Appendix 23

Virus Inactivation Kinetics

Soren Alexandersen

Danish Institute for Food and Veterinary Research, Department of Virology, Lindholm, DK-4771 Kalvehave, Denmark

At the session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease in Chania, Crete, Greece, FMDV inactivation kinetics were again discussed and it was concluded that more experimental data was needed but that existing data should be thoroughly reviewed and be the basis for subsequent specific recommendations for experimental studies to be performed. It was agreed that Prof. Matthias Greiner would facilitate a review of the available data on FMDV inactivation in meat and meat products and that Prof. Soren Alexandersen would facilitate a review of existing data on FMDV inactivation in milk and milk products. The findings and conclusions in regard to meat and meat products will be presented separately by Matthias Greiner. The existing knowledge in regard to FMDV excretion, transmission and stability of FMDV relevant for estimating the risk of raw and treated milk and milk products is briefly reviewed below and has been previously reviewed by Donaldson and by Haas (Donaldson, 1997; Haas, 2003). The review below focuses in particular on areas of uncertainty where suitable data are sparse or missing.

Virus and target and host range. Foot-and-mouth disease virus (FMDV) is classified within the Aphthovirus genus in the Picornaviridae family (Bachrach et al., 1957; Newman et al., 1973; Belsham, 1993; King et al., 2000) and is the causative virus of foot-and-mouth disease (FMD), a vesicular disease which with some minor exceptions affects members of the order Artiodactyla, i.e. all cloven-hoofed animals including domestic and wild ruminants and pigs (Thomsen, 1994; Thomson et al., 2003; Alexandersen & Mowat, 2005). The animals which under natural conditions are of greatest significance include cattle, pigs and small ruminants (sheep and goats) and, in particular in Asia and South America, the water buffalo. African buffalo play an important role as the natural maintenance host in Africa, but other wildlife such as impala may also be involved in the natural epidemiology of FMD. Animals which may contribute to the transmission of virus under certain conditions or which cannot be excluded as having some risk of transmission include deer, camels, llamas and alpacas, any animal of the order Artiodactyla and Indian elephants. These animals may not easily become infected but can potentially be of some significance if they get in close contact with livestock, for example when kept under farmed conditions or in zoos (Alexandersen & Mowat, 2005). However, whether such animals, e.g. camels, could play any role in transmission of FMDV by milk and milk products are currently unknown.

FMDV is highly variable with seven serotypes identified and it cannot be excluded that heat stability may vary among serotypes or subtypes.

Transmission and prevalence. FMD virus can be transmitted by many routes, including airborne spread, and may in addition to the usual acute vesicular disease cause a subclinical, persistent infection in ruminants (so-called “carrier” animals) (Van Bekkum et al., 1959a; Van Bekkum et al., 1959b; Sutmoller & Gaggero, 1965; Alexandersen et al., 2002; Alexandersen et al., 2003b). FMD spreads very efficiently when first introduced on a farm, and the prevalence on any given farm is likely to be rather high before disease is noted, in particular if there is free contact among animals. Furthermore, dairy cattle are the animal group having the highest relative risk of becoming infected according to common experience and also recently shown by quantitative modelling studies of epidemiological data (Keeling et al., 2001; Ferguson et al., 2001a; Ferguson et al., 2001b; Keeling et al., 2003). Nevertheless, as the clinical signs of FMD in cattle, in particular in dairy cattle, are usually obvious, it is considered unlikely that more than a few dairy herds may become infected before suspected FMD is reported to the official veterinarian. Consequently, for the purpose of analysis one may assume that only a few dairy herds are affected at the time of reporting, but that the prevalence of FMD may vary.
from 10-90% among dairy cattle in an individual herd. As disease progresses and lesions may develop on the udders, affected animals may become difficult to milk and milk production may drop dramatically. Furthermore, such animals are more likely to develop mastitis, affecting the quality and quantity of milk produced from infected farms. However, the risk of spread of FMDV by milk is mainly in the early phases of the infection before disease is officially reported and procedures put in place. Therefore, one may assume that the production of milk from farms affected early on will be at normal levels and of normal quality.

