Thermal Inactivation of Foot-and-Mouth Disease Virus in Milk Using High-Temperature, Short-Time Pasteurization¹

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ABSTRACT

Previous studies of laboratory simulation of high temperature, short time pasteurization (HTST) to eliminate foot-and-mouth disease virus (FMDV) in milk have shown that the virus is not completely inactivated at the legal pasteurization minimum (71.7°C/15 s) but is inactivated in a flow apparatus at 148°C with holding times of 2 to 3 s. It was the intent of this study to determine whether HTST pasteurization conducted in a continuous-flow pasteurizer that simulates commercial operation would enhance FMDV inactivation in milk. Cows were inoculated in the mammary gland with the field strain of FMDV (01/UK). Infected raw whole milk and 2% milk were then pasteurized using an Arm-field pilot-scale, continuous-flow HTST pasteurizer equipped with a plate-and-frame heat exchanger and a holding tube. The milk samples, containing FMDV at levels of up to 10⁴ plaque-forming units/mL, were pasteurized at temperatures ranging from 72 to 95°C at holding times of either 18.6 or 36 s. Pasteurization decreased virus infectivity by 4 log₁₀ to undetectable levels in tissue culture. However, residual infectivity was still detectable for selected pasteurized milk samples, as shown by intramuscular and intradermal inoculation of milk into naïve steers. Although HTST pasteurization did not completely inactivate viral infectivity in whole and 2% milk, possibly because a fraction of the virus was protected by the milk fat and the casein proteins, it greatly reduced the risk of natural transmission of FMDV by milk.

Key words: foot-and-mouth disease virus, pasteurization, milk, thermal inactivation

INTRODUCTION

Foot-and-mouth disease (FMD) is caused by a small RNA virus of the family *Picornaviridae*, genus *aphtovirus*, foot-and-mouth disease virus (FMDV). Foot-and-mouth disease is not a public health threat but is highly contagious to cattle and other cloven-footed animals. Outbreaks of FMDV have devastating effects on the economies of affected countries (Callis et al., 1968), as demonstrated by the 2001 outbreak in the United Kingdom. Restrictions on the importation of susceptible livestock and animal products, such as fresh meat and certain dairy products, are imposed on countries where FMD is present to protect the disease-free countries. After the terrorist attacks against the United States in 2001, there was concern that FMDV could be used as an agent to target US agriculture.

Foot-and-mouth disease virus is present in all tissues and fluids from infected animals, including the milk. Raw milk has been implicated as a vector for the spread of the virus. Of special concern is that the virus is shed into milk up to 33 h before there are apparent signs of the disease in dairy cows. Cattle may become infected with FMDV if fed the raw, infected milk (Sellers, 1971) or by inhaling aerosol droplets of the infected milk (Donaldson, 1997). Sellers (1971) stated that a 7,570-L (2,000 gal) farm bulk tank with 10⁴ ID₅₀/mL, where ID₅₀ is the virus titer that will cause infection in 50% of animals exposed, would contain 10⁶ infective doses for pigs and 10⁵ infective doses for calves by ingestion. Lax biosecurity measures for handling milk on the farm and during transport of milk by milk tanker trucks have also been implicated in dissemination of the disease (Tomasula and Konstance, 2004.) A review of FMD and a discussion of control measures for infected livestock was given by Grubman and Baxt (2004).

In the event of an outbreak of FMDV, consumer confidence in the food supply may be undermined because

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uncooked or subprocessed animal products that possibly contain the virus, although not harmful to humans, may already be in the food supply. Consumers may question the safety of all animal products (e.g., milk) on store shelves. In this study, our focus was to examine the effectiveness of HTST pasteurization as a measure to eliminate or reduce the risk of transmission of FMDV by milk and dairy products.

In a series of experiments that were conducted to determine the effectiveness of pasteurization on elimination of FMDV in milk, researchers at the Plum Island Animal Disease Center (**PIADC**), Orient Point, New York (Hyde et al., 1975; Blackwell and Hyde, 1976) determined the heat resistance of FMDV type A in naturally infected whole milk. A batch-type experimental system was used to simulate HTST pasteurization by heating milk to 72°C for a holding time of 15 s, the legal temperature and time conditions for pasteurization of milk as mandated by the Pasteurized Milk Ordinance (FDA, 2003). Results showed that up to 6 log₁₀ FMDV were eliminated in naturally infected whole or skim milk when detected by cell culture. However, residual infectivity of the virus was demonstrated by inoculation of the pasteurized samples into steers.

