Inactivation of foot and mouth disease virus in skimmed milk with propionic acid, citric acid and hydrogen peroxide

E. SONDER *, M. ACKERMANN **, K.C. McCULLOUGH * and U. KIHM *

Summary: In order to protect farm animals from infections such as foot and mouth disease (FMD) and tuberculosis, the pasteurisation of milk and milk products designated for the feeding of animals is compulsory in Switzerland. Nowadays, milk products are often treated chemically with acids or with hydrogen peroxide in order to keep bacterial contamination low. The capacity of these chemical treatments to inactivate FMD virus in skimmed milk within 6 h at 5°C was tested in this study.

The results indicated that the addition of 0.1%-0.3% of consumable acids, such as citric acid or propionic acid, could not guarantee the complete inactivation of FMD virus in skimmed milk. Similar results were obtained both with FMD virus deliberately added to skimmed milk and with skimmed milk obtained from naturally infected cows. Hydrogen peroxide in concentrations of 0.1%-0.3% was also an ineffective means of controlling the risk of FMD virus transmission from contaminated milk.

KEYWORDS: Citric acid - Foot and mouth disease virus - Hydrogen peroxide - Inactivation - Propionic acid - Skimmed milk.

INTRODUCTION

Foot and mouth disease (FMD) is an economically significant, highly contagious, viral disease of cloven-hoofed animals. The causative agent, FMD virus, is a member of the family Picornaviridae. FMD is controlled by slaughter and/or vaccination. In enzootic areas such as Africa, Asia and South America, the vaccination option is favoured. At present, most European countries also vaccinate their national cattle herds, even in the absence of the disease, in order to develop a reliable immune barrier against FMD. In Switzerland, an annual vaccination campaign is performed between February and May (1, 21). All cattle aged six weeks and over are vaccinated against the serotypes O, A and C of FMD virus.

Since FMD virus may be excreted in milk before clinical signs of the disease are observed in the infected animal (22), Swiss regulations (Art. 22.5 TSV or Epizootics Decree of 15 December 1967) require that milk products which are designated as

* Institut für Viruskrankheiten und Immunprophylaxe, Hagenaustrasse 74, 4056 Basel, Switzerland.
** Institut für Virologie der Universität Zürich, Winterthurerstrasse 266a, 8057 Zürich, Switzerland.
animal feed should be pasteurised before transportation from the creamery to the farm. During the last major epizootics of FMD in Switzerland (1966 and 1968), milk and milk products played an important role in the distribution of the infection among pigs and cattle, and measures for inactivating the virus in milk were therefore important (Nabholz, personal communication).

Nowadays, milk products are often treated chemically with acids or with hydrogen peroxide in order to keep bacterial contamination low (16, 19). It is well-known that chemical inactivation of biological agents depends highly on the final concentration of the chemical compound, temperature, time of incubation, protein concentration, molarity of salts and also on factors that are not well-defined.

In Switzerland, skimmed milk is frequently used for feeding pigs. For this purpose, fresh milk is centrifuged at the creamery and the skimmed milk (as it is called now), containing less than 0.5% milk fat (Art. 74 LMV or Food Products Decree of 26 May 1936) is cooled to 5°C. Then it is loaded into the tankers, where acids (propionic acid or citric acid), or hydrogen peroxide, are added to a final concentration of 0.1%-0.3% (16 and Jost, personal communication). At least 6 h will elapse after the addition of the chemicals before the skimmed milk is fed to pigs (Jost, personal communication). The cooling conditions are preserved throughout transportation and during the storage of the skimmed milk.

Since these treatments are done with permissible food additives and are, furthermore, much easier to perform, less time consuming and less expensive than pasteurisation, it was important to test their potential for inactivating FMD virus in skimmed milk. In contrast to other picornaviruses, such as the enteroviruses, FMD virus particles can be cleaved into non-infectious 12S protein subunits at pH levels below 7 (7); hydrogen peroxide, on the other hand, is well-documented as an inactivating substance of several infectious agents (19). Nevertheless, little is known about the pH requirements or potential of hydrogen peroxide to inactivate FMD virus secreted into milk or added to milk by contamination.

In this paper, the authors show that neither acid (propionic acid, citric acid), nor hydrogen peroxide treatment under standard conditions (0.1%-0.3%; 6 h at 5°C) was capable of completely and consistently inactivating FMD virus in skimmed milk.

