Effect of Storage Temperature and Sample Volume on *Brucella melitensis* Isolation from Goat Milk

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Authors' contributions

This work was carried out in collaboration between all authors. Author JAZ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors RCM, DVN, and RHG contributed to study design. Author DVN assisted with statistical analysis. Authors JAZ, MS, VA, and ADP managed field activities and data collection. Authors DC and RC managed laboratory protocols and activity. All authors read and approved the final manuscript.

ABSTRACT

Aim: To determine the impact of storage temperature and sample volume on milk culture success under a simulated field setting.
Study Design: Prospective cohort study.
Place and Duration of Study: Centro de Salud Global UPCH, Tumbes, Peru and Naval

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Methodology: We aseptically collected milk from unvaccinated goats, and then experimentally inoculated the pooled milk sample with *B. melintensis* in order to compare the effect of two different sample volumes (2ml and 5ml) and two different storage temperatures (4ºC and -20ºC) on culture success.

Results: We achieved higher culture success in refrigerated (4ºC) versus frozen (-20ºC) samples (OR 4, 95% CI 1.7, 9.6) and with 5-ml versus 2-ml aliquots (OR 9, 95% CI 3.3, 26.6).

Conclusion: In resource-poor field settings where cold-chain and transportation are unreliable, use of ice for sample storage and transport of goat milk is an acceptable method for the purpose of culturing *B. melintensis.*

1. INTRODUCTION

Brucellosis is a worldwide zoonotic disease caused by bacterial pathogens of the genus *Brucella. Brucella melitensis*, the most virulent species in humans, primarily affects goats and can be transmitted to humans by direct contact with fetal tissues or through ingestion of unpasteurized dairy products (Young, 1995; Young and Suvannoparrat, 1975). Mexico, Argentina and Peru have the most reported cases of human brucellosis in Latin America, where goats are important in local agriculture (Gurria, 1998). Human brucellosis is seldom life-threatening, but it does lead to serious morbidity and, as such, is an economic and public-health concern in developing countries (Godfroid et al., 2005; Smits and Kadri, 2005; Pappas et al., 2005; Franco et al., 2007).

Accurate diagnostic tests are necessary to control *Brucella*. Serological methods (such as the Rose Bengal plate test and the lateral-flow assay) have been used to diagnose brucellosis in goats when bacteriological examination is not practical (Abdoel et al., 2008; Nielsen, 2002). Although these methods are preferred for rapid screening, they do not provide direct evidence of pathogen presence. Milk culture is the gold standard for bacterial isolation and definitive diagnosis, and has been previously described for bovine brucellosis (Alton et al., 1988; OIE, 2012). Milk-sample collection also has the added benefit of being safer for goats and less technically challenging for people than blood-sample collection, but disease transmission through aersolization is a risk. Although goats naturally infected with *B. melitensis* shed bacteria in their milk and mammary secretions, there is no published information quantifying the amount of bacteria shed. The concentration of Brucellae in milk likely varies over the course of infection and stage of lactation. In goats experimentally infected with *Brucella abortus*, the concentrations of Brucellae were as high as 1.4×10^7 colony-forming units (CFU)/g mammary- gland tissue, 6.0×10^5 CFU/g supramammary lymph-node tissues, and 3.0×10^8 CFU/ml mammary secretions (Meador et al., 1989).

Brucella-control programs in resource-poor settings are challenged by inadequate cold-chain and unreliable transportation. In some settings, shipping frozen samples is an option, but frozen milk samples give variable milk-culture yields depending on the bacterial species and duration of storage (Schukken et al., 1989; Sol et al., 2002). Furthermore, freezers are less common in resource-poor settings and subject to the availability of electricity. Ice and ice packs are becoming more widely available and might provide a more practical alternative

when used in combination with insulated coolers. In addition to storage and transport challenges, collecting an adequate sample volume from malnourished goats late in lactation can be difficult. Low sample volume can decrease culture yield from food products (Sperber et al., 2001). Therefore, our objective was to determine the impact of storage temperature and sample volume on milk culture success under a simulated field setting.

