from a species other than pigs only once, namely from a bovine in the Philippines. Whether or not this animal showed clinical disease is unclear, but the possibility remains that the virus could frequently infect buffalo or cattle in South-East Asia without being clinically obvious. When this virus was experimentally inoculated intradermolingually into two cattle, it failed to infect one and only caused a local lesion on the other (9, 22). This is an extreme example, but demonstrates that the virus itself can manifest different infectivity and consequent clinical appearance in different susceptible species. The type O virus that caused the 1994 outbreak of FMD in Bulgaria was derived from the Middle East. This virus was claimed not to infect pigs, on the basis that when a group was inadvertently fed milk from an infected bovine the animals failed to develop disease. This claim was never tested experimentally and it was not even confirmed that the milk contained significant titres of virus. The implication was that the virus had not had contact with pigs for a considerable time and had become adapted to sheep. However, the Veterinary Authorities of Israel did report isolating FMDV from wild pigs in the Jordan Valley, suggesting that adaptation of these Middle East strains was by no means absolute. Insufficient experimental work has been conducted on the different strains of FMDV to investigate most of the FMDV host specificity claims, which are often little more than anecdotal. Experience with attenuated strains of FMDV used as vaccines in the past would indicate that any reduced virulence or host adaptation of the virus is likely not to remain inviolate due to the high mutation rate of the virus. The genomic explanation for reduced virulence and possible host species preference is being examined and a connection between attenuation and a deletion in the 3A gene has been postulated (2, 15). However, much still needs to be explained.

Vaccination

In FMD endemic countries that vaccinate against the disease and in countries which vaccinate during an outbreak of FMD, the possibility is high that clinical disease will be masked in those animals which have only partial immunity and which are exposed to live virus. These animals may show some clinical signs that would be detected by a trained clinician, but such signs will usually be missed by owners and untrained animal health personnel. However, these animals are likely to remain a source of infection to in-contact susceptible species and the virus can be maintained unobserved in a vaccinated population. Similarly, animals vaccinated during an outbreak of FMD will pass through a period of partial immunity before the vaccine becomes fully effective, during which clinical signs will be reduced or prevented, but infection and virus transmission can still occur (8). The speed at which vaccination induces protective immunity and prevents transmission depends on the potency of the vaccine against the outbreak strain and the level of viral exposure, but may be as short as four days (6, 19).

Persistent infection

Ruminant animals that have recovered from infection with FMDV and vaccinated ruminants that have had contact with live virus may retain infection in the pharyngeal region for a variable period of time. The carrier is defined as an animal from which live virus can be recovered after 28 days following infection. This is not an exceptional situation and over 50% of ruminants exposed to live FMDV become carriers; pigs do not become carriers (7). The duration of the carrier state depends on the species and individual. The African buffalo (*Syncerus caffer*) may carry virus for over five years, cattle for over three years, sheep for up to nine months (20), goats and wild ruminants for shorter periods of time and for South American camelids, no carrier state exists (4). Eventually the carrier does eliminate the virus.

The virus persists in the basal layer cells of the pharyngeal epithelium, particularly of the dorsal soft palate (23). Existing methods do not permit detection of the virus in the more superficial layers of cells and how the virus is excreted into the pharynx is not clear. Another unknown aspect is how the virus changes from a lytic agent, which destroys the host cell, into one that can establish a persistent infection. A mutation possibly reduces the ability of the persistent virus to shut down host cell metabolism. A further back-mutation may then restore the lytic action of the virus, ultimately resulting in elimination. However, this remains to be proven.

The establishment of the carrier state and the duration of this state depends on the host species, but probably also on the strain and serotype of FMDV and even on the breed of host species. All three serotypes of the South African Territories (SAT) viruses are found in the wild African buffalo populations of Botswana and Zimbabwe, but rarely are the commercially farmed Brahman cattle of the region found to be carrying either SAT 1 or SAT 3. In the last twenty years a series of outbreaks of SAT 2 was observed in the FMD-free zone of Zimbabwe and one Brahman bull in particular remained a carrier of SAT 2 virus for over three years. In addition, during the 1991 outbreak of SAT 2 in Zimbabwe, it was notable that the European cattle, although affected, carried the virus for a shorter period than the Brahman cattle. The SAT viruses occasionally spread out of Africa into the Middle East and most recently, into Saudi Arabia during 2000. However, although the O, A and Asia 1 serotypes persist in this region, in spite of limited attempts at control, the SAT viruses die out.