MATERIALS AND METHODS

Viral strains and cell cultures

FMD virus strain O1 Lausanne (isolated in 1965) was adapted to cell culture by serial passages on BHK-21/C13 cells. The cells were propagated in Eagle’s Minimum Essential Medium, buffered with 0.25% bicarbonate and 30 mM HEPES, and contained 5% (v/v) fetal calf serum (growth medium). Wild type virus was propagated in animals by the intradermolingual inoculation of FMD-negative cattle. Virus containing tissues could be stored at -40°C for several years.

Infection of cattle with FMD virus

Unvaccinated cattle, including two dairy cows (all animals free of FMD virus-neutralising antibodies), were imported from Denmark. Two of these animals were
subsequently inoculated (intradermolingual route) with approximately $10^4$ ID$_{50}$ of FMD virus O1 Lausanne and kept between the two lactating dairy cows.

**Preparation of skimmed milk**

Milk solids comprise approximately 8% of the mass of skimmed milk. The approximate composition of these solids is 56% lactose, 36% milk proteins, 0.5% lipids and 7% other solids (16). Skimmed milk was purchased from various sources on different occasions in order to test the properties of the chemical agents in different batches of the product. Furthermore, in the mornings and in the evenings milk was collected from each of the two cows separately. For this, a milking machine was used.

The fresh milk was heated to 37°C and centrifuged manually at 6,000 rpm (manually operated centrifuge), in order to prepare skimmed milk. FMD virus contaminated skimmed milk was prepared by adding serial dilutions of either cell culture adapted FMD virus or wild type FMD virus to the skimmed milk. The mixtures were vortexed vigorously before the chemical treatment.

**Chemical treatment of skimmed milk**

Citric acid (98.5%) and hydrogen peroxide (35%) were obtained, as were propionic acid (> 99%), fumaric acid (> 99%), acetic acid (> 99%), sulphuric acid (95%-97%), hydrochloric acid (37%) and sodium hydroxide pellets (> 99%). The chemicals were added directly to skimmed milk which either had been contaminated with FMD virus or was virus-free and controlled in order to have final concentrations of 0.1%-0.3%, unless otherwise specified. The mixture was vortexed vigorously before being incubated at 5°C for 6 h, or for the time periods indicated in the Results section. In order to neutralise the acids prior to the inoculation of cell cultures, equal amounts of 1M Tris HCl, pH 7, and 1M NaOH were added until the original pH value (6.8) of skimmed milk was restored. Hydrogen peroxide containing mixtures were diluted 10-fold (for skimmed milk containing 0.1%-0.5% hydrogen peroxide) or 100-fold (for skimmed milk containing 1%-5% hydrogen peroxide) with cell culture medium before inoculating cell cultures.

**Determination of pH and detection of hydrogen peroxide**

The pH was determined by using a pH-meter [Metrom pH-meter 604, electrode No. 6.0212.000 (7.0 Ag; Ag 3m; 200 Mega OHM), 3M KCl electrolyte]. Hydrogen peroxide was traced using Perid-Teststicks.

**Isolation of FMD virus from skimmed milk**

Both before and after chemical treatment, 10 ml samples of virus contaminated skimmed milk were taken and were used to overlay separate, semi-confluent (70%-80%) monolayers of BHK cells in 25 cm$^2$ plastic tissue culture flasks. After one hour for virus adsorption, the inoculum was discarded and the cells were washed twice with phosphate-buffered saline (PBS) before being overlayed with medium. The monolayers were observed daily for cytopathic effects (CPE) due to FMD virus replication. When CPE was observed in > 90% of the monolayer, the infected cell cultures were frozen and kept at -70°C until the tests for identification of the isolated virus were performed. When no CPE was observed after three or four days of incubation at 37°C the cells, plus the supernatant, were given two cycles of freezing and thawing before fresh cells were inoculated. Such blind passage was repeated, if necessary, for up to three passages.
Identification of virus isolates

Virus isolates were identified as FMD virus serotype O by checkerboard neutralisation using monospecific sera and by complement fixation according to standard methods (20).

Detection of antibodies to FMD virus by ELISA

Antibodies to FMD virus in skimmed milk were measured using the sandwich ELISA (9, 10).