Increases in either pasteurization temperature or holding time do not completely eliminate the virus in whole milk but are effective for reducing, and in some cases eliminating, the virus in skim milk (Blackwell and Hyde, 1976). Steers inoculated with samples of whole milk heated at 72°C for 2 min developed FMDV. However, only 1 out of 3 steers developed the disease when inoculated with pasteurized skim milk samples processed under identical conditions. Heating skim milk at 72°C for 2 min, followed by evaporation of the initial volume by 50% at 65°C for 1 h, resulted in elimination of the virus. In addition, samples of whole milk pasteurized at 85°C and held for 15 s were infectious to steers, but samples of skim milk pasteurized under the same conditions were infectious to only 1 out of 5 steers tested. Hyde et al. (1975) and Blackwell and Hyde (1976) concluded that pasteurization was less effective for elimination of the virus in whole milk because the milk fat and CN proteins offered some protection to the virus.

A later study (Walker et al., 1984) showed that the FMDV was inactivated in many samples when infected whole or skim milk was heated at temperatures between 102 and 148°C for under 10 s in a laboratory-scale flow apparatus. It was unclear from the results whether the elevated temperatures alone were responsible for inactivation of the virus or whether the heating of milk under continuous flow conditions contributed to inactivation of the virus. The experiments were not

conducted using the flow apparatus at temperatures <102°C.

Since the aforementioned experiments were conducted, the methods used to simulate HTST pasteurization to determine the inactivation kinetics of pathogens in milk and their influence on the outcome of the experiments have been discussed in the literature (Tomasula and Kozempel, 2004). The methods are classified as batch or continuous heating methods. The standard holder and capillary tube methods are examples of batch methods (Cerf and Griffiths, 2000; Tomasula and Kozempel, 2004). Hyde et al. (1975) and Blackwell and Hyde (1976) used a variation of the standard holder method.

Tomasula and Kozempel (2004) evaluated the batch and pilot-scale continuous flow HTST methods and concluded that the most useful ones for studying pathogen inactivation in milk and predicting pathogen behavior in commercial HTST pasteurization are those for which the fastest moving particle (**FMP**) through the holding tube may be determined. Knowledge of the FMP ensures that the thermal treatment of milk and any pathogens it may contain are known, and the holding tube length is such that any particle in milk will not traverse the holding tube in less than the required holding time according to the Pasteurized Milk Ordinance (FDA, 2003). Problems with evaporative cooling or splashing of milk, often encountered when batch methods are used to simulate HTST pasteurization and which may underestimate the effectiveness of a pasteurization process, are eliminated with continuous flow HTST pasteurization.

For the short holding times encountered in continuous HTST pasteurization of milk, the thermal treatment that milk receives is controlled by adjusting the flow rate through the holding tube, which is of known volume. The come-up and cool-down times are small compared with the holding time and can be distinguished from the holding time. In the batch methods, the come-up and cool-down times are not precisely controlled and are difficult to distinguish from the holding time itself. The level of pathogen reduction for a reported holding time actually includes the pathogen reduction that occurs during the longer come-up and cool-down times and tends to overestimate the time to inactivate the pathogen. With the continuous methods, there is also no human error associated with timing for the short holding times encountered in HTST pasteurization.

The objective of this study was to determine whether continuous flow HTST pasteurization would be more effective than batch pasteurization methods in eliminating or reducing the infectivity of FMDV in naturally infected milk and to determine the temperature-time

conditions, at temperatures <100°C, required for eliminating the virus in whole milk and 2% milk under flow conditions.

MATERIALS AND METHODS

Animals and Animal Care

All experimental protocols involving the animals were approved by the Plum Island Animal and Care Use Committee. Lactating Holstein cows were used as the source for milk naturally infected with FMDV. Naïve steers were used to test for the presence of residual FMDV in pasteurized milk. Animals were acclimated to the biolevel 3 animal rooms for 1 wk prior to the start of experiments. The cows were fed free choice alfalfa cubes, grain supplement, and water. Cows were milked twice per day using a portable mechanical milking machine (Conde Portable Milking Machine, Westmoor Ltd., Sherrill, NY).

Naturally Infected Milk

Lactating cows were inoculated by intranasal and intramammary installation of 10% tongue epithelium macerate containing 10^4 bovine ID₅₀ of FMDV serotype O, 01/UK, passed once in bovine. Cows were milked twice a day for up to 2 wk or until little milk was obtained during the acute phase. Cows were examined for mastitis, their temperature was recorded, and the pH of the raw milk was measured daily. In the first series of experiments, conducted over 3 d, a single Holstein cow was used to provide infected milk. Because milk production was severely reduced by the third day of experiments, 2 lactating Holsteins were used to provide milk for the second series of experiments, which were also conducted with milk obtained over 3 d. The milk obtained after each milking was refrigerated at 4°C prior to use in pasteurization experiments. Milk collected over the weekend was frozen at -70°C, mixed with the milking from the following Monday, and then stored at 4°C.