**Excretion.** FMDV is shed to all secretions and excretions, including semen, during the acute phase of the infection. FMDV can replicate in the squamous epithelia of the mammary glands of dairy cattle (Burrows, 1968; Sellers et al., 1968; Sellers et al. 1969; Burrows et al., 1971; Burrows et al., 1981; Blackwell et al., 1981; Blackwell & Yilma, 1981; Blackwell et al., 1982) and virus is consequently excreted in significant titres in milk from shortly, i.e. up to 2-4 days, before clinical signs appear and through the 4-5 days of the clinical phase, in a pattern that largely mirrors the viraemia profile. After the high level excretion during the acute phase, very low levels of FMDV have been reported to be excreted in milk for up to 3 weeks or more (Burrows et al., 1971). Not much is known about the content of FMDV in milk from other species than cattle, but a positive reaction (RT-PCR) for FMDV in the milk from experimentally infected sheep has been reported (Callens et al., 1998) and work by McVicar and Sutmoller (McVicar & Sutmoller, 1971) showed high levels of virus in the milk from infected goats. Thus, one should assume that the milk from other species than cattle also contain relatively high titres of FMDV. Large amounts of virus are excreted in vesicular fluid, in desquamated vesicular epithelium and, in cattle, also in saliva (Hyslop, 1965; Scott et al., 1966; Cottral, 1969). There is also excretion, but to a much lesser extent, in faeces and urine (Burrows, 1968; Parker, 1971; Garland, 1974), in a pattern that also reflects the peak of viraemia, lesions and clinical disease. A sharp decline in viral excretion and load occurs around day 4-5 of clinical disease, when a significant circulating antibody response is detectable.

In regard to levels of FMDV shed in milk, Donaldson et al 1982 (Donaldson et al., 1982) mention individual milk titres of $10^{0.7} - 10^{6.6}$ TCID50/ml in samples collected from six clinically normal cattle during the 1981 Isle of Wight outbreak and in a single bulk tank milk (the herd had 32 milking cows) of $10^{5.2}$ TCID50/ml. Thus, we may assume an average FMDV titre of $10^2$ TCID50/ml milk from infected farms and we may also assume that the bulk tank milk from a single infected herd go through a further dilution of 10-fold into uninfected farms milk (into a collecting tank), then the average titre would be $10^1$ TCID/ml milk in the collecting tank (bulk). However, it is not unlikely that the titre in certain situations may be much higher, e.g. titres of $10^{5.5}$ TCID/ml and $10^5$ mouse infectious doses (corresponding to approximately $10^5$ TCID50) per ml have been found in a milk chum and in both a milk tanker and a retail bottle of milk (Anonymous, 1969; Hedger & Dawson, 1970; Burrows et al., 1971).

**Interference from vaccination.** Shedding of FMDV in the milk from vaccinated animals is likely to be at a lower level than from non-vaccinated animals (de Leeuw et al., 1978; de Leeuw, 1980). However, as vaccination against FMD in the EU are expected to be reactive, i.e. to potentially be started after the first outbreaks are reported, the risk of excretion of FMDV in the milk of infected ruminants before disease reporting is not affected. It should be reiterated that although vaccination are likely to reduce FMDV excretion in milk, vaccinated cattle may still be susceptible to a low grade infection and shed low amounts of FMDV in the milk, potentially without any clinical signs of disease. Consequently, milk should be treated accordingly, e.g. according to EC1774/2002 (see later).