ELISA plates were coated overnight at 5°C with rabbit anti-FMDV serotype O antibodies at a dilution of 1:5000 in 0.1 M bicarbonate buffer (pH 9.6). The ELISA plates were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBS-Tween), before these antibodies were saturated with FMD virus particles (O\textsubscript{1} Lausanne; diluted in PBS-Tween) for 1 h at 37°C before the samples of skimmed milk were added to the wells. After incubation at 37°C for 1 h, the ELISA plates were washed with PBS-Tween and incubated for 1 h with peroxidase conjugated antiovine antibodies at a dilution of 1:200. After a further washing cycle, the substrate (ABTS and hydrogen peroxide) was added. After 30 min of incubation at room temperature, the absorbance was read at 405 nm (A405) using an Anthos Photo-meter. The values were transmitted on line to an NCR PC 8, where relative values were calculated (percentage reaction in comparison to positive reference serum).

RESULTS

PROPERTIES OF ACIDS AND HYDROGEN PEROXIDE IN SKIMMED MILK

In preliminary experiments, the chemical characteristics of the compounds designated as inactivators of FMD virus were tested in skimmed milk and in protein-free, aqueous solutions. In addition to varying quality and pH values of milk purchased on various occasions from various sources, striking differences between the two systems (aqueous versus milk) were observed:

- Addition of 0.1%-0.3% propionic acid or citric acid to aqueous solutions decreased the pH to 2.5, whereas addition to skimmed milk reduced the pH to between 6.14 (with 0.1% acid) and 4.62 (with 0.3% acid). Although citric acid is tri-acidic whilst propionic acid is mono-acidic, no differences in their behaviour were observed at these low concentrations. In contrast, at higher concentrations of acid (0.3%-1%), the pH change was greater with citric acid.

- The hydrolysation of hydrogen peroxide was slow in skimmed milk. At levels between 0.1% and 0.3%, the hydrogen peroxide was only barely detectable after 24 h. With 1% of hydrogen peroxide the active levels were only slightly reduced after 24 h, but undetectable after 48 h. Increasing the concentration of hydrogen peroxide added to skimmed milk to 3% resulted in only a slight reduction in activity over a three-day period. The decay of hydrogen peroxide in otherwise untreated skimmed milk occurred faster and more efficiently than in the same batch of milk which had been treated with ultra-high temperatures before the addition of hydrogen peroxide.
INACTIVATION OF FMD VIRUS IN CONTAMINATED SKIMMED MILK

In this series of experiments, skimmed milk was contaminated with FMD virus before the addition of various concentrations of different chemical compounds. After incubation at 5°C for either the standard minimal time of 6 h used commercially, or for prolonged periods, the skimmed milk was assayed for residual FMD virus infectivity by the inoculation of cell cultures. Essentially the same results were found with cell culture adapted FMD virus and with FMD virus obtained from vesicular lesions of infected animals.

In order to determine the efficacy of inactivation of FMD virus by the various agents, the contaminated skimmed milk was treated with:

a) between 0.1% and 0.3% propionic or citric acid;

b) between 0.1% and 0.3% hydrochloric, acetic, fumaric or sulphuric acid, or between 0.1% and 4% sodium hydroxide;

c) between 0.1% and 3% hydrogen peroxide;

d) combinations of between 0.1% and 0.3% acid with 0.1% to 0.3% hydrogen peroxide.

Effect of 0.1%-0.3% acid within 6 h

Figure 1 demonstrates that neither propionic acid nor citric acid at concentrations between 0.1% and 0.3% consistently inactivated all of the contaminating FMD virus within a period of 6 h at 5°C.

The pH values of the various samples of skimmed milk used in these experiments varied considerably both before and after the addition of acids. Figure 1 illustrates the correlation between the final concentration of acid, the observed pH values and residual titer of FMD virus obtained from different experiments using different batches of skimmed milk which had been kept in standard incubation conditions with propionic acid and citric acid.