HTST Pasteurizer

An Armfield model FT74P/T HTST/UHT (Armfield Inc., Denison, IA) plate-and-frame continuous pasteurizer was used in these studies. The pasteurizer is an HTST type similar to that found in commercial pasteurization operations and is described in detail in Tomasula and Kozempel (2004). Briefly, it consists of a feed vessel mounted on a progressing cavity pump that leads to a plate-and-frame heat exchanger followed by a holding tube section. Pasteurized milk leaving the holding tube reenters the opposing side of the plate-and-frame

heat exchanger and is used to preheat the entering milk. The pasteurizer has a maximum processing capacity of 60 L/h. Milk was heated to pasteurization temperatures using hot water on the opposing side of the heat exchanger. Two different holding tube assemblies were used in these experiments. One had a nominal holding time of 21.5 s at a milk flow rate of 10 L/h, and the other had a holding time of 52.2 s at a milk flow rate of 20 L/h. Temperature was monitored using thermocouples at the following points on the pasteurizer: between the pump and the inlet to the pasteurizer; at the holding tube exit, and as the hot water entered the heating section of the pasteurizer. Pasteurization temperature was automatically controlled at the holding tube exit. The flow characteristics of the pasteurizer were established prior to use to determine the residence time distribution and the FMP times through the pasteurizer and the holding tubes, and are detailed in Tomasula and Kozempel (2004).

Milk Sample Preparation

Raw whole milk and low-fat (2%) milk were used in the experiments. Low-fat milk was prepared by centrifuging the raw milk using an Armfield FT 15-B disc bowl centrifuge (Armfield Inc.). Fat content was determined using the Babcock test (AOAC, 1998). Raw milk was pasteurized as is and was not standardized prior to pasteurization, as is the common procedure in the fluid milk industry. Feed volumes of milk in each experiment ranged from 500 mL to 1 L, depending on the amount of milk available for the experiments performed that day.

HTST Pasteurization

Prior to using the pasteurizer, the FMP time for both holding tubes was determined to ensure that the apparatus met the Pasteurized Milk Ordinance (FDA, 2003) standards for pasteurization of milk in flow apparatus. Tracer studies (Tomasula and Kozempel, 2004), to establish the time that each particle in the milk spent in the holding tubes, showed that the FMP was 18.6 s for the short holding tube and 36 s for the longer tube, whereas the nominal holding times were 21.5 and 52.2 s, respectively.

Experiments were performed in 2 separate visits to PIADC. In the first, only whole naturally infected milk was pasteurized according to experimental design I described below. In the second, both 2% and whole naturally infected milk were pasteurized according to experimental design II below.

Prior to each experiment, water was fed to the pasteurizer to eliminate temperature fluctuations caused

by heating and cooling of the equipment itself when bringing it from room temperature to experimental operating conditions. The pasteurizer was placed next to a biological hood. For each run, the feed vessel was removed from the pasteurizer, filled with up to 2 L of cheesecloth-filtered infected milk inside the biological safety cabinet, covered, and then mounted over the pump leading to the pasteurizer. The milk was allowed to flow into the pasteurizer when it was noted that the level of the hot water in the pump just disappeared. The cooled, pasteurized milk was pumped through a hose attached to the exit line of the cooler through a penetration in a wall into a second "clean" biological safety cabinet located in an adjacent laboratory to ensure that cross-contamination between the pasteurized product and the feed samples did not occur. The pasteurized samples were collected in sterile 50-mL tubes or 100-mL bottles kept on ice. Samples of the feed milk were also collected for analysis and placed on ice. Samples were then stored at 4°C for analysis the same or the next day.

Experimental Design I

The pH of the milk was measured prior to and after infection of the cows and prior to each pasteurization run. Because naturally infected milk was used, it was not possible to control the amount of virus in the milk prior to each pasteurization experiment. The milk samples for each run contained between 2 to 4 log₁₀ **TCID**₅₀ of infectious virus, where **TCID**₅₀ is the virus titer that will cause infection in 50% of cell culture wells. The effectiveness of pasteurization was tested using the Charm Luminator T (Charm Sciences Inc., Lawrence, MA), which detects the presence of alkaline phosphatase, with sensitivity to 0.005% raw milk.

A simple 2² experimental design was followed in the first visit to PIADC to define the temperature-time region where the virus may be inactivated in whole milk. Two levels of temperature, 72 and 80°C, and 2 levels of holding time, 18.6 s at a flow rate of milk of 10 L/h and 36 s at a flow rate of milk of 20 L/h were chosen. Duplicate trials were run so that 8 experiments were performed each day for 3 d.

Experimental Design II

A second series of experiments were performed to determine the temperature-time region for inactivation of the FMDV in whole milk compared with low-fat milk. The holding time in these experiments was fixed at 36 s. Pasteurization temperature was varied from 72 to 95°C.

Sanitation

The pasteurizer and hose leading to the biological safety cabinet in the second laboratory were sanitized prior to use, between runs, and after use. Prior to use, hot water at the temperature of the experiment was pumped through each section of the pasteurizer to equilibrate it. The pasteurizer was then rinsed with a 10% bleach-water solution for approximately 10 min and then flushed with hot water again for 20 min. All rinse solutions were collected in the second laboratory in a carboy filled with a 10% bleach solution. The biological safety cabinet and the body of the pasteurizer were sanitized using a 10% bleach solution followed by a 70% ethanol solution.