This implies that the cattle, sheep and goats are unable to maintain the SAT serotypes, or conversely, these serotypes require particular host species, either the Brahman for SAT 2 or the African buffalo, to be maintained. Similarly, the distribution of the Asia 1 serotype would suggest that the virus has been constrained from establishing itself outside of Asia.

Whether the geographical restriction of serotypes and even strains of FMDV is related to the ability of the virus to establish the carrier state in particular susceptible species or breeds is not known, but should that be the case, it presents a powerful argument for considering the importance of the carrier in the epidemiology of FMD.

Carriers causing outbreaks

Transmission of FMDV from a carrier bovine to a susceptible in-contact bovine has never been shown under experimental conditions, despite a considerable number of attempts. In one series of experiments, carriers were inoculated with dexamethasone in order to depress their immune systems. These animals were kept in contact with susceptible cattle, but this had the reverse effect of causing the virus to disappear from the pharynx, only to reappear once the treatment was stopped (12). Even dosing the in-contact animals with dexamethasone failed to result in transmission. A further experiment in which carriers were infected with rinderpest virus, which destroys host T cells, also failed to increase the level of pharyngeal FMDV (M.C. Ilott, unpublished data).

An experiment with carrier African buffalo, kept in contact with susceptible cattle and additional susceptible buffalo, did succeed, but the results were difficult to explain (5). A group of three FMD-free buffalo were infected with SAT 2 virus and kept in an enclosure with four susceptible cattle on an island in Lake Kariba. The buffalo developed clinical FMD and recovered without transmitting the disease to the cattle. The buffalo all became carriers and four months later, two further FMD-free buffalo were introduced. Seven weeks after the introduction of the two additional buffalo, the cattle developed clinical FMD, which then spread to the two new buffalo. What triggered the transmission event was not clear, but the cattle were confirmed to be infected with the same virus as that carried by the originally infected buffalo. All the animals were monitored throughout the experiment and regular samples collected from the pharynx to confirm the continuing persistence of the virus. The transmission of SAT 2 virus from carrier buffalo to cattle under controlled conditions was also shown by Vosloo *et al.* (21).

There have been a number of anecdotal accounts of carriers starting new outbreaks of FMD in the field (18). The strongest evidence for the involvement of carriers comes from Zimbabwe in 1989 and 1991. Following an outbreak of SAT 2 FMD in 1987, cattle on affected and neighbouring farms were vaccinated and kept in quarantine for 18 months. Following this, movement off the farms was allowed, but soon there were further outbreaks, shown by nucleotide sequencing as being due to the same virus as the 1987 outbreaks, associated with cattle from the quarantined farms. The same control programme was implemented in this new series of outbreaks and vaccinated farms were quarantined again for 18 months. After this period, cattle were moved from one of the farms on which vaccinated animals were present and which had been close to a known infected farm near Bulawayo, but which itself had not been identified as being infected. Cattle were moved to a feedlot north of Harare, where a new outbreak occurred, shown by nucleotide sequencing to be due to the same strain of SAT 2 virus that had caused the 1989 outbreaks. Although the cattle which had been moved were not individually identified and sampled, samples taken from the farm of origin of the animals showed the presence of carriers. What was particularly interesting was that no vaccine had been used on the farm since 1989 and a new population of susceptible young stock was now present on the farm, together with the carrier animals, but no transmission of virus had taken place. However, when carriers were moved and mixed with cattle from other farms, transmission had occurred. This scenario cannot be proven, but the hypothesis is that the stress of moving and mixing the carrier cattle was sufficient to cause these animals to start excreting sufficient virus to precipitate a new outbreak.

Identifying the carrier and subclinically infected animal

The definitive identification of carrier or subclinically infected animals requires recovery of live FMDV from those animals. The predilection of the virus for the epithelium of the pharynx makes this tissue the most suitable to sample, a procedure which can be carried out using the probang sampling cup (13). This is a hollow metal cup with a slightly sharpened edge, attached from the centre of the bowl by a long wire, approximately half a metre long, to a handle at the free end, which can be pushed into the mouth of the animal being tested, over the base of the tongue into the pharynx. The cup is then withdrawn, collecting as it is pulled out, mucous and superficial cellular material from the pharynx. The contents of the cup are usually mixed with a neutral buffer solution and if not examined immediately, kept frozen over liquid nitrogen or on dry ice (solid carbon dioxide). Live virus can be cultured on sensitive tissue culture such as primary bovine thyroid cells or lamb kidney cells. Carrier animals, which have either recovered from clinical disease, or have been vaccinated and subsequently acquired infection following contact with live virus, will also have high levels of specific anti-FMDV antibody present in the pharyngeal mucous and treatment of the probang sample with chlorofluorocarbon can help dissociate the virus/antibody complexes and increase the possibility of recovering virus on tissue culture. Subclinically infected animals, other than those

with partial vaccinal immunity, will not usually have detectable antibody levels at this stage of infection.

