Effect of Freezing on the Viability of *Mycobacterium paratuberculosis* in Bovine Feces

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Three bovine fecal specimens were cultured for *Mycobacterium paratuberculosis* before freezing and after frozen storage at -70°C for 3 and 15 weeks. The losses in viability from 0 to 3 weeks of storage were significant ($P = 0.01$) for all three samples. The losses in viability between 3 and 15 weeks of storage were not significant ($P > 0.05$) for two specimens in which the *M. paratuberculosis* occurred naturally, but the loss was significant ($P = 0.01$) in a simulated specimen (*M. paratuberculosis* culture added to feces from a healthy cow).

The value of fecal culture in the diagnosis of Johne's disease in cattle and goats is well established (1, 3). The etiological agent, *Mycobacterium paratuberculosis*, is sloughed into the feces from the site of infection, usually the mucosa of the ileocecal valve and adjacent tissues. Fecal specimens for culture are collected from the rectum of animals, transported to the laboratory without refrigeration or chemical preservatives, and processed within 18 h after receipt at the laboratory (6). Any delay in processing enhances the growth of contaminants and reduces the culturability of *M. paratuberculosis*. When large numbers of specimens are collected at one time, delays in processing result. A solution to this problem is to store the specimens at -70°C until facilities are available to complete the culture, permitting a single collection of specimens regardless of herd size. This provides convenience to the herd owner and reduces collection and shipping costs.

The purpose of this study was to obtain information regarding the effect of freezing on the viability of *M. paratuberculosis* in bovine feces.

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MATERIALS AND METHODS

Fecal samples. Three fecal samples were obtained. Sample A was prepared by mixing *M. paratuberculosis* with feces collected from the rectum of a healthy cow. Approximately 125 small, actively growing, primary colonies of *M. paratuberculosis* previously isolated from bovine feces were flushed from the slant with 6 ml of sterile Butterfield buffer (6) and incorporated into 125 ml of fresh feces. The mixture was blended, and 0.5-ounce (ca. 15 ml) portions were transferred to sterile 1-ounce (ca. 30 ml) ointment tins.

Fecal sample B was collected from the rectum of a cow that had clinical signs of Johne's disease and from which *M. paratuberculosis* was isolated 3 months earlier.

Fecal sample C was collected from the rectum of a bull that had clinical signs of Johne's disease, but from which culture had not been attempted. The bull died 5 days after the fecal specimen was collected.

The blending and packaging of samples B and C were the same as for sample A.

Freezing fecal samples. Representative portions of each sample were cultured before freezing and after freezing and storage at -70°C for 3 and 15 weeks. The fecal samples in ointment tins were quick frozen by placing them in a pan containing ethyl alcohol and dry ice. The tins were supported in a manner that prevented the alcohol from entering the tins. This treatment was sufficient to freeze 6 ml of water in 1 min. Each ointment tin remained in the alcohol-ice bath for 5 min before rapid removal to a -70°C freezer.

Thawing fecal samples. The fecal samples were thawed at 3 and 15 weeks after freezing with the same apparatus as that used for freezing, except water at 37°C replaced the dry ice and alcohol. The loaded ointment tins were in contact with warm water for 10 min.

Quantitative culture method. Six replications of each sample were processed separately. Approximately 1 g of feces was weighed in a sterile test tube to which sterile distilled water was added to make a 1:10 dilution. The tube was shaken horizontally for 30 min at 23°C and then set upright for 1 h to allow larger and heavier particles to settle. A 1-ml portion of the suspension was taken from just under the surface and transferred to a sterile screw-cap test tube containing approximately 45 ml of a 0.3% Zephiran (benzalkonium chloride manufactured by Winthrop Laboratories, New York, N.Y.) solution. The tube was inverted several times and left in an upright position for 18 h at 23°C .

The fecal suspension was centrifuged for 30 min at 2,400 rpm ($1,000 \times g$). The supernatant fluid was decanted, and the sediment was suspended in a volume

of sterile 0.5% Tween 80 solution equivalent to the volume of sterile water used in the original suspension. This established a 1:100 dilution, each milliliter of which contained 0.01 g of feces and resulted in a colony count factor of 500. Two successive 10-fold dilutions were made in 0.5% Tween 80 to provide a 1:1,000 dilution and a 1:10,000 dilution of the original fecal suspension.

Three slants of Herrold egg-yolk agar medium (6) containing mycobactin (mycobactin is a growth stimulant extracted from *Mycobacterium phlei*; slanted in 30-ml plastic screw-cap tissue culture flasks) and one slant without mycobactin were each inoculated with 0.2 ml of one of three dilutions of the six replicates of each sample. The inoculum was distributed evenly over the surfaces of the slants, which were maintained in the horizontal position for 24 h with lids loose to facilitate the drying of the inoculated medium surfaces. The slants were then inverted, with caps tightened, and incubated at 37°C for 9 weeks when the first reading was made. Incubation was continued at 37°C until the final reading at 13 weeks.

Colony counts. Colonies of *M. paratuberculosis* were illuminated, and their images were cast on a ground-glass screen for counting. Colonies growing on the film of medium that adhered to the sides of the flask were observed with a simple $\times 7$ magnifier. The number of colonies observed on each slant was recorded at 9 weeks and again at 13 weeks. A colony-forming unit is used here to represent a single bacillus or a clump of bacilli that results in the formation of a colony. Tween 80 was used in the preparation of dilutions to minimize clumping. Smears of 50 isolated colonies from separate slants were prepared and stained by the Ziehl-Neelsen method (6).