Detection of Virus in Milk

The raw and pasteurized milk samples were tested first for FMDV by cell culture. In this method, undiluted and 10-fold dilution samples of the milk were inoculated into monolayers of BHK-21 cells grown in 96-well culture plates, incubated for 3 d, and examined for cytopathic effect, which is indicative of viral replication. At the end of the 3-d period, the cells and the surrounding medium were frozen, thawed once, and inoculated onto another set of BHK plates for a second 3-d period. Samples from the first and second blind passages were tested for viral antigen by capture ELISA to confirm the identity of the virus causing cytopathic effect (House and House, 1989). Although cell culture is useful in enumerating viral plaques at high virus concentrations, it is not sensitive when virus titers are low. Other methods such as real-time reverse-transcription PCR are more sensitive than virus isolation but fail to distinguish viral RNA originating from infectious or inactivated virions (Callahan et al., 2002).

Therefore, we used steer inoculation because it is the most sensitive method described to detect infectious FMDV. Milk samples negative for virus isolation in cell culture were inoculated into a single steer as described in Blackwell and Hyde (1976). Two milliliters of the sample was inoculated intradermally into 20 sites in the tongue and 35 mL was inoculated intramuscularly in 4 sites in the gluteal muscles. The steer was observed for febrile response, presence of the virus in blood (viremia), and development of tongue or foot vesicles. If the animal did not show symptoms of FMDV, a second steer was inoculated with the same original sample that had been kept at -70°C to verify that the sample was free of FMDV.

For the second set of experiments, if pasteurized milk samples tested negative by tissue culture, 0.1 mL was inoculated by the intraperitoneal route into 10 suckling mice. Mice were observed for 1 wk for debility or death.

If the sample did not cause symptoms of FMDV in mice, it was then inoculated in a steer as described above.

RESULTS AND DISCUSSION

This study was conducted to determine whether the flow component of continuous HTST pasteurization, in addition to temperature-time parameters, would have an effect on the rate of inactivation of the virus in milk and to determine whether the temperature-time conditions used to pasteurize milk in continuous HTST equipment similar to that used by the dairy industry would be sufficient to eliminate FMDV in naturally infected milk.

The methodology used for detecting residual infectious virus in pasteurized milk samples is the most sensitive available. Although other methods to detect viral antigen (such as ELISA tests) or viral RNA (such as real time reverse-transcription PCR) exist, these methods fail to distinguish inactivated from infectious virus (Callahan et al., 2002). The FMDV serotype O (UK/2001) used in this study can also grow and kill suckling mice (House and House, 1989), but the most sensitive method is the inoculation of naïve steers. Therefore, to rule out the presence of even residual infectious virus, we used this technique, as previously described by Blackwell and Hyde (1976).

The temperature-time conditions used in the first phase of this study were chosen to match as closely as possible those used by the dairy industry—a minimum temperature of 72°C and a holding time of 15 s (18.6 s was used in this study, as discussed previously in Tomasula and Kozempel, 2004). These conditions (FDA, 2003) ensure the safety of the fluid milk supply while maintaining the quality and flavor attributes of milk (Holsinger et al., 1997). The pasteurization process is generally effective for reducing pathogen levels by 5 log₁₀. Because some milk-processing plants use higher temperatures, longer holding times, or both, the upper bound of the temperature-time conditions used in this phase of the study was 80°C, with a 36-s holding time. Even higher temperatures were used to pasteurize whole milk in the second phase of this study because the virus was not inactivated at the lower temperatures.

Whole Milk Pasteurization, Temperature ≤ 80°C

High-temperature, short-time pasteurization experiments conducted at temperatures of 72 and 80°C at holding times of either 18.6 or 36 s (experimental design I) were not effective for inactivating FMDV in naturally infected whole milk with a fat content of 6%. The fat content of whole milk used in this study was approximately twice that of commercial whole milk.

Six replicates of each temperature-time pair were measured (Table 1) for the pasteurization experiments. The infected whole milk had a pH of 7.1, compared with a pH of 6.7 prior to infection of the cow with the FMDV. Samples pasteurized at 72°C and 18.6 s, although negative by virus isolation in tissue culture, were positive by steer inoculation tests. (Only samples PA1 and PA2 were used.) Even in milk containing the lowest titer of virus [1.92×10^2 plaque-forming units (pfu)/mL], there was still enough residual viral infectivity to cause disease in inoculated steers. The reduction of the virus in whole milk as a result of HTST pasteurization ranged from 99.9 to 99.99% (3 to 4 log₁₀), respectively, as determined by tissue culture and depending on the initial titer of virus.