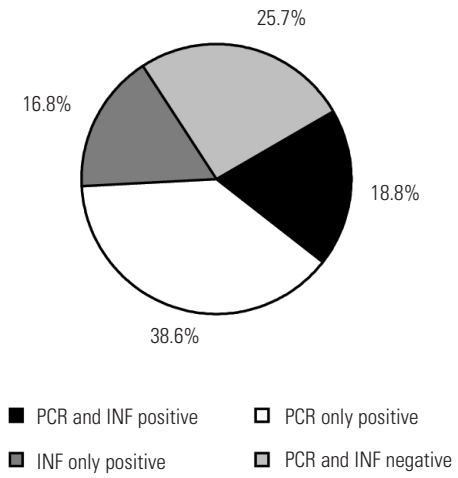
The quantity of virus present in the pharynx of carrier animals can vary considerably over time and the successful recovery of virus will depend on this and other factors, such as the subsequent handling of the sample and the skill of the operator. Possibly only 50% of carrier animals will be identified from the examination of a single probang sample, but this percentage can be increased by repeating the sampling procedure at two weekly intervals. Table I shows the results of regular sampling of a group of vaccinated cattle on a farm in Saudi Arabia soon after an outbreak of FMD. Some of these animals failed to yield a positive sample on every occasion, but the identification of live virus in later samples indicated that they would have been falsely declared negative. The sensitivity of the test can be improved by using the polymerase chain reaction (PCR), which identifies small quantities of viral genome present in the sample. However, the PCR itself can also give false negative results due to the presence of non-specific inhibitors. A comparative study using both tissue culture and PCR on probang samples demonstrated that some samples could give positive results using one method and negative results using the other and that both tests should ideally be used together (Fig. 1). There is also the unresolved question on the significance of a positive PCR result. The PCR identifies only part of the viral genome and would be positive even if the genome was itself fragmented and unassociated with any live virus. While a positive PCR is therefore highly suggestive of previous infection, the animal from which the sample was collected could no longer be carrying live virus and no longer represent any risk of causing a further outbreak.

Testing of animals suspected of having subclinical infection may also include animals that have only recently been infected and have not yet developed clinical disease. Mucous samples from the nose and mouth can be collected to detect the low levels of virus present, but because tissue culture techniques for virus isolation may take up to 96 hours to complete, by which time these animals would show clear clinical signs, the more rapid PCR test can be used. Methods have been designed to carry out large numbers of PCR tests on 96-well microtitre plates, which would allow rapid screening of at-risk animals at the start of an outbreak, or to determine the extent of a rapidly spreading outbreak. In addition, blood samples can be collected from suspect animals for identification of viraemia, either by PCR or inoculation of tissue culture.

Table I
Result of isolation of foot and mouth disease virus from probang samples serially collected from 15 heifers after the occurrence of an outbreak caused by serotype A in October 1992

Serial number and date of probang collection		Number of heifer														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
First	13.12.92					x					x	x	x	x	x	
Second	16.01.93		x											x	x	x
Third	13.02.93							x		x				x	x	x
Fourth	16.03.93	x			x			x		x				x	x	x
Fifth	13.04.93	x			x				x					x	x	x
Sixth	16.05.93				x					x				x	x	x
Seventh	29.06.93		x		x											x

x: positive for foot and mouth disease type A isolation



PCR: polymerase chain reaction
INF: virus positive in tissue culture

Fig. 1
Pie chart showing the percentage distribution of results following infectivity and polymerase chain reaction assays on 96 probang samples
Source: C. Amaral-Doel, personal communication

During the 2001 outbreak of FMD in the UK, the spread of disease in subclinically infected sheep was responsible for the widespread dissemination and persistence of the virus. Advantage could have been taken of the use of blood samples to help identify infected animals, for although the isolation of virus from blood is restricted to a three day viraemic period, the samples could have been simultaneously tested for the presence of specific anti-FMDV antibody, as a sheep, like any other susceptible species that is or has been recently infected, will either be virus or antibody positive – or sometimes both.