The results indicate that the addition of acid to 0.1% affected only marginally the pH values and the final virus titers in treated skimmed milk. The addition of acid to 0.2% had a more marked effect on both parameters; with propionic acid, complete virus inactivation was achieved in 9 out of 16 experiments (56%), whereas in the remaining 7 experiments residual titers of up to 10^4 TCID_{50} of FMD virus per 10 ml of skimmed milk could be detected; with citric acid, complete virus inactivation was achieved in 10 out of 17 experiments (59%), whilst residual virus titers of up to 10^3 TCID_{50} per 10 ml were found in the remaining experiments. Upon addition of the acid to 0.3%, the pH values were further lowered, more markedly with citric acid than with propionic acid. Yet, while 0.3% of propionic acid led to apparently complete inactivation of FMD virus in 12 of 15 experiments (80%), complete inactivation with 0.3% of citric acid was observed in only 11 of 17 experiments (65%). Residual titers of FMD virus of 10^2 TCID_{50} with propionic acid and 10^3 TCID_{50} with citric acid were detected per 10 ml of skimmed milk in the experiments with incomplete inactivation.
Fig. 1a: Effect of 0.1%-0.3% propionic acid

Effect of incubating skimmed milk for 6 h at 5°C with various concentrations of propionic acid

Fig. 1b: Effect of 0.1%-0.3% citric acid

Effect of incubating skimmed milk for 6 h at 5°C with various concentrations of citric acid

Fig. 1

FMD virus inactivating effect of 0.1%-0.3% acid in contaminated skimmed milk within a period of 6 h at 5°C

FMD virus titers in skimmed milk are shown in relation to the pH values following the addition of various amounts of organic acids. Each symbol refers to a single experiment. Repeated experiments using the same experimental conditions are encircled.
Kinetics of FMD virus inactivation in skimmed milk following the addition of acids

In order to determine if inactivation of FMD virus in skimmed milk with propionic or citric acid occurred as a linear function of time, virus contaminated skimmed milk was analysed for the presence of infectious FMD virus at different times after acidification, and compared with untreated contaminated skimmed milk. The results (Fig. 2) show clearly that, under the applied conditions, FMD virus inactivation was not linear and even after two days of treatment, residual virus infectivity remained. Similar results were obtained with both propionic and citric acid.

![Log virus titer vs. hours of incubation for untreated control, 0.1% citric acid, and 0.2% citric acid](image)

**FIG. 2**

Kinetics of FMD virus inactivation in skimmed milk following the addition of citric acid

FMD virus titers in skimmed milk are shown in relation to time following the addition of citric acid in the concentrations indicated on the right of the figure.

Effect of extreme pH conditions

In order to explore more extreme pH conditions and, in addition, the effect of inorganic compounds, FMD virus contaminated skimmed milk was treated with 0.1%-0.3% of hydrochloric acid, acetic acid, fumaric acid, sulphuric acid and sodium hydroxide before being tested for residual FMD virus by the inoculation of cell cultures.
The results, summarised in Table I, demonstrate that complete inactivation of FMD virus in skimmed milk could be obtained, but that the more common situation was that a proportion of the virus remained infectious for at least 6 h under these extreme pH conditions.

**TABLE I**

<table>
<thead>
<tr>
<th>Acid</th>
<th>Incomplete inactivation due to insufficient acidification</th>
<th>Complete inactivation</th>
<th>Incomplete inactivation due to flocculation of milk proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic acid</td>
<td>5.54-6.13</td>
<td>5.16-5.60</td>
<td>5.16-5.37</td>
</tr>
<tr>
<td>Citric acid</td>
<td>5.44-6.14</td>
<td>4.62-5.76</td>
<td>4.88-5.24</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>6.10</td>
<td>-</td>
<td>5.10-5.42</td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td>-</td>
<td>5.23</td>
<td>1.97-5.12</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>5.84-6.33</td>
<td>5.29</td>
<td>5.16-5.59</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>5.81</td>
<td>5.46</td>
<td>4.82-5.29</td>
</tr>
</tbody>
</table>

The acids were added to skimmed milk in concentrations of 0.1%-0.3%. The numbers in the Table represent ranges of pH values. — = no results obtained under the applied conditions.

At pH values above 5.5, no reliable virus inactivation could be observed. When the pH fell below 5.2 the virus was apparently protected by precipitating milk components. It could still be detected after 6 h at pH 1.97 (0.3% sulphuric acid) and only a very narrow pH range allowed complete inactivation of FMD virus.

