No foot-and-mouth disease virus transmission between individually housed calves

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Abstract

The foot-and-mouth disease outbreak in The Netherlands in 2001 most likely started on a mixed veal-calf/dairy-goat farm. The outbreak among the 74 calves on this farm appeared to be limited to four animals, and no clinical signs of FMD were reported. Also on a second veal-calf farm minor clinical signs and limited virus transmission were observed. Since FMD is known to be a very contagious disease, and can cause severe lesions, these observations were disputed. Therefore, we carried out two experiments to determine whether the Dutch FMD virus isolate from 2001 does spread among individually housed calves with limited contacts, either indirect (experiment 1) or direct (experiment 2). In experiment 1, four pairs of calves were housed in an individual box at 1 m distance from each other. In experiment 2, two groups of three calves were housed in individual boxes, directly bordering each other. We infected one animal per pair in experiment 1, and the calf in the middle in experiment 2. We recorded clinical signs, virus shedding in saliva and the development of antibodies. In addition, we determined whether the virus was transmitted from the inoculated calves to the neighbour(s). All inoculated calves showed mild signs of FMD—fever, and some vesicles on hoofs and/or in the mouth—but only one calf showed signs that were visible without physical examination. All inoculated calves shed virus in the saliva and developed neutralising antibodies. None of the contact animals seroconverted, indicating that virus transmission did not occur. These experiments showed that no virus transmission among individual housed calves can occur. This finding supports the hypothesis of the route of virus introduction to The Netherlands in 2001 and show that the observations on the two veal-calf farms were not impossible.

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1. Introduction

In March 2001, a suspicion of foot-and-mouth disease (FMD) on a mixed dairy-goat/veal-calf farm in The Netherlands was reported. The diagnosis was confirmed after a few days by laboratory diagnosis. This farm (index case 2001/3) had received 74 calves from Ireland, 19 days before the suspicion was reported. The
calves had been housed individually. The first clinical signs of FMD, however, were reported in the goats, 19 days after arrival of the calves. Neither the farmer nor the practitioner reported signs of FMD in the calves. At the time of culling, serum samples were taken from 100 of the approximately 500 goats present and from all calves (74) on that farm. From the 100 goats sampled, 87 were serological positive, indicating that the disease had been present for some weeks. From the 74 calves, 4 were serological positive, indicating an infection of these calves with FMDV. Based on the observation that no disease signs were seen, this infection had been ‘subclinical’.

Tracing of the source of virus introduction indicated that the most likely route of introduction of FMDV to The Netherlands was via the transport of these calves, which was later supported by sequencing the isolate (Dekker et al., 2001). The calves must have been infected at an FMDV-contaminated resting point in Mayenne, France, where they arrived on 23 February, from Ireland. The calves arrived at the farm in The Netherlands on 24 February. The same sequence of the 1D part of the genome was found in the virus isolated on the index herd, in Mayenne and in the English farm responsible for the infection of the farm in Mayenne. This 1D sequence was only found in two other Dutch farms that had a direct epidemiological link to the index case. All other isolates found in The Netherlands were different (Dekker et al., 2001).

The outbreak among the calves on the index case appeared to be limited to four animals, and no clinical signs of FMD were reported. Later during the epidemic, FMDV was isolated on a second veal-calf farm (2001/8). Limited clinical signs were seen in one calf and no laboratory confirmation of spread to the other calves was established.

Since FMD is known to be a very contagious disease, which can cause severe lesions, it was questioned whether the hypothesised route via Mayenne was likely or even possible. It would normally be considered unlikely that FMD would have been present in calves without a major clinical outbreak.

One explanation for the minor outbreak among the calves could be the limited contact structure between the calves. The contacts between the calves had remained limited due to individual housing during the first weeks after arrival. Probably, the calves infected one or a few goats. Because the goats could mingle freely, the virus probably spread easily and extensively amongst the goats. In theory, infection of animals housed in a row will stop spreading, if transmission relies on direct contact between animals only, because the chance of transmission will always be smaller than 1 (Ball et al., 1997). Only limited quantitative data about the transmission of FMDV were available, and the effect of limited contact structure on the transmission of FMDV was unknown.

Thus, the observations on the veal-calf farms gave rise to the question whether no transmission of virus between individually housed calves can occur. We, therefore, wanted to investigate experimentally how clinical signs and virus transmission among the calves would present itself. We carried out some experiments to falsify the hypothesis that an FMD virus strain among individually housed calves with limited contact (direct or indirect) spreads extensively.

2. Materials and methods

2.1. Animal experiments

Conventionally reared calves of 4–9 weeks of age were randomly allocated to the experimental groups. The animals arrived at the “high containment” facilities of ID-Lelystad 5–7 days before the experiment started. Two experiments were conducted: one with a limited contact and a distance of 1 m between the calves (indirect contact) and one in which the animals were individually housed but close to the inoculated calf (direct contact). All stables used in the experiments were mechanically ventilated at a rate of 400 m³/h.