Carrier animals also have specific antibodies to FMDV. This is true whether they have recovered from infection or have been vaccinated. In countries that identify vaccinated animals by a brand or an ear tag, there should not be a problem in distinguishing animals that are antibody positive as a result of vaccination from those that are positive following recovery from infection. However, the difficulty lies in identifying those vaccinated animals that have had contact with live virus and become carriers.

Antibody in saliva

Carrier animals have antibodies to FMDV. These can be detected in the serum (16) and also in the saliva. Specific immunoglobulin A (IgA) is present in saliva of recovered or vaccinated cattle and is elevated in the carrier animal, probably because of the constant low levels of virus maintaining the antigenic stimulus to the mucosal immune system. An enzyme-linked immunosorbent assay (ELISA) has been developed to quantify this elevated level of specific IgA, to indicate the possibility that the animal from which the sample was collected could be a carrier (1). Some carrier cattle, however, fail to produce a level of IgA in their saliva significantly higher than non-carrier cattle and while this test has potential as a herd test, further refinement and increased sensitivity is required.

Non-structural proteins

Foot and mouth disease virus has a positive sense, single-stranded ribonucleic acid (RNA) genome of 8,400 nucleotides that codes for twelve proteins, four of which are structural and make up the capsid of the virus and eight of which are non-structural, which together allow the virus to replicate in an infected cell. The structural genes are identified as 1A, 1B, 1C and 1D, the non-structural as L, 2A, 2B, 2C, 3A, 3B, 3C and 3D. The functions of the proteins for which the non-structural genes code have not all been fully identified and it is beyond the scope of this paper to describe current opinions (17). However, the 3D gene should be mentioned as coding for the viral polymerase, and precipitating antibodies to this protein are detected in the viral infection-associated antigen (VIAA) test. The vaccine used to help control outbreaks of FMD is an inactivated preparation of whole virus particles in an oil or aluminium hydroxide/saponin adjuvant. There is no replication of the virus following vaccination and the vaccinated animal develops antibodies to the structural proteins of the virus present in the viral capsid. Some of these antibodies are neutralising and will protect the animal from subsequent infection. No viral replication means that there is no expression of the non-structural proteins (NSPs) and the animal will not develop antibodies to these proteins – although some vaccines do contain low levels of these NSPs depending on the manufacturing process, in particular 3D, and a low antibody response to the NSPs has been observed. This response is more obvious in animals that have been vaccinated several times.

Animals that have recently recovered from infection will have antibodies to the NSPs, because as the virus replicates in the tissues of the animals, these proteins will be expressed and stimulate the production of specific antibodies by the host. The detection of these antibodies can therefore be used to identify those animals that have been infected with FMD and which may still be carrying live virus. A variety of tests have been developed to detect these antibodies, including ELISA and enzyme-linked immuno-electrotransfer blot (EITB) (16), using pure NSP antigens expressed in viral (baculovirus) or plasmid (*Escherichia coli*) expression systems. These tests have been predominantly designed to detect NSP antibodies in cattle and are less useful in sheep and pigs. Sheep, in particular, probably because of the frequently subclinical nature of FMD, may fail to develop detectable levels of these antibodies. Even in cattle, considerable individual variation can be seen in the amount of antibody produced to each of the NSPs and consequently in the period of time after infection that antibody may be detected. The 2C antibodies may be detectable for twelve months, while the 3ABC antibodies persist for longer periods. The severity of the infection is likely to be the major influence on the levels and the subsequent duration of detection of the NSP antibodies.

In South America, the EITB, which uses a western blotting technique to detect the antibodies to five of the NSPs, 3A, 3B, 2C, 3D and 3ABC, was used very successfully to support the local FMD control programmes and the ultimate recognition by the OIE of freedom from FMD, particularly for regions of Brazil (3). A 3ABC ELISA was used to define the limits of the 1996 outbreak of FMD in the Balkans and antibody to the 3ABC polyprotein is considered the single most reliable indicator of infection (14).

