The Survival of *Mycoplasma bovis* at Different Temperatures

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Abstract

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The survival of *Mycoplasma bovis* in milk samples was investigated at three storage temperatures (5°C, –30°C and –80°C) during 5 weeks. For the storage temperatures –30°C and –80°C, the respective samples were prepared weekly and those for culture by repeated defrosting. At 5°C the total number of *M. bovis* CFU/ml decreased from the initial of 1.13×10^7 CFU/ml to the final of 3.92×10^6 CFU/ml. The development in the frozen samples prepared for each week was as follow: (1) at –30°C – from the initial of 1.13×10^7 CFU/ml to 5.69×10^6 CFU/ml; (2) at –80°C – from the initial of 1.13×10^7 CFU/ml to 9.75×10^6 CFU/ml. The decrease in *M. bovis* colony count was more evident in the samples that were repeatedly defrosted: (1) at –30°C – the initial of 1.13×10^7 CFU/ml to the final of 2.18×10^6 CFU/ml; (2) at –80°C – the initial and final values were 1.13×10^7 CFU/ml and 7.89×10^6 CFU/ml, respectively.

Keywords: *Mycoplasma bovis*; raw cow milk; temperature

The bacteria of the *Mycoplasma* genus are known as the smallest free-living organisms. They have a worldwide distribution as free living saprophytes or as parasites of humans, mammals, reptiles, fish, arthropods, and plants. They have no cell wall, only a simple genome, and a limited metabolism in comparison to other groups of bacteria. The absence of the cell wall and cell wall-associated proteins makes mycoplasmas resistant to antibiotics that interact with these proteins. Regardless of their simple genetic potential, they are important microorganisms and are considered to be one of the main pathogens in zoopharmacy and food industry the world over. They are able to degrade milk as the raw material for milk products. The situation in the Czech Republic is presently good (Vyletělová 2006a).

Species like *M. bovis, M. californicum, M. canadense, M. bovigenitalium, M. alcalescens, M. argini, M. bovihirnis, M. dispar*, bovine groups 7 and F-38, can cause mastitis (Alexander *et al*. 1985; Kumar & Garg 1991). Of these, *Mycoplasma bovis* is the species most often isolated. It is also associated with other bovine diseases such as abortion, arthritis, conjunctivitis, pneumonia, and synovitis (O'Berry *et al*. 1966; Hjerpe & Knight 1972; Langsford 1977; Jasper 1982).

In practice, bulk milk samples from only 10 to 20 cows are collected and investigated because of the very expensive analytical procedure. Next to these samples, also individual samples are collected from dairy cows which are stored at temperatures 5°C or –30°C and analysed in the case of positive results in bulk milk samples (Vyletělová 2006b).

The aim of this study was to investigate the survival and total count of *Mycoplasma bovis* colonies in milk samples during 5 week storage at temperatures of 5°C, –30°C and –80°C, as well as the possible changes of the total count of colonies and repeatability.

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MATERIALS AND METHOD

Bulk milk samples. The incidence of *Mycoplasma bovis* was investigated at different farms in the state of New York. The bulk milk samples were collected by the farmers themselves in such cases where the milk showed an abnormal composition (flaky structure, watery appearance, blood). The samples were transported to the laboratory and investigated immediately.

Milk samples preparation for the growth at different temperatures. Eleven bulk milk samples with a positive incidence of *Mycoplasma bovis* were selected to examine the growth curves. The samples were distributed into 5 sterile tubes per 5 ml for each week, and into 1 tube per 20 ml for repeated freezing and defrosting (samples were repeatedly defrosted and analysed). The tubes containing the milk samples were stored at temperatures of 5°C, –30°C, and –80°C for 144 hours. The total count of colonies was determined in regular 1 week intervals.

Cultivation and colony count determination. The frozen milk samples were defrosted at room temperature (22°C). The milk samples or their dilutions were inoculated on the surface of *Mycoplasma* Medium (Oxoid) enriched with horse serum, yeast extract, DNA, thallous acetate, and penicillin, and were cultivated at a temperature of 37°C and 7% CO_2 for 72 hours. The control reading was made after 48 hours. The presence of *M. bovis* was determined using a microscope (exp. 16×). The species of the suspected colonies were then identified (Razin 1992). The results were processed as the logarithms of 11 sample averages.