With the addition of between 1% and 4% (v/v) 1M sodium hydroxide, the pH values ranged from 7.39 up to 10.51, but the inactivation of the FMD virus in the skimmed milk again was incomplete. In contrast, when the skimmed milk was treated with 1% (w/v) (pH 12.88) and 2% (w/v) (pH 13.78) sodium hydroxide pellets, virus infectivity was no longer detected in inoculated cell cultures. However, the milk was completely unfit for consumption.

**Effect of 0.1%-0.5% hydrogen peroxide within 6 h**

No reduction of the FMD virus titer could be detected at 6 h after the addition of 0.1%-0.5% hydrogen peroxide to contaminated skimmed milk. Furthermore, no synergistic effect between the addition of 0.1%-0.3% acids and 0.1%-0.3% hydrogen peroxide was observed (data not shown).

**Kinetics of FMD virus inactivation in skimmed milk following the addition of hydrogen peroxide**

In order to determine if inactivation of FMD virus in skimmed milk with hydrogen peroxide was possible at all and, if so, whether it occurred as a linear function of time or not, contaminated skimmed milk was mixed with various concentrations of \( \text{H}_2\text{O}_2 \) (0.1% to 3%) and samples were tested for the presence of infectious FMD virus at various times thereafter.
The results (Fig. 3) show clearly that FMD virus was inactivated following the addition of hydrogen peroxide. At low concentrations (0.1%-0.5%), the first indications of virus inactivation were observed only after a minimum of 48 h had elapsed. The inactivation capacity increased at higher concentrations of hydrogen peroxide. At levels between 1% and 3% (v/v) of H₂O₂, a decrease in virus infectivity could be seen as early as 30 min after the addition of the chemical.

Nevertheless, under the current test conditions, FMD virus inactivation was not linear and complete inactivation was not possible, even after two days of incubation with the hydrogen peroxide. In fact, with the highest concentrations of hydrogen peroxide employed, maximum inactivation was found after only 3 1/2 h, but thereafter a residual quantity of what was apparently resistant virus infectivity remained.
INACTIVATION OF FMD VIRUS IN SKIMMED MILK OBTAINED FROM FMD VIRUS-INFECTED COWS

In order to test the potential of chemical inactivation of FMD virus in skimmed milk obtained from naturally infected cattle milk was collected from two cows, kept in close contact to FMD virus infected animals. Skimmed milk was assayed for FMD virus and, when positive, separate samples were mixed with either 0.2% or 0.3% propionic acid before the inoculation of cell cultures for the detection of residual infectivity.

Course of FMD virus infection in contact-infected cows

Two dairy cows which had no previous contact with FMD viral antigens were kept between two cattle which had been infected intradermalingually with FMD virus. Five days after the infection of the contact animals, both the dairy cows developed a rise in body temperature (Fig. 4). Clinical signs of FMD were observed only after the virus was detectable in the skimmed milk, i.e. between one and two days after the onset of fever. With both cows, antibodies to FMD virus were detected in the skimmed milk, beginning on the third day after the onset of clinical signs of FMD. The titer of FMD virus in skimmed milk decreased following the increase of antibody titers. Nevertheless, FMD virus could be isolated from skimmed milk as late as eight days after the appearance of these antibodies. The infectivity of this virus was not lost, even after repeated freezing and thawing of the milk samples (data not shown).

Detection of FMD virus in skimmed milk obtained from infected cows despite treatment with 0.2% propionic acid

FMD virus positive skimmed milk, obtained from the naturally infected cows on several occasions, was treated with 0.2% propionic acid for 6 h at 5°C before the inoculation of cell cultures.

Up to 10 TCID$_{50}$ of FMD virus per 10 ml skimmed milk remained infective in 8 of 9 assays with skimmed milk containing peak levels of FMD virus. In only one assay was infective FMD virus no longer detected following the acidification. The virus titer in the corresponding, non-acidified control skimmed milks ranged between $10^3$ and $10^5$ TCID$_{50}$ per 10 ml. No residual virus was detected after acidification of skimmed milk containing only trace amounts of FMD virus.

Following the addition of 0.3% propionic acid to skimmed milk containing FMD virus, the milk proteins precipitated immediately. Therefore, these samples were not tested further.