Whole milk samples pasteurized at 80°C and held for either 18.6 or 36 s were negative by cell culture as well. Of the 3 samples pasteurized at 80°C for 18.6 s and tested by steer inoculation, samples PG1 and PG2 (Table 1), containing raw milk titers of 9.25×10^3 and 1.92×10^2 pfu/mL, respectively, contained residual virus even though sample PG2 had a lower viral titer prior to pasteurization. Sample PB2, which had an initial viral titer of 1.92×10^2 pfu/mL and was also held for 18.6 s, tested negative in a first inoculation of one steer, but a second steer inoculated with additional sample to confirm this negative result tested positive for the virus.

Sample PC2, with an initial titer level of 1.92×10^2 pfu/mL, was pasteurized at 80°C for 36 s. It was not infectious when tested in one steer, but a similarly treated sample, PF2, although negative in a first inoculation, was infectious in a second steer inoculation.

We did not demonstrate here that the residual infectivity of whole milk was significantly reduced when temperature was increased from 72 to 80°C at a fixed holding time because the 2 samples, PA1 and PA2, were tested only once for residual infectivity by steer inoculations. However, for the samples with an initial titer level of 1.92×10^2 pfu/mL pasteurized at 80°C, the results suggested a reduction in infectivity as the holding time was increased from 18.6 to 36 s.

Hyde et al. (1975) and Blackwell and Hyde (1976) used a batch method to simulate HTST pasteurization of naturally infected whole milk. Hyde et al. (1975) showed that the virus was still present in milk (initial titer level of 6 log₁₀), as indicated by tissue culture, after heating at 72 or 80°C, with a 5 log₁₀ reduction in virus. These samples were infectious to steers even though they were evaporated at 65°C for 1 h to 50% of initial volume after pasteurization. Blackwell and Hyde (1976) heated naturally infected milk with initial titer levels of 5 to 6 log pfu/mL of virus at 72°C for up to 5 min. The samples were negative for the virus by tissue culture but all were infectious when inoculated into

Table 1. Inactivation of foot-and-mouth disease (FMD) virus in naturally infected whole milk by HTST pasteurization¹

Temperature (°C)	Sample	Holding time (s)	Titer level ² (pfu/mL)	Cell culture	Steer inoculation
73	PA1	18.6	9.25×10^3	—	+
72.6	PH1	18.6	9.25×10^3	—	ND
72.3	PA2	18.6	1.92×10^2	—	+
72.7	PH2	18.6	1.92×10^2	—	ND
72	PA3	18.6	2.20×10^3	—	ND
72.6	PH3	18.6	2.20×10^3	—	ND
80.6	PB1	18.6	9.25×10^3	—	ND
80.7	PG1	18.6	9.25×10^3	—	+
80.2	PB2	18.6	1.92×10^2	—	—, +
80.5	PG2	18.6	1.92×10^2	—	+
80.9	PB3	18.6	2.20×10^3	—	ND
80.5	PG3	18.6	2.20×10^3	—	ND
74.3	PD1	36	9.25×10^3	—	ND
74.8	PE1	36	9.25×10^3	—	ND
73.7	PD2	36	1.92×10^2	—	ND
74.1	PE2	36	1.92×10^2	—	ND
72	PD3	36	2.20×10^3	—	ND
72	PE3	36	2.20×10^3	—	ND
81.3	PC1	36	9.25×10^3	—	ND
82.7	PF1	36	9.25×10^3	—	ND
80.7	PC2	36	1.92×10^2	—	—, ND
82.5	PF2	36	1.92×10^2	—	—, +
80	PC3	36	2.20×10^3	—	ND
80	PF3	36	2.20×10^3	—	ND

¹ND = not done; — = negative reaction by cell culture; —* = steer did not develop FMD after inoculation with the sample; + = positive reaction by cell culture; +* = steer developed FMD after inoculation with the sample.

²pfu/mL = plaque-forming units/mL.

steers. They also did not observe a decrease in infectivity of whole milk when pasteurizing it at 72°C, holding it for 3 min, and then following pasteurization with evaporation under vacuum at 65°C for 1 h.

The results from this study indicate that the continuous HTST method used in this experiment may be more effective than the batch method used previously (Hyde et al., 1975; Blackwell and Hyde, 1976) for elimination of the virus in milk. In this study, steer inoculation results for whole milk pasteurized at 80°C and held for 18.6 s showed that 2 out of 3 steers tested were positive for the virus, whereas at 80°C and a holding time of 36 s, 1 steer out of 3 was positive. In the previous studies (Hyde et al., 1975; Blackwell and Hyde, 1976), all steers inoculated with samples of milk batch pasteurized at 80°C and held for 15 s tested positive for the virus; however, they used naturally infected milk with a much higher titer level.

Pasteurization of Whole Milk, Temperature > 80°C

Additional experiments were conducted to determine whether the FMDV was eliminated by HTST pasteurization of whole milk at temperatures ranging from 80 to 95°C and of milk with a 2% fat content at temperatures in the range from 72 to 95°C. The holding time

for these experiments was 36 s. The experimental pasteurization temperatures, prepasteurization treatment received, and titer levels of milk prior to and post pasteurization are shown in Table 2. Only the results for samples tested for residual infectivity using steer inoculation are shown.