- **Experiment 1—indirect contact.** Eight calves were allocated to four groups of two calves each. The calves were housed in an individual box at 1 m distance from each other as illustrated in Fig. 1 (with middle box absent). The four groups of two calves were each housed in separate stables. The calves did not have direct contact with each other.

- **Experiment 2—direct contact.** Six calves were allocated to two groups of three calves each. The calves were housed in individual boxes, directly bordering each other (see Fig. 1). The two groups were housed in separate stables. The three calves in each group
could have direct contact with their neighbour: the calf in the middle with both neighbours, the other two calves only with the calf in the middle as illustrated in Fig. 1.

An infection chain was started in each group by inoculation of one calf per group with FMD virus intranasally. In experiment 2 the calf in the middle was inoculated. The other animal(s) in each group were in-contact calves.

Infection was performed by intranasal inoculation with 1.5 ml of FMD virus (see Section 2.2) in each nostril. The body temperature of all calves was recorded daily. After inoculation heparinised blood samples were taken from all calves daily until 7 days, saliva samples were taken by insertion cotton gauze on a forceps in the buccal cavity. In the laboratory the cotton gauze was put into 4 ml of maintenance medium EMEM containing 5% foetal bovine serum and 10% antibiotics. Medium and saliva were centrifuged and the sample was stored at $-70^\circ C$ until analysis. Saliva samples were collected until 14 days after inoculation, and serum samples weekly. Animal caretakers first took care of the contact calves, and changed gloves, shoes and clothing before taking care of the infected calves. Calves had their own feeding and drinking bucket.

The animals were culled at the end of the experiment: in experiment 1 after 30 days in groups 1 and 2, and after 43 days in groups 3 and 4, and in experiment 2 after 71 days.

2.2. Virus

Samples from outbreak NET/2001/1 and NET/2001/3 were grown in the tongue of FMD serological negative calf. After approximately 25h all vesicles were collected and used to prepare the challenge virus, which was stored at $-70^\circ C$. The challenge virus was titrated on cattle tongue before use (Henderson, 1949), and a titre of $10^{5.9}$ cattle-ID$_{50}$/ml was found.

In the experiments, the virus was diluted 1500 times and 1.5 ml was inoculated in each nostril, resulting in approximately 1500 cattle-ID$_{50}$ per calf (500 cattle-ID$_{50}$/ml). This dose is lower than often used for challenge. The reason is that we want to mimic a natural infection. The choice of 1500 cattle-ID$_{50}$ is based on concentration of virus in swabs with saliva from infected calves. After inoculation samples of the freshly prepared inoculum as well as the inoculum used in the stable were titrated on secondary porcine kidney cells as well as on secondary lamb kidney cells (De Leeuw et al., 1979).
2.3. Laboratory tests

The virus neutralisation test was performed as described previously (Dekker and Terpstra, 1996) using O1 Manisa virus and secondary porcine kidney cells. Eight twofold dilutions per serum sample were tested to determine the titre of VN antibodies. VN titres are expressed as the reciprocal of the highest dilution that inhibited the cytopathic effect in 50% of the cell cultures. Serum samples with titres >2.4 (10 log) were not diluted further, because knowing the exact VN titer beyond 2.4 was not relevant for interpretation of the experiment.

Virus isolation was carried out using plasma obtained from the heparinised blood samples and the saliva samples by inoculating 200 l of tenfold dilutions of the sample on two wells of secondary lamb kidney cells grown in a collagen coated 6-well plate (Greiner®). After 1 h absorption the cells were overlaid with maintenance medium containing 1% methylcellulose. After 2 days the plates were dipped in a citric acid solution to kill FMD virus, and the monolayers were stained with amido-black (0.1% amido-black in 1 M acetic acid, 0.09 M sodium acetate, 10% glycerol). All incubation were made at 37°C in a humidified atmosphere containing 5% CO2. Monolayers were examined macroscopically and titres were expressed as plaque-forming units (p.f.u./ml).

2.4. Estimation of virus transmission

The reproduction ratio $R_0$, which is the average number of secondary cases caused by one typical infectious individual during its whole infectious period (Diekman et al., 1990) was calculated with the maximum likelihood method described by Kroese and De Jong (2001). Suppose $m$ transmission trials have been performed each with ($i_0$, $s_0$) as initial state. Let $X$ be the number of contact-infections in the $j$th trial. The likelihood function of the outcomes $X_1, \ldots, X_m$ in $m$ trials is (Vélthuis, 2002):

$$f(x_1, \ldots, x_m|R_0) = \prod_{j=1}^{m} p(s_0 - x_j, i = 0|R_0)$$

The estimate of $R_0$ is obtained by maximising this function for each possible value of $R_0$. For the estimate of $R_0$, a two-sided 95% confidence interval (95% CI) is calculated.