DISCUSSION

For economic reasons, FMD is considered most important among the viral infections of veterinary concern. In Switzerland, pigs are highly susceptible to FMD virus infection, since they are usually not vaccinated. Although most cattle are vaccinated annually against serotypes O, A and C during a national vaccination
FIG. 4

Course of FMD virus infection in contact-infected cows

Observations on the progression of foot and mouth disease in two contact-infected dairy cattle (4a: animal 1; 4b: animal 2). The parameters analysed were:
- rectal temperature in °C
- anti-FMD virus antibody levels in the skimmed milk measured using a sandwich ELISA and recorded as a percentage of the value obtained using a positive reference serum
- infectious FMD virus in the skimmed milk measured by inoculating BHK cells (histogram). The numbers above the histograms indicate the titre of FMD virus in 0.1 ml skimmed milk, when such a titre could be found. Where the histogram is marked with a "+", this indicates that the samples contained FMD virus which could be detected only when the BHK cell monolayers were inoculated with 10 ml of skimmed milk. The arrows indicate the time point at which the first clinical signs of FMD were observed.
campaign between February and May (21), calves younger than six weeks at this time of year will not be vaccinated until the following year.

Transmission of the disease occurs by direct and indirect contact of susceptible animals as well as by inanimate vectors, such as meat and milk obtained from infected animals (15). Consequently, the role of meat, meat products, milk, and milk products in the spread of FMD has been studied thoroughly (4, 22). FMD virus may be present in the milk of infected cows before clinical symptoms of the disease are observed (22). In addition, FMD virus in milk seems to be remarkably resistant to inactivation (22), considering the otherwise high degree of chemical and thermal sensitivity of the virus.

With pasteurisation of milk, a broad range of micro-organisms, including the agents of foot and mouth disease, tuberculosis and brucellosis, may be inactivated (6, 11, 14). The importance of inactivating infectious agents in milk (e.g. by pasteurisation) has been shown under field conditions in connection with periods of FMD (Nabholz, personal communication).

In order to protect the susceptible animals from infection by FMD virus which may be present in either whole milk or skimmed milk, the pasteurisation of milk and milk products destined for feeding purposes is compulsory in Switzerland (Art. 22.5 TSV). However, heating installations for pasteurisation are required. Furthermore, this and the associated processes are both time and labour intensive. In contrast, chemical inactivation of micro-organisms can be achieved by relatively inexpensive methods, e.g. routinely consumable acids or hydrogen peroxide are added to transported skimmed milk (16, 19).

Since it is well-known that FMD virus is susceptible to chemical inactivation (8, 12, 18, 23), it was important to test if the chemical methods routinely used for conservation of skimmed milk were reliable for inactivation of FMD virus. The direct comparison of pasteurisation and chemical treatment, however, was excluded from this study because it was not our intention to suggest the replacement of a valuable field method by a more or less promising laboratory method.

In a first series of experiments, the properties of various acids and of hydrogen peroxide in skimmed milk were tested.

As expected, the acids led to a less pronounced drop of pH value in skimmed milk than in aqueous, protein-free solutions. Interestingly, the final pH values after the addition of constant amounts of acid varied with skimmed milks obtained from different sources and on different occasions. Furthermore, the pH value where the precipitation of milk proteins was initiated also varied considerably.

Similarly, the properties of hydrogen peroxide in skimmed milk were difficult to standardise. Hydrogen peroxide was surprisingly stable in both pasteurised and ultra-high temperature treated milk. It was hydrolysed more easily in raw skimmed milk. This is probably due to the viable enzymes (peroxidases) which are apparently present in skimmed milk (19).

In a second series of experiments, skimmed milk was contaminated with FMD virus and the inactivating effects of various acids and hydrogen peroxide were tested. According to the literature (16 and Jost, personal communication), consumable acids or hydrogen peroxide are added at levels between 0.1% and 0.3% (v/v) to skimmed milk in the tanks of lorries. At least 6 h incubation with the chemicals should be allowed before the treated milk is fed to animals.
It is well-known that FMD virus is inactivated at high as well as at low pH levels (4), and the capacity of hydrogen peroxide to inactivate other infectious agents, including both viruses and bacteria, is broadly accepted (19). With contaminated skimmed milk, however, several problems were obvious:

- With only a narrow range of pH (between pH 5.2 and 5.5), and then only in certain experiments, was complete inactivation of the contaminating FMD virus possible. At pH values greater than pH 5.5, a large proportion of virus infectivity remained. Depending on the batch of skimmed milk, precipitation of milk proteins started to occur at pH levels below pH 5.5. As a consequence, FMD virus inactivation was less efficient than would have been expected from results obtained in aqueous solutions. Below pH 5.2, the precipitation of milk proteins was always observed. In contrast to others (2, 24, 25), who reported a higher efficiency of the acids at elevated temperatures, the authors observed that the milk protein precipitated, and thus "protection" of the virus against acidification occurred at higher pH values when the skimmed milk was not sufficiently cooled (data not shown).