Sample PK1 (Table 2) of whole milk (initial titer 3.1×10^4 pfu/mL), pasteurized at 95°C for 36 s, was negative for the virus by tissue culture. The sample was negative when inoculated into one steer but residual virus was indicated when a second inoculated steer developed FMD.

This result and the results obtained for whole milk pasteurized at 80°C (Table 1) are in contrast to the results obtained using the batch method. Blackwell and Hyde (1976) found that all samples of naturally infected whole milk (initial titer of $5.3 \log_{10}$) heated at 85°C and held for 15 s were infectious when inoculated into steers. This confirms that HTST pasteurization conducted in a flow pasteurizer may be more efficient in eliminating the virus in milk than the batch methods because of the additional flow component. When Cunliffe et al. (1979) treated naturally infected whole milk with titer levels ranging from 3.7 to 6.4 $\log_{10}$ to several UHT treatments for 2 to 5 s in a continuous flow apparatus, whole milk showed a drop in infectivity after treat-

Table 2. Effect of higher pasteurization temperatures on inactivation of foot-and-mouth disease (FMD) virus in naturally infected whole milk and in naturally infected 2% milk¹

Temperature (°C)	Sample	Fat content	Titer level ² (pfu/mL)	Cell culture	Steer inoculation
95	PK1	Whole	3.1×10^4	—	+, -*
95	PR1	2%	4.5×10^3	—	+, -*

¹- = negative reaction by cell culture; -* = steer did not develop FMD after inoculation with the sample; + = positive reaction by cell culture; +* = steer developed FMD after inoculation with the sample.

²pfu/mL = plaque-forming units/mL.

ments at 123 and 130°C (holding time of 2.3 s), respectively, when the ratio of (number of steers inoculated that developed FMD)/(total inoculated) dropped from 2/3 at 123°C, to 1/5 at 138°C, and to 0/2 at 148°C.

Pasteurization of Low-Fat Milk, Temperature = 95°C

Milk naturally infected with FMDV was centrifuged to reduce the fat content to 2% and then pasteurized using HTST pasteurization. Milk sample PR1 (Table 2), containing 2% fat with an initial titer level of virus of 4.5×10^3 after skimming, was pasteurized at 95°C and then held for 36 s. Although the sample was negative by cell culture, 1 out of the 2 steers that were inoculated tested positive for the virus.

Overall, the results of Tables 1 and 2 show that for whole or low-fat (2%) milk infected with FMDV, pasteurization temperatures >72°C removed all infectivity detectable in cell culture, indicating a reduction of at least 4 log₁₀ in virus infectivity. However, sufficient residual infectivity remained to cause FMDV when steers were inoculated with the infected samples. It was evident that flow HTST pasteurization enhanced virus inactivation relative to the batch methods that simulated HTST pasteurization but, as suggested by Hyde et al. (1975) and Blackwell and Hyde (1976), the virus may have been protected by the milk components, especially the milk fat.

Blackwell and Hyde (1976) observed a decrease in infectivity of skim milk naturally infected with FMDV (4.6 to 6 log₁₀) after heating at 72°C for 2 min. The residual fat content of the skim milk was not stated. Two out of 6 steers inoculated with the pasteurized sample developed FMD compared with 6/6 steers that developed FMD when inoculated with a sample of skim milk pasteurized at 72°C but held for <2 min. When pasteurization of skim milk at 72°C with a holding time of 15 s was followed by evaporation at 65°C for 1 h, the virus survived and only 1 out of 3 steers inoculated with the samples developed the virus. The virus was eliminated in milk when pasteurized at 72°C with a holding time of 30 s followed by evaporation. Blackwell and Hyde (1976) found that pasteurizing naturally in-

fectured skim milk (5 to 6 log₁₀) at 85°C for 15 s decreased infectivity of the milk even further. Only 1 out of 5 steers inoculated with the pasteurized skim milk sample developed FMD compared with 6 out of 6 developing FMD when inoculated with whole milk samples pasteurized under the same conditions.

Effect of Fat Content

The results of this study and those of Blackwell and Hyde (1976) show that the pasteurization process is most efficient for milk with reduced fat content and that increases in temperature or holding time are required to reduce the infectivity of the milk. Using electron microscopy, Blackwell et al. (1981) demonstrated that FMDV replicates in the mammary gland, and proposed that 3 virus-release mechanisms operate in infected mammary gland secretory cells. The virus that is incorporated in CN micelles and fat droplets is protected from environmental inactivants such as heat.