However, a problem persists with the NSP tests on an individual animal level. Some cattle that have been vaccinated, particularly with a high potency vaccine as might be used in an outbreak in a previously FMD-free country, will fail to develop antibodies to the NSPs should they have contact with live virus. This is because their level of immunity prevents any significant viral replication and therefore expression of the NSPs. These animals could, however, become carriers of live virus. On a herd basis, even potent FMD vaccine will not protect 100% of the cattle and should the herd become exposed to live virus, some will support replicating virus, even though they do not show clinical disease and sero-convert to some of the NSPs, in particular to 3ABC. Thus, testing an entire herd makes it possible to diagnose a previous encounter with live virus and determine the potential for the presence of carriers, assuming, of course, that the entire herd was exposed to the same challenge. The test may fail if only a few animals were in contact with live virus, perhaps as an aerosol from a neighbouring infected farm and were all sufficiently immune to prevent the expression of the NSPs.

Conclusion

The test for antibodies to NSPs is a significant advance in the detection of carrier animals. However, the test has limitations and cannot be used reliably on individual animals to exclude the possibility that the animal may be a carrier of live virus. Even when used on an entire herd, the test does not constitute a guarantee. The possibility of carrier animals creating fresh outbreaks is probably extremely small and this can be further reduced by probang and serological testing. Nevertheless,

however small the risk, if importing countries have a choice, they are likely to choose to import their live animals and animal products from areas where there is no FMD vaccination or possibility of the presence of carrier animals. Until the identification of carrier animals is 100% certain, FMD will remain the most significant constraint to trade of susceptible animals and their products. ■

Identification des animaux porteurs du virus de la fièvre aphteuse infectés de manière subclinique et différenciation par rapport aux animaux vaccinés

R.P. Kitching

Résumé
Lors de l'apparition d'un foyer de fièvre aphteuse, les pays indemnes hésitent à recourir à la vaccination ; en effet, celle-ci risque de compliquer le recouvrement du statut de pays indemne requis par leurs partenaires commerciaux. Le problème ne réside pas dans la distinction entre les animaux vaccinés et les animaux guéris, les animaux vaccinés pouvant être reconnus par la pose d'une boucle auriculaire ou par une autre marque ; il s'agit plutôt d'identifier les animaux vaccinés qui sont devenus porteurs du virus à l'issue d'un contact avec le virus sauvage. Outre son manque de sensibilité, la méthode traditionnelle de la curette œsophagienne est très exigeante en temps et en moyens matériels de laboratoire. Toutefois, les épreuves sérologiques de remplacement, par exemple les tests fondés sur les anticorps dirigés contre les protéines non structurales ou sur des IgA spécifiques, ne sont pas elles non plus sensibles à 100 %. Ces nouvelles méthodes n'en offrent pas moins des garanties supplémentaires, puisqu'elles réduisent les risques de commercialisation d'animaux porteurs de virus et peuvent contribuer à circonscrire les limites d'un foyer. La vaccination utilisée comme méthode d'appoint pour juguler un foyer de fièvre aphteuse apparu dans un pays précédemment indemne a toujours des répercussions considérables sur le commerce des animaux sensibles à la fièvre aphteuse et de leurs produits.

Mots-clés
Diagnostic – Échanges internationaux – Fièvre aphteuse – Infection subclinique – Porteurs – Vaccination.

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Detección de ejemplares portadores del virus de la fiebre aftosa o con infección subclínica, y discriminación entre estos animales y los vacunados

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Resumen
Los países libres de fiebre aftosa son reticentes a servirse de la vacunación en la hipótesis de que surgiera un brote, ya que luego pueden tener dificultades para recobrar la condición de país libre requerida por sus interlocutores comerciales. El problema no estriba en distinguir entre los animales vacunados y los que se

han recuperado de la infección, ya que los ejemplares vacunados son identificados mediante crotales u otro tipo de marcas, sino más bien en detectar a los animales vacunados que, habiendo estado en contacto con el virus, se han convertido en portadores. La clásica prueba de la sonda esofágica carece de sensibilidad suficiente y requiere laboratorios bien dotados y mucha dedicación, pero las pruebas serológicas alternativas (como las que detectan anticuerpos contra proteínas no estructurales o reconocen la IgA específica) no presentan tampoco un 100% de sensibilidad. Estas nuevas pruebas, sin embargo, al reducir la probabilidad de que animales portadores penetren en el circuito comercial, ofrecen un mayor nivel de seguridad y pueden ayudar a definir los límites de un brote. En un país previamente libre de la enfermedad, el uso de vacunas como instrumento de lucha contra un brote de fiebre aftosa sigue teniendo importantes repercusiones sobre el comercio de animales susceptibles a la enfermedad y de sus derivados.

Palabras clave
Diagnóstico – Fiebre aftosa – Infección subclínica – Intercambios internacionales – Portadores – Vacunación.

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