Statistical analysis. The Student's *t* test was used to test for differences between colony counts made at 9 weeks and those made at 13 weeks of incubation (5). The analysis of variance was used to test for differences in colony counts made on samples before frozen storage and after 3 and 15 weeks of storage and to compute the variance of colony counts between replications and between slants (5).

RESULTS

M. paratuberculosis was isolated from each of the three fecal samples, with colonies occur-

ring only on slants containing mycobactin, an important characteristic of *M. paratuberculosis*. The average colony counts per slant for the six replications of each sample listed by storage time, observation time, and dilution are shown in Table 1.

The detrimental effect of freezing on viability was apparent in all replications of each sample. The reduction in average colony counts for sample A (1:100 dilution at 13 weeks of incubation) was very conspicuous, dropping by 83.7% between 0 and 3 weeks of storage, by 91.7% between 3 and 15 weeks of storage, and by an overall loss of 98.5% (Fig. 1). The reduction was less marked in sample B (1:10,000 dilution at 13 weeks of incubation): 69.5% between 0 and 3 weeks of storage, an apparent increase of 24.4% between 3 and 15 weeks, and an overall loss of 62.1%. Losses for sample C (1:1,000 dilution at 13 weeks of incubation) were 50.4% between 0 and 3 weeks of storage, 7.6% between 3 and 15 weeks, and an overall loss of 54.2%.

The loss in viability from 0 to 3 weeks of storage at -70°C was significant ($P = 0.01$) for all three samples. The loss in viability was not significant ($P > 0.05$) when frozen storage of the two natural samples was extended from 3 to 15 weeks; however, a significant loss ($P = 0.01$) was noted in the simulated sample for the same storage period.

Average colony counts were predominantly greater at 13 weeks than at 9 weeks of incubation (Table 1). Of 54 trials in sample A (three dilutions, six replications, three storage times), 49 gave higher counts, 2 were equal, and 3 were lower at 13 weeks. For sample B, only one dilution (1:10,000) was examined. Of a possible 18 average counts, 16 were greater and 2 were smaller at 13 weeks of incubation. The 1:1,000 and 1:10,000 dilutions were examined for sample C for a total of 36 average counts, of which 32 were higher, 2 were equal, and 2 were lower at week 13. Colony counts made at 13 weeks were

TABLE 1. Effects of freezing and increased incubation time on colony counts

Sample	Observation time (weeks)	Colony count ^a at dilution of feces of:								
		1:100			1:1,000			1:10,000		
		0 ^b	3	15	0	3	15	0	3	15
A	9	117.5	16.7	0.0	12.4	1.3	0.1	0.8	0.0	0.1
	13	126.1	20.5	1.7	13.3	2.0	0.5	1.2	0.0	0.0
B	9	TNC ^c	TNC	TNC	TNC	TNC	TNC	162.6	46.0	53.9
	13	TNC	TNC	TNC	TNC	TNC	TNC	169.2	51.6	64.2
C	9	TNC	TNC	TNC	70.3	36.9	32.7	7.1	3.8	3.2
	13	TNC	TNC	TNC	82.1	40.7	37.6	7.7	4.4	4.3

^a Average number of colonies per slant for six replications on each sample.

^b Storage time at -70°C (weeks).

^c TNC, Too numerous to count.

FIG. 1. Comparative reduction of colony-forming units of *M. paratuberculosis* in each of three fecal samples stored at -70°C . Each point on the graph indicates the average number of colonies per slant (six replications per sample) before storage and after 3 and 15 weeks of storage. Vertical brackets indicate the range of counts in six replications. Counts were made at 13 weeks of incubation. (a) Change in colony counts from 0 to 3 weeks of storage; (b) change in colony counts from 3 to 15 weeks of storage; (c) change in colony counts from 0 to 15 weeks of storage.

significantly greater by the Student's *t* test (5) than those made at 9 weeks.

DISCUSSION

The results of this study indicate that at least one phase of frozen storage is detrimental to the viability of *M. paratuberculosis* in bovine

feces. The particular phase (freezing, storage, or thawing) of frozen storage responsible for the loss in viability was not determined. Although many theories and facts have been reported on the specific effects of physical conditions on the freezing process, a single ideal method cannot be recommended for freezing all microorganisms (4). Kim and Kubica (2) reported that *Mycobacterium tuberculosis* and *Mycobacterium bovis* (including BCG) tended to lose appreciable numbers of viable bacterial cells when stored in Tween-albumin liquid medium at -20°C , with losses often reaching 99.99%, whereas, virtually 100% of the population was protected by storage at -70°C . However, their *M. paratuberculosis* strain 1613 was nonviable after storage for 21 months at -20°C , and it had lost 90 to 99.99% of its viability after 21 to 24 months of storage at -70°C .

In this study, resistance to loss of viability during extended frozen storage was significantly greater for *M. paratuberculosis* organisms occurring naturally in feces than for those in the simulated sample. Although the protective factors remain unidentified, there were two characteristics inherent in the *M. paratuberculosis* organisms in the natural samples that may have been conducive to their resistance: (i) many of them occur in macrophages (1), and (ii) none had been subcultured.

Although losses in viable numbers were demonstrated, it was noted that in no instance was viability completely lost when a practical diagnostic procedure (6) was simulated (e.g., 1:100 dilution, 3-week storage at -70°C , and 13-week observation time).

Information presented here suggests that *M. paratuberculosis* in feces from infected cattle shedding moderate to high numbers of organisms would survive storage at -70°C in sufficient numbers to assure detection by routine fecal culture after storage. The advantages inherent in sampling all animals in large herds at one time seem to warrant the storage of surplus fecal samples at -70°C when field or laboratory conditions are not conducive to immediate culture.

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