- The protection of the FMD virus by the acid-precipitated milk proteins was very efficient: even after 6 h of incubation at pH 1.97, residual infectivity was observed. If protected by abundant amounts of non-viral proteins, FMD virus is known to survive harsh conditions for long periods of time; e.g. residual FMD virus infectivity is a major problem in formaldehyde-inactivated FMD vaccines which have been prepared from epithelial tissues of infected bovines according to the Waldmann method (13).

- The inactivation rate of FMD virus in skimmed milk after acidification was apparently non-linear. Thus, in contrast to vaccine production, where linear inactivation of FMD virus is achieved by binary ethylenimines (17), it is not possible to calculate the required amount of acid to be added and the time of incubation necessary for complete inactivation of FMD virus in skimmed milk.

- The addition of 0.1%-0.3% (v/v) of hydrogen peroxide to contaminated milk had absolutely no effect on the survival of FMD virus within the standard "inactivation" period of 6 h. In contrast, after 48 h incubation with 0.5% hydrogen peroxide, or 72 h with 0.2% hydrogen peroxide (data not shown), a dose-dependent inactivation of FMD virus was observed. Furthermore, high concentrations of hydrogen peroxide (1%-3% v/v) reduced the infectivity of FMD virus in skimmed milk by 99% (1% hydrogen peroxide) or 99.999% (3% hydrogen peroxide) within 4 to 5 h of incubation.

Nevertheless, residual infectivity measuring between 10 and 10^4 TCID\textsubscript{50} per 10 ml remained. The inactivating effect could be attributed to the hydrolysation of hydrogen peroxide to H\textsubscript{2}O and O\textsubscript{2}, since no effect could be observed with hydrogen peroxide solution which had been hydrolysed prior to the experiment (data not shown).

Although a number of infectious agents can be inactivated by hydrogen peroxide (19) and although it is added to skimmed milk as a food preserving agent at concentrations of 0.1%-0.3%, it is a totally ineffective means of controlling the transmission of FMD virus.

- It could be conceived that the viral RNA would become more exposed to the action of hydrogen peroxide following the acid treatment. However, no additional effects were observed when acids and hydrogen peroxide were used simultaneously.
Unlike others (7), the authors therefore speculate that it is not the viral RNA which represents remaining infectivity following the chemical inactivation of FMD virus with acids.

The effects of inorganic acids and sodium hydroxide were tested for scientific reasons only. In contrast to organic acids, such as citric acid or propionic acid, which may act simultaneously as preservatives and taste enhancers, it is not desirable to have inorganic acids in food products. Although they could be neutralised, one might expect problems with contaminating metals, such as arsenic.

After the addition of sodium hydroxide, the milk was no longer suitable for consumption. Nevertheless, it is worth remarking that contaminating FMD virus remained infectious in skimmed milk for more than 6 h at pH 10.51, whereas it was apparently inactivated at pH levels above 12.88.

In a third series of experiments, skimmed milk obtained from cows which had been naturally infected with FMD virus was used. As expected, the virus was detected in the milk before clinical signs of FMD were observed. Although the milk appeared grossly unaffected during the excretion of FMD virus, slightly increased pH values were observed. Interestingly, in the skimmed milk obtained from cow 1, the highest values (pH 7.11) coincided with the highest titers of FMD virus in the milk. Antibodies to FMD virus were first detected in the skimmed milk three days after the onset of clinical signs of the disease. An apparent decrease in the virus titer was noted in the skimmed milk following the appearance of antibodies. Nevertheless, infectious FMD virus was still detected eight days later, when neutralising antibodies in the serum exceeded titers of 1:3000.