Table 1. Geometric mean *M. bovis* (CFU/ml) and log CFU/ml (value log CFU/ml = log of the geometric mean of 11 samples) – storage temperature 5°C

Week	Geometric mean	log geometric mean
0	1.13×10^7	7.05
1	8.87×10^6	6.95
2	8.19×10^6	6.91
3	6.38×10^6	6.80
4	6.10×10^6	6.79
5	3.92×10^6	6.59

RESULTS AND DISCUSSION

Survival at a temperature of 5°C. The total count of *M. bovis* colonies generally declined with all tested temperatures and the way of the analyses at the temperatures of –30° and –80°C. The results for CFU/ml, geometric means of 11 samples stored at a temperature of 5°C and their logarithms are shown in Table 1. This shows the decreasing growth from the initial CFU/ml of 1.13×10^7 to 3.92×10^6. A marked decrease is evident after the 3rd week where the count of CFU/ml fell to 6.38×10^6 CFU/ml (Table 1, Figures 1–3).

Survival at temperatures –30°C and –80°C in the samples prepared weekly. The count of *M. bovis* colonies decreased differently depending on the temperature and sample preparation. *M. bovis* survival in the samples prepared for each week was as follows: (1) at –30°C – the total count of colonies fell from the initial CFU/ml 1.13×10^7

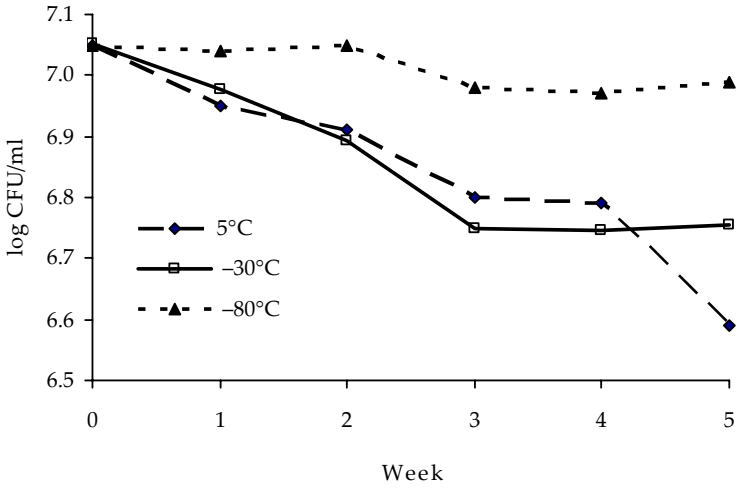

Figure 1. The growth of *M. bovis* at temperatures of 5°C, –30°C, and –80°C. Identical samples – for each week a frozen sample was analysed. The value log CFU/ml = log of the geometric mean of 11 samples

Table 2. Geometric mean *M. bovis* (CFU/ml) and log CFU/ml – storage temperature –30°C (value log CFU/ml = log of the geometric mean of 11 samples)

Week	Geometric mean (W)	log geometric mean (W)	Geometric mean (FD)	log geometric mean (FD)
0	1.13×10^7	7.05	1.13×10^7	7.05
1	9.50×10^6	6.98	9.50×10^6	6.98
2	7.80×10^6	6.89	7.93×10^6	6.90
3	5.57×10^6	6.75	4.17×10^6	6.62
4	5.59×10^6	6.75	3.23×10^6	6.51
5	5.69×10^6	6.75	2.18×10^6	6.34

W – weekly samples; FD – samples repeatedly frozen and defrosted)

to 5×69.10^6 (Tables 2 and 3, Figures 1 and 3); (2) at –80°C – the decreasing growth of *M. bovis* was slower than at –30° and the count varied from CFU/ml 1.13×10^7 to 9.75×10^6 (Tables 2 and 3, Figures 1 and 3). From these results it is evident that the count of colonies was higher after the end of the test in the case of the temperature of –80°C than for the temperature of –30° after the first week – CFU/ml 9.75×10^6 and 5.69×10^6 (Tables 2 and 3, Figure 3).

Survival at temperatures of –30°C and –80°C in the samples that were repeatedly frozen and defrosted. The continual decline in the colony count was more demonstrable in the samples that were weekly repeatedly frozen and defrosted. The geometric mean of CFU/ml was 2.18×10^6 after 5 weeks at –30°C and 7.89×10^6 at –80°C (Tables 2 and 3, Figures 2 and 3). The value of CFU/ml $2.18.10^6$ for the temperature –30°C was lower than the values CFU/ml 3.92×10^6 (5°C) and 5.69×10^6 (–30°C – samples prepared weekly) – Tables 2 and 3, Figure 3.

Compare** M. bovis **colony count with the storage time and temperature. It is evident from the results that *M. bovis* colony count decreased depending on the storage time and temperature. We discovered that the temperature of –80°C appears to be optimal for *M. bovis* survival, and then the temperature of –30°C in the case that the samples are prepared for each analysis (Figures 1–3). The survival at the temperature of 5°C is not viable.