**Infection and minimum doses.** Susceptible livestock may be infected by FMDV as a result of direct or indirect contact with infected animals or with an infected environment. When infected and susceptible animals are in close proximity, the aerial transfer of droplets and droplet nuclei is probably the most common mode of transmission. Long-range airborne transmission of virus is an uncommon but important route of infection, requiring the chance combination of particular factors, including (1) the animal species, (2) the number and location of the transmitting and recipient animals, and (3) favourable topographical and meteorological conditions (Alexandersen et al., 2003b). Studies have been carried out in animals infected by simulated natural methods (direct or indirect contact with infected donors or virus aerosols from such donors) or in animals infected by artificial methods,
including subcutaneous, intradermal, intramuscular and intravenous inoculation, intranasal instillation, and exposure to artificially created aerosols. It should be mentioned however, that studies carried out to establish minimum infective doses for the main livestock species, with various serotypes and strains of FMDV delivered by different routes, summarized in the Table below, all have an important reservation concerning the statistical significance of the numerical values as a result of the practical and cost constraints on the number of animals that could be used for the experiments and the number of variables that could be investigated. In addition, the several methods used for titration of virus were of varying sensitivity and may not be directly comparable. The results should therefore be taken as indicators and not as absolute values (Alexandersen et al., 2003b). The origin of FMD epidemics in countries normally free from the disease is frequently difficult to identify with certainty, but several recent outbreaks have been linked to the entry of virus in contaminated material that has subsequently been fed to animals. For example, the South Africa 2000 and UK 2001 epidemics have been attributed to the feeding of unheated waste food to pigs, and the Japan 2000 epidemic to the feeding of contaminated fodder (Knowles et al., 2001; Alexandersen et al., 2003a). It should be noted that animals are relatively insensitive to experimental infection by the oral route; the dose for pigs being about $10^4$ - $10^5$ and for ruminants about $10^6$ - $10^7$ TCID50 (Sellers, 1971). These doses are much higher than those required to infect by the airborne route (Donaldson, 1987; Alexandersen et al., 2003b). It should also be noted, however, that animals with abrasions of the epithelium in and around the mouth may be infected by smaller doses (Donaldson, 1987). Sharp objects, such as pieces of bone, may therefore facilitate infection by contaminated waste food or facilitate the infection by other contaminated feed such as milk or milk products.

*Selected estimated minimum doses *for various species and routes of exposure*

<table>
<thead>
<tr>
<th>Species</th>
<th>Inhalation</th>
<th>Intradermal</th>
<th>Intramuscular</th>
<th>Nasal</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>10</td>
<td>100</td>
<td>$10^4$</td>
<td>$10^4-10^5$</td>
<td>$10^5-10^6$</td>
</tr>
<tr>
<td>Sheep</td>
<td>10</td>
<td>100</td>
<td>$10^4$</td>
<td>$10^4-10^5$</td>
<td>$10^5-10^6$</td>
</tr>
<tr>
<td>Pigs</td>
<td>$&gt;800$</td>
<td>100</td>
<td>$10^4$</td>
<td>Unknown</td>
<td>$10^5-10^5$</td>
</tr>
</tbody>
</table>

*The estimated minimum doses are those reported to cause clinical disease. It is emphasized that these are not absolute values but represent estimates based on different experiments that are not necessarily directly comparable. It is possible that even smaller doses might produce infection if large numbers of animals were exposed. Doses are given as TCID50 (bovine thyroid tissue culture 50% dose end-point estimates). For further information see Alexandersen et al., 2003 (Alexandersen et al., 2003b). It should be noted that for intradermal and intramuscular inoculation, doses from 5 to 10 fold lower are cited in the literature, but without details of the assay systems used (Sellers, 1971).

**Risk of spread by milk and milk products.** As mentioned above, excretion of FMDV in the milk is likely to occur if dairy cattle, and probably also other ruminants, are infected and such milk is definitively a risk in the period before disease is confirmed. Donaldson (Donaldson, 1997) and Haas (Haas, 2003) have described the risks of spreading FMD with milk and dairy products and describes examples where such spread has happened. Sellers (Sellers, 1971) mentioned three mechanisms by which animals could potentially be infected by milk or dairy products containing FMDV: 1. Drink the milk; 2. Inhale infected droplets or aerosols generated from the milk or product; 3. The milk or dairy product may contaminate people who may subsequently handle animals.