2. MATERIALS AND METHODS

Goats used in this study were cared for in compliance with the Institutional Animal Care and Use Committee (IACUC) of Cornell University and Universidad Peruana Cayetano Heredia. All goats were owned, and all farmers provided oral informed consent to collect biological specimens from their goats.

Milk samples were collected from 24 goats in Tumbes, Peru in May of 2010. A total of 100 ml of milk was collected from each goat. All milk samples were aseptically collected via previously described methods to lessen bacterial contamination (National Mastitis Council, 1999). Blood samples were collected from each goat at the time of milk sample collection. The serum samples from each goat (n = 24) were tested via the Rose Bengal plate test (RBT) and the lateral-flow assay (LFA) within 4 hours of collection. The sensitivity of the RBT for *B. abortus* in cattle is reported to be 21.0 - 98.3% and the specificity is reported to be 68.8 - 100% (Nielsen, 2002). Given that the sensitivity of the LFA in goats is 100%, and that the tests were performed in parallel, the false-negative risk for the samples tested was 0% (Abdoel et al., 2008). All goats were seronegative for *Brucella* on both tests. Following serum testing, the milk samples (n = 24) were pooled, for a total volume of 2.4 L, and stored at 4°C for 5 ds. From this volume, 1 L was removed and 60 ml were set aside to serve as a negative control. We inoculated the remaining 940 ml with *B. melitensis* strain 16M to achieve a final concentration of 1 x 10^4 CFU/ml. Milk was divided into 2-ml and 5-ml aliquots to simulate collection of different sample volumes from goats. The aliquots were then either refrigerated (4ºC) to simulate storage on ice or frozen (-20ºC). A total of 200 aliquots were evaluated, with 50 aliquots in each of the 4 possible volume and temperature groups (2 ml and 4ºC, 2 ml and -20ºC, 5 ml and 4ºC, or 5 ml and -20ºC). Two negative controls were also evaluated for each volume and temperature group (8 total negative controls). Samples were stored at their designated temperature for 7 d to simulate field transport time. Samples stored at -20ºC were thawed at 37ºC. From the aliquots, 1 ml was removed and instilled into Eugon Broth for enrichment and incubated for 4 d at 37ºC (Atlas, 2010). Then 100 μl was subcultured onto modified Farrell's medium with *Brucella* selective supplement (one subculture per plate) and incubated at 37ºC for 4 to 5 d (Farrell, 1974). All groups and samples were run concurrently.

Data were analyzed using descriptive statistics and multivariable logistic regression in JMP 7.0 (SAS Institute Inc., 1989-2007). The dichotomous outcome of interest was *Brucella* growth or not in the sample. The predictor variables explored were storage temperature, sample volume, and their interaction. Manual backwards stepwise removal of variables was performed to arrive at the final model. All predictors were entered into the model and those with *P* < .05 were selected for inclusion.

3. RESULTS AND DISCUSSION

The 2-ml aliquots stored at -20ºC had the lowest culture success, with 29 (58%) out of 50 plates exhibiting growth, followed by the 2-ml aliquots stored at 4ºC in which 42 (84%) out of

50 plates exhibited growth (Table 1). Among the 5-ml aliquots, those stored -20ºC had 46 (92%) out of 50 plates exhibiting growth, while those stored at 4ºC had 49 (98%) out of 50 plates exhibiting growth (Table 1). There was no evidence of bacterial contamination. All 8 controls exhibited no growth. In addition, no important bacterial overgrowth was observed on the plates in the other groups. In the logistic regression, the interaction between temperature and volume was not statistically significant (P=.93) and was therefore excluded. Homogeneity of the bacteria present in the milk aliquots and the impact of thawing on the spiked milk samples were assumed to be randomly distributed across the samples and was therefore not factored into the analysis.