To inactivate the virus encapsulated in milk fat globules, the thermal process applied to the milk fat must equal that applied to the bulk of milk. Milk fat droplets or globules range in size from 0.1 to 10 µm (McPherson and Kitchen, 1983) in diameter and are surrounded by a thin milk fat globule membrane. For heat to penetrate the milk fat globule, it must first transfer from the bulk of the milk to the surface of the milk fat globule by convection. There may or may not be a resistance to heat transfer at the surface of the fat globule. The heat then transfers through the thickness of the milk fat membrane and to the center of the milk fat globule. During the heating process, the fat in the fat globule liquefies. Therefore, additional time may be required above that of the holding time alone for heat to penetrate the milk fat globule membrane and inactivate the virus.

Operating at an increased pasteurization temperature will increase the rate of heat transfer through the fat globule membrane, and increasing the holding time at a fixed pasteurization temperature may provide the additional time required for heat transfer through milk fat globules. For example, in this study, at 80°C for

pasteurization of whole milk, increasing the holding time from 18.6 to 36 s resulted in fewer animals developing FMD after inoculation with the pasteurized product. Also, Blackwell and Hyde (1976) reported that the virus was not inactivated in skim milk (which contains residual fat) pasteurized at 72°C and held for 15 s, but virus levels were reduced for skim milk held at the same temperature for 2 min.

In whole milk that is not homogenized, as in this study, the fat globules exist as aggregates, and thus should also require a longer heating period. We would then expect that the rate of inactivation of a virus in an oily medium is decreased relative to that of the virus in an aqueous environment, as shown in the earlier studies by Senhaji and Loncin (1977). They showed that the heat resistance of spores of *Bacillus subtilis* and vegetative cells of *Pseudomonas fluorescens* was greater in the presence of oil without added water.

Effects of pH

In early research, Bachrach (1961) and Bachrach et al. (1957) demonstrated the effects of pH and temperature on the FMDV (the virus was not suspended in milk). They found that heating the virus from 61 to 85°C resulted in the release of infectious RNA from the virus and that the virus was most stable between pH 7 and 7.5 (Bachrach et al., 1957). At pH <7, the virus dissociated into 12 pentamers, releasing viral RNA.

In this study, milk infected with FMDV had a pH of 7.1, compared with a pH of 6.7 prior to infection of the cows with FMDV. The increase in pH of milk from infected cows has been observed previously (Hyde et al., 1975). Sellers (1969), in experiments using milk inoculated with FMDV, showed that the time to reduce the viral titer upon pasteurization is reduced if the pH of the milk is 6.7 compared with a pH of 7.6. Sellers (1969) showed that at a temperature of 72°C, the time to inactivate FMDV to a level of 0.00001 in milk was 17 s and was 40 s at pH 7.0. This study and that of Blackwell and Hyde (1976), conducted under conditions of pH >7 where the virus was assumed to be stable (Bachrach et al., 1957), demonstrated that virus inactivation was more efficient under conditions of increased temperature or at a longer holding time.

Lethality of Pasteurization for FMD

Pasteurization is most effective against microorganisms that have *z*-values, where the *z*-value is defined as the temperature change needed to reduce the *D*-value 10-fold and the *D*-value is defined as the time in minutes to kill 90% of the organism, less than that of alkaline phosphatase (*z* = 4.8°C), and it reduces the load

of microorganisms by about 5 log₁₀, the level observed in our experiments and those of Blackwell et al. (1981). As pointed out by Tomasula and Konstance (2004), the *z*-value, calculated from the thermal death curve of Walker et al. (1984), is approximately 20°C. However, as discussed above, our studies showed a reduction of viral titer of 3 to 4 log₁₀ and previous studies have shown a reduction of viral titer up to 7 log₁₀ at *T* > 72°C and a holding time <20 s. This suggests that the *z*-value for the FMDV in milk is most likely closer to that of the value for alkaline phosphatase.

It is intuitive that the curve presented in Walker et al. (1984) represents not only the inactivation kinetics for the most heat-resistant fractions at the elevated pH of milk because of viral infection, but also the fraction that may be encapsulated by milk fat or bound to other milk components.

Recommendations

Processing plants in the United States typically use HTST pasteurization to process milk at temperatures in the range of 72 to 81°C at holding times up to 40 s. Ultrapasteurized milk, which is sold in aseptic packaging, is processed at temperatures ranging from 137 to 143°C with holding times of 2 to 3 s. This is in the range at which Walker et al. (1984) found complete destruction of the FMDV in whole and skim milk.

This study and those of Hyde et al. (1975) and Blackwell and Hyde (1976) are not entirely representative of commercial milk- and milk product-processing operations because the milk used in these experimental studies was not blended with noninfected milk, which would lower the pH of the milk, and was not subjected to all of the preprocessing steps and processing protocols used in commercial operation.

In the event of an outbreak of FMDV, before the animals exhibit clinical symptoms of the virus, milk transported to the processing plant would be pumped from the tanker truck [with capacities of up to 10,000 gal (37,850 L)] to holding tanks that may be as large as 50,000 gal (189,250 L), and would be diluted further assuming that no other milk is infected, diluting the virus and lowering the pH of the milk. Donaldson (1997), Hedger and Dawson (1970), and Sellers (1971) noted that the maximum titer of virus in the milk in farm bulk tanks on farms in England during the outbreak of FMDV was on the order of 2.5 log₁₀.