**A. Raw milk.** As mentioned above, milk from affected animals may contain up to $10^{6.6}$ TCID50/ml or possibly more, as this value is based on a single finding in the field (Donaldson et al., 1982). Thus, the risk of animals becoming infected if directly fed such milk is substantial, as the dose required to infect by the oral route, as indicated above, is only $10^4-10^5$. Furthermore, if the milk is fed to animals with pre-existing cuts or abrasions the infective dose may be less. This is naturally only the case for untreated products in which the FMDV is likely to be rather stable provided the pH is around neutral and the temperature does not exceed 30°C for long periods. However, as also mentioned above, the concentration in the raw milk is likely to be much less due to the dilution occurring during collection. Based on the assumptions given above, an average titre of FMDV in a milk tanker containing milk...
from one infected herd diluted by milk from another 10 farms may be $10^4$ TCID/ml milk. However, this value is based on a single finding in the field, and in certain circumstances the milk from an individual infected herd may be significantly higher, perhaps as high as $10^5$-$10^6$ TCID/ml milk if some animals have clinical signs but are not diagnosed with FMD. In this latter case the diluted milk tanker may thus contain up to $10^5$ TCID/ml milk and thus easily spread FMD if fed to susceptible animals. However, in the situation where the milk may only contain around $10^1$ TCID/ml milk a healthy pig may have to drink as much as 1 litre to become infected. However, if the animal has pre-existing cuts or abrasions in and around the mouth then less volume may be needed, perhaps as little as 10 ml of milk. If the milk contained around $10^5$ TCID/ml and somehow was aerosolised, the dose needed to infect a calf may only be 1 ml, which appears to be a very large and unlikely amount of aerosol to be produced under natural conditions. However, if the milk contains $10^5$ TCID/ml milk containing 10 ml of such infected milk may establish infection through damaged mucosa.

**B. Milk treated by heat.** High temperature-short time pasteurisation (HTST = 72°C for 15 sec) is reported to give a $10^4$-$10^5$-fold reduction in FMDV infectivity (Donaldson, 1997). However, one should take into account that the inactivation of many agents, including FMDV, is biphasic in nature and that a small fraction of virus will often survive (Donaldson, 1997). Furthermore, the thermal inactivation kinetics of FMDV may also be affected by the pH, and milk with a pH above 7 may be inactivated slower by heat that milk with a pH below 7. However, the mixing of milk from several sources at most processing sites results in raw milk of average pH around 6.5-6.8 (de Leeuw et al., 1978; de Leeuw, 1980). Also, it should be realised that virus within the cellular fraction of the milk may be more heat resistant than other fractions, e.g. in the study by Blackwell et al. (Blackwell et al., 1982) it is mentioned that HTST treatment resulted in approximately a $10^{3.4}$-fold reduction in infectivity of the whole milk while the same treatment of the skim milk or the pelleted cellular fraction resulted in a $10^2$-fold and $10^3$-fold reduction in infectivity, respectively. In the examples mentioned earlier, i.e. with raw tank milk having an initial titre of $10^1$ TCID/ml or up to maybe $10^5$ TCID/ml milk, after HTST treatment such milk may contain from trace amounts and up to around $10^1$-$10^2$ TCID/ml of pasteurised milk. Although such milk has a low probability of infecting susceptible species and may require 1 litre to infect a pig, it may, if the animal has pre-existing cuts or abrasions in and around the mouth, perhaps only need as little as 10 ml of milk. Independently, if fed to many susceptible animals (Sutmoller & Vose, 1997), there may be a small but significant risk of causing an outbreak of FMD with single HTST pasteurised milk.

Ultra-high temperature (UHT) treatment (minimum treatment of 132°C for at least 1 sec) is reported to cause a 10-fold higher inactivation of FMDV than HTST treatment (Donaldson, 1997), and consequently, the risk of UHT treated milk or products are likely to be reduced 10-fold compared to single HTST treated milk or products.

Heating to 80-90°C for 30 sec, often used for production of milk powder, is reported to reduce the FMDV infectivity by $10^{3.4}$-$10^{6.0}$ and the subsequent drying process is likely to further reduce the infectivity (Donaldson, 1997), perhaps by a factor of 10. Thus, products treated in this way are unlikely to represent a risk in regard to spread of FMD.

Sterilisation by heat normally requires a process giving an $F_c$ or $F_0$ greater or equal 3.00, which, according to EC 1774/2002 “laying down rules concerning by-products not intended for human consumption”, is equivalent of at least 121°C at the coldest point for 3 min. Such a treatment may inactivate $10^{12}$ spore forming pathogens, e.g. mesophilic *Clostridium botulinum* (Gaze, 2005), and is thus likely to also inactivate FMDV by a factor of at least $10^{12}$.

