Concentration of Foot-and-Mouth Disease Virus in Milk of Cows Infected Under Simulated Field Conditions

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ABSTRACT

Susceptible lactating grade dairy cows were infected with foot-and-mouth disease by exposure to infected pigs. The virus was detected during asymptomatic state in esophageal fluids, blood, and milk. Foot-and-mouth disease virus not only was present but also persisted in whole milk components, skim milk, cream, and reconstituted cellular debris, with titers of highest infectivity usually in the reconstituted debris. Survival of the virus in milk after high-temperature short-time pasteurization suggests thermal stability comparable to that of progeny virus resulting from direct inoculation of foot-and-mouth disease virus into the mammary gland.

INTRODUCTION

The pH and thermal stability of foot-and-mouth disease (FMD) virus in the milk of infected lactating cows are greater than those for the virus in buffer solutions (3, 4, 5, 6, 7, 13, 14, 19, 20). In addition stability of the virus under these cited conditions is greater for virus present from infection than for virus added to milk (19). These findings suggest that origin of the enhanced stability occurs in the milk secretory cell of mammary gland, and, thus, a possible explanation for the results is interaction of synthesized milk components with progeny virus within the secretory epithelial cell of mammary gland. Demonstrations of replication of FMD virus in the bovine mammary gland as a result of systemic infection is essential to proof of interaction of FMD virus with milk component.

In the field, FMD is transmitted primarily from infected animals by aerosolization of the virus (1), and simulation of these conditions is necessary to substantiate mammary gland replication.

Thus, in our studies milk of cows exposed to FMD-infected pigs, which are generators of large aerosols of FMD virus (16, 24), was examined for content and persistence of virus and partial characterization of thermal resistance of FMD virus found.

MATERIALS AND METHODS

Virus

The Brugge (Br) strain of the 0 serotype, subtype 1 [0\(^1\) (Br)] of FMD virus was used. Original vesicular material was isolated by Leunen during a field outbreak in Belgium in 1963 (21, 22). At the Plum Island Animal Disease Center (PIADC), cell culture passaged material was obtained through the courtesy of J. G. van Bekkum, Centraal Diergeneeskundig Instituut, Lelystad, The Netherlands, and passaged in primary bovine kidney (BK) cell culture six additional times. An additional viral passage in BK cells was made for this study. Infected material was harvested, clarified, titered as described by Bachrach (2), and stored at -70°C until use. Serial 10-fold dilutions were prepared in Hepes (N-2-hydroxyethylpiperazine-N\(^1\)-2-ethane sulfonic acid) buffered lactalbumin hydrolysate in Hanks’ balanced salt solution (HLH) containing antibiotics (100 units of penicillin, 100 μg streptomycin sulfate/ml of fluid). An inoculum of
.05 ml of respective virus dilution was adsorbed onto randomized wells of disposable six well plastic plates (Linbro Chemical Co., Hamden, CT) containing confluent BK cells. Cells were held at 37°C for 1.0 h with redistribution of the inoculum with gentle rolling of plates at each 15-min interval, followed by addition of an overlay medium of .8% methyl cellulose suspension in minimum essential medium, F-15 (Grand Island Biological Co., Grand Island, NY). The 24-h monolayers were stained with a .05% crystal violet solution in 20% formalin for 20 min, and plaques were enumerated macroscopically. Swine inoculum was prepared just before use by dilution of stock virus in HEPES buffered HLH containing antibiotics to yield 7 log10 plaque forming units (pfu)/ml.

Animals

Lactating grade dairy cows and Tamworth (13 to 68 kg) pigs were obtained commercially and had no previous experience with FMD. Each of six sets of donor-recipient animals was composed of one cow and two pigs and was housed in a standard PIADC isolation unit (20). Donor pigs were infected by intradermal inoculation with 7 log10 pfu of FMD virus contained in 1 ml of suspension into the foot pad of each front foot. The recipient cows remained in contact with the infected pigs for the duration of the study, usually 15 days. Although pigs were not restrained, only the larger pigs would venture to come into close contact with the cows.

Cows were examined daily for clinical signs of FMD, e.g., excessive salivation, development of vesicles, and cessation of eating (9).

Collection of Samples

We also monitored onset of infection by examining body fluids such as blood, esophageal-pharyngeal (EP) fluids, and milk for virus. Samples were collected from each cow immediately before swine inoculation and at 24-h intervals throughout the infection, usually 15 days.

Samples of EP fluids, consisting of saliva, mucus, desquamated epithelial cells, and food, were collected from the oral pharynx and anterior esophagus by a probang, a round bottom stainless steel cup, 3 cm in diameter (24). Samples were processed by three repetitive freezing and thawing cycles and then homogenized in a Potter-Elvehjem type glass tissue grinder followed by clarification by centrifugation at 800 × g for 20 min at 4°C. Supernatant fluid portions were collected, frozen at −70°C, thawed, then assayed for infectivity.