At first sight, the titers of excreted FMD virus in skimmed milk appear quite low compared to published data (3). However, it should be remembered that the virus would also have been associated with the milk components removed from the skimmed milk; relatively high titers of FMD virus were found in association with both milk sediments and milk fat after centrifugation (3).

In conclusion, neither acidification nor hydrogen peroxide treatment was reliable for the inactivation of FMD virus in skimmed milk. Either the virus was incompletely inactivated or the consistency of the milk was destroyed, making it unfit for consumption. Although current methods used commercially for the chemical preservation of milk might reduce the efficiency of transmission of FMD, they are not reliable as inactivators of FMD virus. One might speculate that under these conditions the acidification of the skimmed milk would be sufficient to block transmission of FMD. However, sooner or later, the worst possible set of circumstances could occur and FMD would be transmitted (5).

ACKNOWLEDGMENTS

This work was funded by grants from the Zentralverband Schweizerischer Milchproduzenten (ZVSM) and by the Bundesamt für Veterinärwesen. The authors acknowledge with thanks the helpful comments of Dr M. Jost, Grangeneuve, concerning the use of consumable additives to skimmed milk. We thank Prof. Dr Nabholz (Director of the Federal Veterinary Services from 1967 to 1977) for the communication of unpublished observations concerning the role of skimmed milk in the transmission of FMD virus. Furthermore, we thank L. Meyer, F. Perlini and
G. Meier for taking care of the experimental animals, R. Tschudin and M. Farkas for the supply of cell cultures and media, and S. Grüninger, W. Fraefel, and R. Schaffner for their technical assistance.

INACTIVATION DU VIRUS DE LA FIÈVRE APHTEUSE DANS LE LAIT ÉCRÉMÉ PAR L’ACIDE PROPIONIQUE, L’ACIDE CITRIQUE ET LE PEROXYDE D’HYDROGÈNE.

Résumé : Afin de protéger les animaux d’élevage des infections telles que la fièvre aphteuse et la tuberculose, la Suisse a rendu obligatoire la pasteurisation du lait et des produits laitiers destinés à l’alimentation des animaux. Les produits laitiers font aujourd’hui fréquemment l’objet d’un traitement chimique par des acides ou du peroxyde d’hydrogène pour limiter la faible contamination bactérienne. Dans le présent travail, la capacité de ces traitements chimiques à détruire le virus aphteux dans le lait écrémé a été étudiée sur six heures à 5°C.

Les résultats ont montré que l’addition de 0,1 à 0,3 % d’acides propres à la consommation humaine tels que l’acide citrique ou l’acide propionique ne peut garantir l’inactivation totale du virus aphteux dans le lait écrémé. Des résultats similaires ont été obtenus avec du lait écrémé auquel des virus aphteux avaient été ajoutés ainsi qu’avec du lait écrémé provenant de vaches naturellement infectées. A des concentrations de 0,1 à 0,3 %, le peroxyde d’hydrogène s’est également révélé inefficace pour supprimer les risques de transmission du virus aphteux par le lait contaminé.


Resumen: Para proteger el ganado de infecciones tales como la fiebre aftosa y la tuberculosis, Suiza ha establecido la pasteurización obligatoria de la leche y sus subproductos destinados a la alimentación de animales. Los productos lácteos suelen ser en la actualidad objeto de tratamientos químicos con ácidos o peróxido de hidrógeno para limitar la contaminación bacteriana a un bajo nivel. En este trabajo se ha estudiado la capacidad de estos tratamientos químicos para destruir el virus aftoso en leche descremada durante seis horas a 5°C.

Los resultados obtenidos mostraron que la adición de 0,1% a 0,3% de ácidos como el ácido cítrico o el ácido propiónico no puede garantizar la inactivación total del virus aftoso en la leche descremada. Se obtuvieron resultados similares con leche descremada a la que se habían agregado virus aftosos y con leche descremada proveniente de vacas infectadas naturalmente. A concentraciones
de 0,1% a 0,3%, el peróxido de hidrógeno se mostró asimismo ineficaz para suprimir los riesgos de transmisión del virus aftoso por leche contaminada.

PALABRAS CLAVE: Ácido cítrico - Ácido propiónico - Inactivación - Leche descremada - Peróxido de hidrógeno - Virus aftoso.

** REFERENCES **