**C. Treatment by changing pH.** From the literature it is clear that FMDV is only stable within a narrow pH range around 7-8. Outside this pH range the virus starts to be inactivated at an increasing speed depending on how far away from neutral. Although the exact rate of inactivation is naturally depending on many parameters, including the content of organic material and the ionic strength of the product, a guiding rule of thumb is as follows: FMDV half-life, or under optimal conditions the 10-fold reduction time, equals roughly 12 hours at pH 6.5; 1 min at pH 6; and 1 sec at pH 5 (Fellowes,
1960; Bachrach, 1968). Thus, the effect of lowering pH will depend on the actual pH achieved and the time of the treatment. However, it should be remembered, as mentioned above under heat treatment, that also pH dependant inactivation of FMDV is biphasic in nature and that a small fraction of virus will often survive for a longer period. In milk products in particular, the exact quantitative effect on virus infectivity of lowering pH may be difficult to assess. Sellers (Sellers, 1968) reported a $10^{15.6} > 10^5$ log reduction of FMDV titre after lowering pH to 6 for 30 min at 4°C unless the material contained milk (40%) after which he only found a $10^{-2}$ log reduction in titre. However, this being said, long term infectivity of FMDV, even in milk, is unlikely to occur at a pH level of under 6.2 (Henderson & Brooksby, 1948).

D. Drying. The effect of drying can be difficult to quantify exactly and will depend on the actual process involved. The drying itself is likely to reduce the FMDV infectivity by a factor of 10-fold or more, however, the drying process often includes some initial heat treatment to facilitate the drying process adding to the inactivation and thus often result in a further reduction in infectivity. In 2003/85/EC on “Community measures for the control of FMD”, it is specifically mentioned that the drying process should again heat the product to at least 72°C and as mentioned above, such products often involve heating the product to 80-90°C for 30 sec.

B. Combination treatments. Combination treatments may consist of repeated heat-treatments, e.g. double HTST or HTST followed by UHT treatment. As an example, Donaldson (Donaldson, 1997) mention the situation during the outbreaks of FMD in Denmark in 1982, where a treatment consisting of initial HTST treatment was followed by 80°C for 3 sec and, in some cases, by lowering pH to below 4.5 (Danish Veterinary Service, 1982a; Danish Veterinary Service, 1982b). It was estimated that 18 million kg of milk treated in this way was fed to domestic animals during the epidemic without causing any outbreaks (Danish Veterinary Service, 1982a; Danish Veterinary Service, 1982b). Other possibilities of combination treatments often used are heat treatment followed by lowering pH or drying. Various EU regulations describe such treatments, e.g. EC1774/2002 “laying down rules concerning by-products not intended for human consumption” in which it for milk and milk-based products is mentioned that such products should be first treated by HTST pasteurisation followed by a drying process for dried milk or dried milk products, and for acidified milk products, a process by which the pH is kept below pH 6 for at least an hour. In regard to milk or milk-products from third countries, EC1774/2002 stipulates that the same treatment, or double heat treatment, may be used from FMD free regions while milk and milk-based products from third countries or regions where FMD have been present within the last 12 months should undergo either the same treatment or double heat treatment or a sterilisation process to an Fc>3. Similar treatments are also described in the Foot-and-mouth disease directive 2003/85/EC which for milk and milk products not intended for human consumption and milk and milk products for animal consumption, mention a sterilisation process to an Fc>3, double HTST pasteurisation, or HTST or UHT followed by a drying process (it is specifically mentioned that the drying process should again heat the product to at least 72°C and as mentioned above, such products often involve heating the product to 80-90°C for 30 sec) for dried milk or dried milk products and for acidified milk products, a process by which the pH is kept below pH 6 for at least an hour. The combined effects of the mentioned treatments is likely to be a $10^{6}$-fold or more reduction in FMDV titre and additionally, either have the added safety of relying on two different principles of inactivation or at least a repeated process. Consequently, products from such combination (double) treated milk or milk-products are unlikely to spread FMD. It should be noted however, that although treatment of products at pH 6 for an hour results in a reduction in the potential infectivity content, it is wise, as indicated in the SCAHAW Report on “Strategy for Emergency Vaccination against Foot and Mouth Disease (FMD)” of 10 March 1999 for feeding of whey to pigs, that the pH treatment is rather prolonged, assuring that the product is kept or transported at the lower pH for several hours (rather than only 1 hour) to ensure full inactivation.