Blood samples (ca. 5.0 ml) were obtained from the jugular vein in heparin-treated vials to prevent clotting. Samples were frozen and stored at −70°C, then thawed before use. Milk samples (10 ml) were collected from individual quarters. The remaining contents of the gland thereafter were collected and pooled. Milk fractions were prepared by centrifugation of whole milk at 1000 × g for 10 min at 0°C. A cannula was placed beneath the cream layer in the centrifuge tube, and the skim milk then was drawn up by a syringe. The sedimented debris was resuspended in reconstituted dried skim milk to the original volume. Cream that adhered to the sides of the centrifuge tube was removed with an applicator stick and resuspended in 5.0 ml skim milk. Samples were held no longer than 24 h at 4°C before virus assay and then stored at −70°C.

Virus Detection

Blood, EP fluids, and milk samples were tested for infectious virus by the plaque assay method as described above. When vesicular lesions developed, the viral specificity of this material was determined by complement-fixation test (12).

Direct Inoculation of Virus into Mammary Gland

In a companion study, an additional cow was inoculated with FMD virus suspension (7 log10 pfu) directly into the right front and left rear teats (papilla mammae). The cow was examined daily for clinical signs of FMD, and its fluids, blood, EP, and milk were assayed for FMD virus.

Pasteurization of Milk

Whole milk and components, skim milk, cream, and reconstituted cellular debris, were heated under high-temperature short-time (HTST) pasteurization conditions (72°C for 15 s) described earlier for milk of cows that were infected by direct inoculation of virus into the mammary gland (7) with the modification that
TABLE 1. Temporal response in lactating cows after exposure to pigs infected with foot-and-mouth disease.

<table>
<thead>
<tr>
<th>Cow no.</th>
<th>Secretions and body fluids</th>
<th>Days after exposure(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Virolactia</td>
<td>&lt;.4(^b)</td>
</tr>
<tr>
<td></td>
<td>EP fluids</td>
<td>&lt;.4</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>&lt;.4</td>
</tr>
<tr>
<td>2</td>
<td>Virolactia</td>
<td>&lt;.4</td>
</tr>
<tr>
<td></td>
<td>EP fluids</td>
<td>&lt;.4</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>&lt;.4</td>
</tr>
<tr>
<td>3</td>
<td>Virolactia</td>
<td>&lt;.4</td>
</tr>
<tr>
<td></td>
<td>EP fluids</td>
<td>&lt;.4</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Blood</td>
<td>&lt;.4</td>
</tr>
</tbody>
</table>

\(^a\) log\(_{10}\) pfu/ml.  
\(^b\) 4 Plaques not observed.  
\(^c\) FMD virus detected in cream component only.  
\(^*\) Appearance of vesicular lesions.
tubes containing milk were placed under a 15-
cm hydrostatic head pressure.

RESULTS

Response of Animals

Pigs responded to inoculation with FMD
virus as early as 24 h but no later than 48 h,
and each cow became infected after exposure
to FMD-infected pigs. Typical signs of FMD
developed in pigs and cattle. Onset of clinical
signs of disease and detection of FMD virus in
EP fluids, blood, and milk were variable and
not influenced totally by generation of rela-
tively larger aerosols of infectious virus by the
larger pigs or by the degree of intimate contact
between cows and pigs (Table 1). Fever was not
detected in cow 3; however, it was in the other
cows coincident with appearance of clinical
signs of FMD (Figure 1).

The virus was detected readily in EP fluids,
and peak infectivity titers were in three of six
cows before appearance of clinical signs of
disease (Table 1). The virus was detected in
blood from 1 day before to 2 days after detec-
tion in EP fluids. Peak infectivity titers were
detected in blood of three cows before appear-
ance of clinical signs and in blood of two cows
concomitant with appearance of clinical signs
of FMD. A viremia was not detected in cow 3
(Table 1).

Detection of Virus in Milk

As in Table 1, initial virolactias were de-
tected from 1 to 7 days after exposure. The
virus persisted for 7 days. Five log_{10} plaque
forming units of virus were detected in the milk
of cow 1 24 h before appearance of clinical
signs of FMD.

Maximum infectivity titers in whole milk
varied from 2.3 to 5.4 log_{10} pfu/ml and
occurred 1 to 3 days after onset of virolactia
(Table 1, Figures 2 and 3). A second peak of
infectivity was on day 7 in milk of cow 1 and
on day 6 in the milk of cow 5 (Figure 2a–e).
Infectivity curves, which suggest replication
induced by progeny virus from infected secre-
tory epithelial cells of mammary gland (tem-
poral increase in virus titer), were observed in
all milk samples.