3. Results

3.1. Inoculation

Titration of the inoculum on secondary pig and lamb kidney cells resulted in a dose of, respectively, 6800 plaque-forming units (p.f.u.) and 9800 p.f.u. per calf (in 3 ml).

3.2. Clinical signs and laboratory tests

3.2.1. Experiment 1: no direct contact

One inoculated calf developed vesicles in the lips, tongue and gums (Fig. 2a) and on the hoofs during

![Fig. 2. Clinical signs in inoculated calves (a, experiment 1; b, experiment 2). In (a), this is the only calf with signs that could be seen without physical examination (calf no. 5903). The other calves showed less visible signs. In (b), the calf on the right was the calf with the most prominent signs of FMD.](image)
days 6–10 p.i. This calf had fever on day 5 p.i. One inoculated calf only had fever on day 8 p.i. Body temperatures of the calves during the experiments are shown in Fig. 3. Lameness was not observed, but due to the housing system also unlikely to be observed easily. One of the inoculated calves did not have visible signs of FMD, but had fever on day 8 p.i.

Three of four inoculated calves shed detectable levels of FMD virus in the saliva (Table 1) after infection and had viremia between 3 and 6 days after infection. All inoculated calves had a fourfold increase in neutralising antibodies against FMDV between 7 and 15 days after infection. The highest VN titre observed was between 1.05 and >2.4 (10 log) (Table 2).

None of the contact calves showed visible signs of FMD. The contact calves did not develop viremia, no virus was detected in the saliva samples, and no detectable VN titer was developed.

### 3.2.2. Experiment 2: direct contact

Both inoculated calves developed vesicles on the hoofs, and in one of the two calves also mouth

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**Table 1**

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* Titres are expressed as 10 log p.f.u./ml of diluted saliva.

* Not positive.
lesions consisting of mainly lesions on the gums, and a small lesion on the tongue (Fig. 2b). Lameness was not observed. One inoculated calf had fever on days 5–7 p.i., and one had fever on day 5 p.i. Body temperatures of the calves during the experiments are shown in Fig. 3.

Both shed detectable levels of virus in saliva samples (Table 1) and had viremia on days 4–6 and 3–6 p.i., respectively. Both developed neutralising antibodies against FMDV from day 7. The highest VN titre was 2.1, and 2.4 (10 log), respectively.

The contact calves did not show visible signs of FMD, nor did they develop viremia or a VN titre. All VN titres were below 0.3 (10 log) TCID₅₀. One calf was tested positive in the virus isolation test on day 6 p.i. Since no other signs were recorded, and the saliva sample was positive for only 1 day, we regarded this calf as not infected with FMDV.

### 3.3. Quantification of virus transmission

In neither of the experiments with either direct or indirect contact the contact calves became infected, because no detectable levels of virus or (neutralising) antibodies were measured. Therefore, we concluded that no virus transmission occurred between inoculated and contact calves. The 95% confidence interval for the reproduction ratio in both experiments was estimated to be [0–3.03]. Thus, the point estimate of \( R_0 = 0 \) was not significantly below 1. Combining the two experiments also did not result in an estimate of \( R \) significantly below 1 (upper limit 95% CI 1.39) (Kroese and De Jong, 2001; Vethuis, 2002).

### 4. Discussion

The outbreak of FMD on two veal-calf farms appeared to be limited to a few animals (minor outbreak) and no clinical signs of FMD in infected calves were reported. Since FMD is known to be a very contagious disease, which can cause severe lesions (e.g. Kitching, 2002), it was questioned whether a minor, subclinical outbreak could occur among veal-calves housed individually. The purpose of this study was to determine whether FMD virus could spread among calves with limited contact, direct or indirect. In our experiments, inoculated calves seem to be infectious as virus was isolated from saliva samples, and also viremia was detected. Nevertheless, none of the contact calves became infected with FMDV, not even the animals with direct contact with infected calves. Apparently, virus isolation from saliva is not a good indication for the ability to transmit virus or the amount of virus was not sufficient to induce seroconversion in in-contact calves. At least, the relation between amount of virus in the saliva and infectiousness appears not to be straightforward. Even calves, from which the saliva sample contained 10⁴.₉ p.f.u./ml, did not transmit virus to the contact calf. These experiments made it clear that virus transmission can be absent with this FMD strain in individual housed calves, and that the observations of subclinical minor outbreaks on the two veal-calf herds (outbreaks NET 2001/3 and NET 2001/8) during the Dutch FMD epidemic in 2001 are possible.