F. Risk of post-processing contamination and possibility to survive during transport and storage. EC1774/2002 “laying down rules concerning by-products not intended for human consumption” lists the safeguards necessary for safe processing of milk and milk-based products: every precaution must be taken not to contaminate the products and the final product must be packed in new containers or transported in vehicles or containers that have been disinfected and approved. Thus, the risk of
contamination is likely to be low and would most likely, if it happened, only result in a very low level of infectious FMDV in the finished product. Nevertheless, it is of utmost importance that treated products are adequately shielded from subsequent contamination. Any infectious FMDV not inactivated during the processing may be relatively stable during storage and transport if the pH of the product is around neutral and the temperature is not above 30°C for extended periods. Similarly, any infectivity still present in dried products is likely to be stable.

**Summary of main uncertainties.** Main uncertainties in relation to the risk of milk and milk can be summarised as below and could individually or combined be the target of specific studies:

- Minimal dose to infect - may give a $10^3$-$10^4$ fold level of uncertainty in animals with cuts or abrasions
- Excretion levels in milk, prevalence on farms and number of farms affected at time of reporting - may give a $10^3$-$10^4$ fold level of uncertainty at the herd or area level
- Uncertainties about heat treatment:
  - Cellular fraction more stable - $10^3$ fold uncertainly
  - Biphasic inactivation
  - pH dependant
  - UHT inactivation not well described
- pH inactivation - biphasic, small fraction may survive
- Drying - not well described by itself
- May be differences in the stability of various serotypes or subtypes
- Even less is known about other species than cattle

**Discussion**

As evident from the review of known data given above, and already mentioned at the 2003 meeting in Gerzensee, Berne, Switzerland in September 2003, the scientific literature on FMDV inactivation kinetics under relevant conditions is fragmented and with major inherent difficulties for making quantitative comparisons on the data from various sources and of various quality and statistical significance. As explained by Have (Have, P. An assessment of guidelines for treatment of meat from a FMD vaccination zone. Session of the Research Group of the Standing Technical Committee, European Commission for the Control of Foot-and-Mouth Disease 2003: 149-152), the effect of heating is determined by a combination of time and temperature and the inactivation kinetics are often assumed to be first-order although this does not appear to be the case for FMDV inactivation and consequently, tailing effects should clearly be considered. The decimal reduction time $D_T$ is the time needed to reduce the viable population by 90% at the temperature $T$. Semi-log plots of $D$-values against temperature yield near linear relationships, from which estimated $z$-values can be calculated as the number of degrees temperature required to change $D$ by one log unit. Heat treatment includes a heating phase and a cooling phase and to account for the combined effect during heating and cooling, the temperature/time relationship data can be used to calculate lethal rates over the entire process and integrating into a cumulated lethal effect, expressed relative to a standard treatment at a chosen reference temperature and taking advantage of knowing the value of $z$ (Peleg, 2003).

**Conclusions**

Clearly, detailed studies of FMDV inactivation in milk and milk products from infected animals should be supported and designed to provide $D$ and $Z$-values in milk and relevant milk products and should also provide additional evidence on the quantities of FMDV excreted in the various fractions of milk from infected animals and the minimum doses to infect.

**Recommendations**

- A small working group should urgently draft detailed plans for studies of FMDV inactivation in milk and milk products from infected animals and funding and input from industry should be sought.
• The studies should be designed so they can provide D and Z-values of FMDV inactivation in milk and relevant milk products. Special attention should be put on the inactivation kinetics in the cellular fraction versus the aqueous (skim milk) and cream fractions and also particular attention should be put on selected treatments such as HTST (72°C for 15 sec), UHT (132°C for at least 1 sec), 70°C, 80°C, 90°C, drying and pH.

• Clearly also other conditions apart from the temperature, such as product type, pH, ionic strength and a number of other factors are also of importance for the kinetics of inactivation and should be an integrated part of the studies as should established combination treatments.

• The studies should be complementary to existing knowledge and done under conditions making comparison to earlier and coming studies feasible.

• The studies should also provide additional evidence on the quantities of FMDV excreted in the various fractions of milk from infected animals at various stages of the infection.

• Studies looking at the actual dose needed to establish infection by the oral or aerosol route by infected milk is also needed, in particular in animals with abrasions or cuts in the oral epithelia.

• Relatively large animal experiments are needed in order to provide statistically significant data.

References