Prior to pasteurization, milk is clarified to remove debris. Blackwell and Hyde (1976) showed that the cellular debris removed from milk prior to separation is infectious to cattle and is a potential source of FMDV. (They noted that processing plants should include decontamination of this debris as part of their biosecurity

measures.) Milk is then preheated to 50°C and separated into the cream and skim milk fractions. In many plants, the cream is ultrapasteurized at 138°C for 2 s, which extends the shelf life of the cream. It is unknown at this time whether this temperature will eliminate the virus in cream, but it will reduce the virus titer significantly. Blackwell and Hyde (1976) pasteurized cream with initial titers of 1.8, 5.6, and 6.3 log₁₀, respectively, at 93°C for 16 s. All samples were negative by cell culture but when inoculated in steers caused FMD. In fluid milk plants, some of the cream is added back to the skim milk to standardize it to make milk products with various fat contents. Vitamins and flavors also are added. The skim milk alone or milk with varying percentages of fat is then pasteurized. The standardized milk stream is also homogenized prior to entering the regeneration section of the pasteurizer. Homogenization reduces the size of the fat globules and increases their surface area. This step alone should accelerate heating of the fat globules and facilitate virus inactivation.

For other dairy products, such as in cheese manufacture, the cheese milk for full-fat or low-fat cheese is pasteurized at 72°C for 15 s. The milk is cooled and then processed further through addition of cultures or acid to facilitate curd formation. Cheese manufacture was investigated by Blackwell (1976), who found that in Cheddar made from raw or subpasteurized milk, the FMDV survived at the final curd pH of 5.1. However, the FMDV did not survive Mozzarella manufacture. The survival of FMDV in other dairy products is described in Tomasula and Konstance (2004).

Guidelines from the OIE (2007) propose more aggressive mitigation procedures for HTST-treated milk to eliminate FMDV from the milk, such as HTST pasteurization applied twice or double pasteurization if pH >7, but this would be difficult for the dairy industry to implement. Double pasteurization, as defined above, implies 2 consecutive pasteurizations, which could be just as easily implemented by increasing the length of the holding tube or by reducing the flow rate of milk to the pasteurizer, or implemented most efficiently by increasing the pasteurization temperature. In the event of an outbreak, however, it is unlikely that the pH of milk in the bulk tank would exceed 7.0 because of mixing of infected milk in the bulk tank with noninfected milk. Additional recommendations by the OIE of milk for animal consumption, such as lowering the pH of milk to 6 for 1 h following HTST pasteurization, are difficult to implement practically.

Although we were able to detect residual infectivity in milk by directly inoculating relatively large volumes of milk intradermally and intramuscularly into steers, the ability of this milk to infect by the oral or other

routes is unlikely. Donaldson (1997) estimated that a single pig would have to ingest 125 to 1,250 L of pasteurized milk to have a high probability of consuming an infectious dose of virus, whereas a calf would have to consume 1,250 to 12,500 L. From the data obtained in this study, for HTST pasteurization conducted at 80°C and a 36-s holding time, 2 direct intramuscular inoculations of steers were required before infectivity in milk was detectable. It is unlikely that milk processed under these conditions poses a significant risk to animals. Infection of cattle is generally through the respiratory route by aerosolized virus, and infection through abrasions in the skin or mucous membranes can occur but is inefficient, requiring almost 10,000 times more virus (Donaldson, 1987).

A risk assessment conducted by the Animal Health and Plant Inspection Service (USDA/APHIS/Centers for Epidemiology and Animal Health/Center for National Animal Health Surveillance, personal communication, Aaron Scott) also concluded that the risk of infection from FMDV for either cattle or hogs is very low, if not impossible, from pasteurized milk or cheese. Calf intake of either milk or cream would need to be extraordinarily high to present even a one-in-a-million risk. A calf would need to drink 65.5 L of milk or 16.3 L of cream.

CONCLUSIONS

Foot-and-mouth disease is not a public health threat but is a threat to cloven-footed animals. It was the intent of this study to determine whether HTST pasteurization that simulated the commercial operation would be effective in eliminating FMD from naturally infected whole and 2% milk. High temperature, short time pasteurization is more efficient than batch pasteurization methods in eliminating the virus in whole and skim milk, but the results of this study largely corroborate those of Hyde et al. (1975) and Blackwell and Hyde (1976), who used batch processing techniques to simulate HTST pasteurization of milk.

Our work and that of Blackwell and Hyde (1976) indicates that the FMDV encapsulated by milk fat is resistant to pasteurization. Modern milk-processing plants with efficient skimming operations and homogenization as part of pasteurization may release the residual encapsulated virus, ensuring its destruction during pasteurization, or may reduce residual virus to much lower levels than observed in this and previous laboratory-scale studies.

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