However, although the point estimates of the reproduction ratio are \( R_0 = 0 \), the estimates were not

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**Table 2**

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* The VN titres of all contact calves remained <0.3 during the experiment. Titres are expressed as 10 log TCID₅₀. The exact titre of serum samples with a titre >2.4 were not determined, as a twofold increase in titre was assumed evidence of FMDV infection.
to estimate one infectious and one susceptible animal are optimal iment all produced the same result. Experiments with must be noted that four replications of the same exper-

sion is possible. When *R* is not significantly below 1, this implies that major outbreaks can still occur. Al-

ough the *R*-value was not significantly below one it must be noted that four replications of the same exper-

iment all produced the same result. Experiments with one infectious and one susceptible animal are optimal to estimate *R*. Therefore, we started the experiments according to this set-up: in experiment 1, we had four groups of one infectious and one susceptible contact animal in total. Knowing the outcome of these experi-

ments it would have been more logical to perform an experiment where calves could mingle freely (maxi-

mal contact). However, we did not have quantitative knowledge about the transmission between naturally infected calves beforehand, and we assumed a priori that FMD virus would be transmitted easily over short distances. We therefore started the experiment with a distance of 1 m between animals. If no transmission would occur, it would be unlikely that major FMD outbreaks would be seen in stables with individually housed calves. In experiment 2 we expected some transmission, but even when direct contact (Fig. 2b) was possible, although limited, no transmission oc-

urred. Recently, Orsel et al. (in preparation) showed that calves infected the same way but mingling freely transmitted FMD virus to all their contacts.

The results of our experiments showed that the virus did not spread to contact calves. Moreover, the inoculation in most of the calves did not result in clearly visible clinical signs of FMD, easily detected by farmer or his veterinarian without physical examin-

ation. This implies that the farmer can easily miss an FMD virus infection, as the farmer normally does not closely examine his veal-calves. Moreover, since virus transmission between these individually housed calves remained limited, as shown in these experi-

ments and on two veal-calves during the epidemic in 2003, the infection may remain unnoticed. Although no transmission occurred in our experiments, it cannot be excluded, of course, that transmission cannot occur at all (Sutmoller and Casas, 2002). The out-

break on the index farm showed that transmission to the dairy goats occurred. As transmission in free mingling calves does occur (Orsel et al., in prepara-

tion) the main explanation for non-transmission despite virus excretion is the limited contact.

Another explanation is the low infectiousness of the calves, despite measurable amounts of virus in blood and saliva samples. It seems that the amount of virus shed was not sufficient to infect the pen mates, not even after direct contact. Donaldson et al. (1987) demon-

strated that using an aerosol with low amounts of virus, not all calves became infected nor developed clini-

cal signs. Perhaps the contact calves became infected but due to non-humoral immune defences, the virus was cleared quickly. Such a response has been demon-

strated for example for HIV virus (Clerici et al., 1992). It may be possible that contact with a low amount of virus results in a quick immune response. This may prevent extended replication of the virus, resulting in a lack of sero-response or visible signs of FMD.

The results of our experiments do question the role of airborne transmission in FMD infection in calves with mild clinical signs, although, of course, extrap-

olation of the results is difficult. Mars et al. (1999) found that bovine herpes virus 1 could be transmitted over a distance of 3.9 m. However, their experimen-
tal conditions differed from our experiments with re-
gard to the amount of ventilation. And, of course, also the ventilation on farms differs from the experimental units.

Extrapolation and generalisation of these results from these experiments to the field situation is always dangerous. In our experiments, for example, the animal caretakers took additional measures to prevent spread of disease. This was because they handled the calves more often than normal in such a husbandry system. In the housing system applied in these ex-
periments calves have their own feeding and drinking bucket, which is cleaned daily. Whereas in traditional housing systems calves used to be fed by drinking out of the same through or by sharing the drinking buckets. Also in housing systems for dairy cattle shar-
ing drinking and feeding places is common. In the outbreaks in The Netherlands there was no indication that the virus was not able to spread on dairy farms or goat farms. The most likely explanation is that the contact structure of the housing system applied in these experiments is the major cause of the reduction in transmission. Sharing feeding, drinking, milking machine and, e.g. injection needles probably causes
transmission in other housing systems. Although ingestion of FMD virus is not known to be a very efficient route of infection, in those cases small wounds in the mouth or on teats might be a port of entry.

We are aware of biological variation; the reason for repeating experiments. Of course, extrapolation of the results from experiments is always questionable, but that is applicable for experiments in general. Moreover, even under field conditions the infection process is highly variable. We used a low infectious dose, to mimic a natural infection. Extrapolation and generalisation of these results from these experiments to the field situation, indicating a very low risk of infection if young cattle are involved, is very dangerous, because other isolates of FMD might behave differently. Nevertheless, the experiments make it clear that no virus transmission can occur with this FMD strain in individual housed calves.

References