Virolactias were detected 0 to 3 days after
detection of virus in the blood of four of the
six cows (Table 1). Viremia occurred 1 day
after detection of virolactia in the milk of cow
1; however, cow 3 did not have detectable
viremia.

The virus was distributed throughout milk
components: skim milk, cream, and pelleted
deb里斯 (Figure 2). In most instances, the virus
concentration was slightly higher in cream sam-
ples than in the skim milk component. How-
ever, the highest concentration of virus was re-
covered from sedimented debris: fragmented
secretory epithelial and other cells, milk fat
globules and membranes, leucocytes, bacteria,
and debris not removed by filtering the milk
through gauze.

Cow Response to Direct Inoculation into
Mammary Gland

Twenty-four hours after intramammary
inoculation with 7.3 log_{10} pfu FMD virus, the
cow had a temperature of 41.4°C but no other
signs of FMD. The cow still was eating and did
not appear depressed; however, almost 7.0
log_{10} pfu of virus/ml were recovered in the
milk. Virus was detected in the blood and EP
fluids 1 day later and persisted for 3 and 4
days, respectively (Figure 4).

A second virolactia infectivity peak occurred
at 4 days after inoculation and was of 1 day’s
duration. A similar biphasic infectivity curve
was observed with the whole milk components
with the cream component having a slightly
higher infectivity titer than the skim milk com-
ponent. Milk from the front quarters had higher
titers of infectivity than milk from the rear
quarters (Figure 3). The virus persisted in the milk for 5 days after inoculation as detected by cell culture infectivity assay.

**Detection of Virus in Milk after Pasteurization**

Foot-and-mouth disease virus was detected in whole milk and reconstituted pelleted debris after HTST pasteurization at 72°C for 15 s by cell culture infectivity assay.

Two $\log_{10}$ pfu/ml of FMD virus was detected in the HTST pasteurized milk of cow 1 at 3 days after exposure (Table 2). This sampling period coincided with appearance of clinical signs of FMD (Figure 2).

Virus concentration in the reconstituted pelleted debris of milk from cow 5 six days after exposure was reduced by 2.0 $\log_{10}$ pfu/ml after HTST pasteurization.

**DISCUSSION**

Involvement of the respiratory tract in aerosol transmission of FMD virus was reported by Burrows (10), Donaldson et al. (15), Graves et al. (16, 17), and Sellers et al. (23).

In our study, FMD-infected pigs housed under conditions described proved to be satisfactory sources of virus for contact animals. Although the amount of virus shed would be reflective of the size of the donor pig (15, 23), this difference was not a factor in the rapidity of onset of infection in the cows. Virolactias were detected 1 day after exposure to 14-kg donor pigs (cow 1), 5 days after exposure to 27-kg donor pigs (cow 4), and 4 days after exposure to 68-kg donor pigs (cow 5).

In four of the six cows, virolactias were detected before clinical signs of FMD ap-
peared, and it is this clinically inapparent state that is of greatest concern for transmission of FMD. With cow 1, FMD virus was detected in

the cream component before virus could be detected in the blood. This observation, in addition to absence of a detectable viremia in cow 3, suggests that the virus concentration during the primary viremic stage may be below that detectable by cell culture assay methods used. Differences in virus titers in parallel milk and blood samples (Table 1) suggest active virus replication in the mammary gland, as was demonstrated earlier by Burrows (11) after direct inoculation of FMD virus into the bovine gland and by Blackwell and Yilma (8), who used immunofluorescence to localize virus in frozen tissue sections of bovine mammary gland.

Higher concentrations of FMD virus in the cream component than in the skim milk component were noted (7). In our studies, however, viral titers were consistently slightly higher in

Figure 3. Temporal detection of foot-and-mouth disease virus in quarter milk samples of infected cows.

Figure 4. Response of lactating cow to intramammary inoculation with foot-and-mouth disease virus.
reconstituted cellular debris of milk of cows infected by contact exposure.

Results of earlier studies on survival of FMD virus in milk after heating were based upon direct inoculation of virus into the mammary gland (3, 4, 5, 6, 7, 18, 19). In the field, transmission of FMD in this manner would involve suckling of dams by infected calves, and during disease outbreaks a significant number of infections probably occur by this route. However, FMD is transmitted routinely by aerosols (1), and one only could speculate as to the comparableness of the stability of the two respective virus populations in milk. Thus, survival of FMD virus after HTST pasteurization in milk of cows infected by contact exposure suggests that FMD virus in milk of such cows has thermal stability comparable to that reported for virus obtained from milk of cows that had been inoculated directly into the mammary gland (7, 18).

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REFERENCES