It is interesting to compare the temperatures 5°C and –30°C for the samples repeatedly frozen. We expected that the colony count would be higher in the case of the temperature of –30°C. The geometric mean was higher for the temperature of –30°C after the first analysis (9.50×10^6 CFU/ml vs $8.87.10^6$ CFU/ml) but after the 2nd re-freezing the count was $7.93.10^6$ CFU/ml vs $8.19.10^6$ CFU/ml

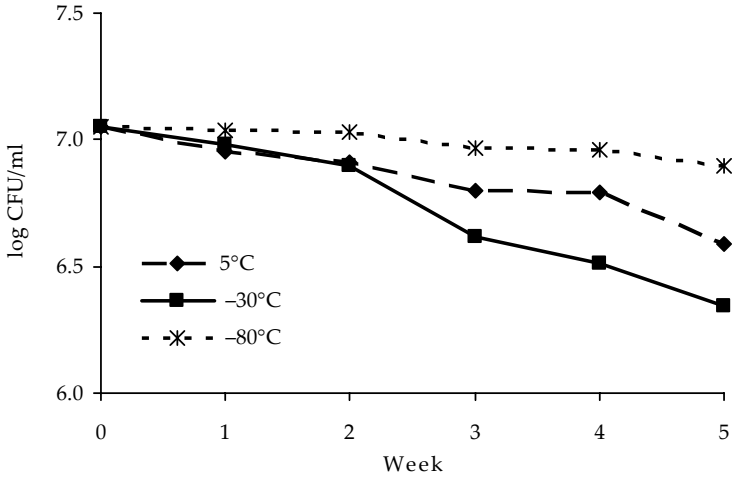

Figure 2. The growth of *M. bovis* at temperatures of 5°C, –30°C, and –80°C. The samples for temperatures of –30°C and –80°C were repeatedly frozen and defrosted. The values CFU/ml = log of the geometric mean of 11 samples

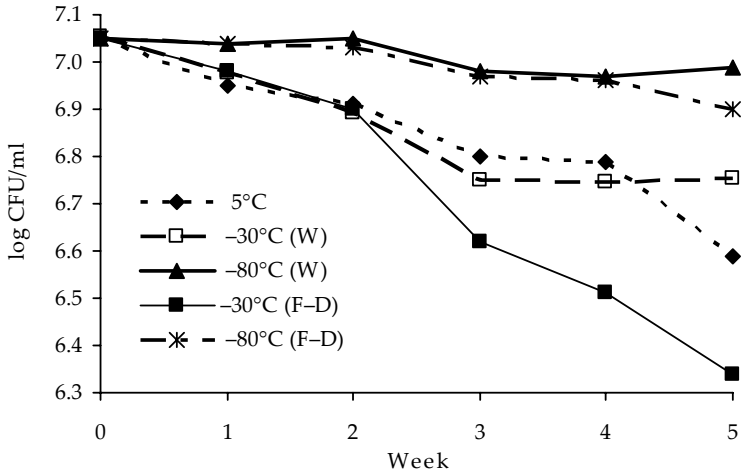

Figure 3. The growth of *M. bovis* at storage temperatures 5°C, –30°C, and –80°C (samples marked as W were prepared for each week and samples marked as F-D were repeatedly frozen and defrosted)

Table 3. Geometric mean *M. bovis* (CFU/ml) and log CFU/ml – storage temperature –80°C (value log CFU/ml = log of the geometric mean of 11 samples)

Week	Geometric mean (W)	log geometric mean (W)	Geometric mean (FD)	log geometric mean (FD)
0	1.13×10^7	7.05	1.13×10^7	7.05
1	1.11×10^7	7.04	1.11×10^7	7.04
2	1.11×10^7	7.05	1.08×10^7	7.03
3	9.62×10^6	6.98	9.34×10^6	6.97
4	9.44×10^6	6.97	9.20×10^6	6.96
5	9.75×10^6	6.99	7.89×10^6	6.90

W – weekly samples; FD – samples repeatedly frozen and defrosted

(Tables 1 and 2). The difference between the total counts of colonies was CFU/ml 2.18×10^6 CFU per ml and 3.92×10^6 after the end of the test (Tables 1 and 2). The difference can be explained by repeated thawing having caused damage to the cytoplasmatic membrane which was attended by the cell destruction.

The difference in the count of colonies between the temperatures –30°C and –80°C shows that the lower temperature is more suitable for the cell survival as freezing is achieved faster and without crystals formation. The count of colonies was always higher in the case of the samples which were prepared weekly (Tables 2 and 3, Figure 3).

Similar results were found by Biddle *et al.* (2004) who used a storage temperature of –20°C. They discovered a continual fall in the colony count from the initial log CFU/ml 6.29 to 4.64 during 1 week, and to 4.14 after 5 weeks. They recommend to apply for thawing the room temperature which was used also in our case. From these and our results is it evident that the decisive factors in *M. bovis* survival involve not only the temperature but also the manner of storage and analysis (storage and repeatability of results).

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