Table 1. Percent of positive *B. melitensis* cultures from goat milk obtained in each test group

Test Group	Fraction Positive	% Positive & (95% CI)
2ml & 4°C	42/50	84 (0.7, 0.9)
2ml & -20°C	29/50	58 (0.4, 0.7)
5ml & 4°C	49/50	98 (0.9, 1.0)
5ml & -20°C	46/50	92 (0.8, 1.0)

Table 2 shows that while controlling for sample volume, a storage temperature of 4ºC had 4 times greater odds (95% CI: 1.7, 9.6) of yielding a positive culture than a storage temperature of -20ºC ($P = .002$). While controlling for storage temperature, a sample volume of 5 ml had 9 times greater odds (95% CI: of 3.3, 26.6) of yielding a positive culture than a sample volume of 2 ml (P < .001).

Table 2. Final model of multivariable logistic regression on the affects of temperature and volume on *B. melitensis* culture positivity

Predictor Variable	Level	Regression Coefficient	SE	P value	OR	95% CI
Intercept	-	-2.1	0.27	< .0001	-	-
Volume (ml)	2ml	-	-	-	referent	-
	5 ml	1.1	0.26	< .0001	9	3.3, 26.6
Temperature (ºC)	4ºC	0.68	0.22	.002	4	1.7, 9.6
	-20ºC	-	-	-	referent	-

These results demonstrate that a 4ºC storage temperature can provide a high positive milk culture success, with minimal bacterial contamination, if an adequate sample volume is collected using aseptic collection methods. When interpreted in light of the constraints of resource-poor settings, ice or ice packs are preferred over freezing for sample storage and transport, and the importance of working with the local community to collect samples at times of day when goats are likely to have full udders should be included when formulating a field plan. In addition, these results allow researchers to approximate anticipated culture success after accounting for these four combinations of field conditions, and to more accurately interpret their own data in reference to these conditions. This was a simulated field study in which we had access to reliable refrigeration, therefore allowing us to maintain a constant storage temperature for all of our samples. In a field setting, thermometers should be used to monitor variations in storage temperature. As ice melts or ice packs thaw, the storage temperature may fluctuate.

We used a relatively low concentration of bacteria to account for variation in the bacterial load in milk at different stages of infection and lactation. Given the strong positive culture results attained at a concentration of 1 x 10^4 CFU/ml, we anticipate that these results will be repeatable at higher concentrations, but do not know whether they will be repeatable at lower concentrations.

Because freezing can stress some species of bacteria and diminish culture yield, refrigeration is preferred for sample storage. Since refrigeration it is not widely available in the developing world, this research calls attention to the importance of finding other means of milk storage that are feasible for use in resource-poor settings. Liquid nitrogen has become more widely available and has no demonstrated impact on milk-culture yield; future studies should compare the effect of ice in comparison to liquid nitrogen on milk-culture yield (Sanchez et al., 2003). Although milk does have the added benefits of being easier to collect and requiring less technical skill than phlebotomy, appropriate biosafety precautions remain necessary when handling milk samples because of the risk of infection through aerosolization. Furthermore, since *Brucella* spp. can still be isolated from frozen milk, frozen milk should be handled with the same precautions as fresh milk.

4. CONCLUSION

This study demonstrated that the odds of obtaining a positive culture were greater for samples stored at 4ºC than for samples stored at -20ºC at the sample volumes tested. Therefore, ice and ice packs are acceptable methods for storing and transporting goat milk samples for bacterial culture of *Brucella melintensis* which is particularly important in developing countries where there are insufficiencies in the cold-chain. As other options become available for the maintenance of cold-chain, such as liquid nitrogen, they could be evaluated for their impact on culture yield.

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DISCLAIMER

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the US Government. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

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CONSENT

All authors declare that oral informed consent was obtained from all study participants prior sample collection from their owned livestock.

ETHICAL APPROVAL

All authors hereby declare that "principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee (protocol number 2009-0097).

COMPETING INTERESTS

No competing interests exist.

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