Interpretive summary: ***Short communication*: Recovery of pathogens from frozen meat sheep milk**. By Smith et al. Freezing sheep milk prior to microbiological analysis might reduce the probability of detecting mastitis pathogens. Whilst culture of a fresh milk sample is optimal, the addition of glycerol to milk before freezing might improve recovery of bacteria by offering protection to Gram-negative isolates.

SHORT COMMUNICATION: RECOVERY OF PATHOGENS FROM FROZEN EWE MILK

Short communication: Preliminary investigation into the effect of freezing and a cryopreservant on the recovery of mastitis pathogens from ewes' milk

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ABSTRACT

The objective of this study was to investigate the recovery of bacteria from ewe milk after freezing for 4 or 8 weeks with and without the addition of glycerol as a cryopreservant. A total of 50 udder-half milk samples with a known range of bacterial species were selected, stored and analyzed in 5 treatment-groups: time zero, frozen for 4 weeks with, and without, glycerol; and frozen for 8 weeks with, and without, glycerol. There was a lower recovery of all bacterial species studied after freezing. Samples containing fewer than 100 cfu/mL came from ewes with a lower somatic cell count and were more likely to be bacteriologically negative after freezing than those above this threshold. The addition of glycerol increased recovery of Gram-negative bacteria after freezing, although this requires further study to draw strong conclusions. The effects on Gram-positive species were inconsistent. We conclude that whilst the addition of glycerol had a small beneficial impact on the sensitivity of detection of bacteria from frozen sheep milk, sensitivity was highest in cultures from fresh milk.

Keywords

sheep milk, mastitis, freezing, cryopreservant.

Mastitis, inflammation of the mammary gland, is a common disease of dairy and meat sheep, which presents as a clinical disease or sub-clinical intra-mammary infection. Both forms can impact milk production and lead to reduced weight gain in suckling lambs (Fthenakis and Jones, 1990). Antibiotic therapy is used to treat clinical disease but is rarely used as a routine treatment for sub-clinical infection at drying off in meat sheep. As a consequence, the udders of meat ewes are exposed to many pathogens over a lifetime, and the prevalence of sub-clinical infection is estimated to range from 5.5 to 7.0% in the UK (Watkins et al., 1991) and is 28.8% in Canada (Arsenault et al., 2008).

The bacterial species isolated from intra-mammary infections in meat sheep are similar to those isolated from other livestock, and include *Escherichia coli*, *Staphylococcus aureus*, coagulase-negative staphylococci (**CNS**) and streptococcal species (Watkins et al., 1991). In addition, *Mannheimia* (formerly *Pasteurella*) *haemolytica* is a common cause of intra-mammary infection (Watkins et al., 1991). Bacteriological culture of milk samples remains the gold standard for identification of pathogens in milk, and freezing samples before analysis is common practice. A variety of freeze and culture techniques have been investigated to determine their effects on the recovery of pathogens from bovine and caprine milk. The only study to date reporting the impact of freezing sheep milk on recovery of bacteria using culture was by Clements et al., (2003), who froze 60 samples for one week. Despite somewhat conflicting reports, where an effect is detected, freezing generally results in an increase in the isolation rate of staphylococci (Schukken et al., 1989, Sol et al., 2002, Villanueva et al., 1991), has variable effects on the isolation rate of streptococci (Sol et al., 2002, Villanueva et al., 1991), and leads to a reduction in the isolation rate of *E. coli* (Schukken et al., 1989) from bovine milk. In contrast to studies of bovine milk, *S. aureus* was not isolated from 2 (100%) known positive ovine milk samples after storage at -21°C for 1 week (Clements et al., 2003). One possible method of attenuating this negative effect of freezing is to use a cryopreservant such as glycerol, which has been shown to significantly improve the recovery of

viable bacteria from cow milk (Bradley et al., 2002). The objective of the current study, therefore, was to investigate the impact of freezing and a cryopreservant on the culture of bacteria in milk from meat sheep.

The study animals were a pedigree Charollais flock of 120 breeding ewes reared extensively on a UK lowland farm and lambed indoors. All samples were collected on a single day from a cohort of 50 ewes between 1 wk and 3 wk post-lambing. On average, the ewes were 3.3 yr old and produced 1.76 lambs. Seven cases of clinical mastitis were detected in this lactation in this flock, giving a period prevalence of 5.8%. Of these, 5 cases were acute and detected within 4 d post-lambing, the remaining 2 cases were chronic infections detected at weaning.

A total of 99 duplicate udder-half milk samples were collected using standard techniques (Hogan et al., 1999) and placed on ice. One sample, preserved with Bronopol, was submitted to a commercial laboratory (Quality Milk Management Services Ltd., UK) for somatic cell count (**SCC**) analysis using the Fossomatic method (Delta CombiScope – Model FTIR 400, Drachten, the Netherlands) according to the FIL International Dairy Federation 148 A: 95 norm (International Dairy Federation, 1995); and the second was collected aseptically for microbiological analysis. An aliquot of this second sample was cultured immediately, with the remainder divided into 2 further samples, with approximately 10 % (vol/vol) sterile glycerol added to one. Two 110 µL aliquots were taken from each of these samples and stored at -20 °C; a total of five aliquots were therefore made from each ewe half.

Milk samples were cultured according to recommended protocols (Hogan et al., 1999). Briefly, 100 µL of milk was spread across the surface of a brain heart infusion (**BHI**) agar plate supplemented with 5 % sterile sheep blood and incubated at 37 °C, with colony morphology recorded at 24 h and 48 h. Samples with 5 or more distinct colony phenotypes were classed as contaminated.

Microbiological growth was characterized using the KOH string, catalase and tube-coagulase tests. Gram-positive, catalase-negative cultures were classified as *Streptococcus*-like; Gram-positive, catalase and tube-coagulase positive isolates as *S. aureus*; and Gram-positive, catalase-positive, tube-coagulase negative isolates as CNS. *Mannheimia haemolytica* and *E. coli* were identified by species-specific PCR (Guenther et al., 2008, Riffon et al., 2001). Bacterial genomic DNA was isolated using a modified Chelex protocol (de Lamballerie et al., 1992), 1 mL of overnight culture was pelleted and resuspended in 100 µL 5 % Chelex (Bio-Rad, Hertfordshire, UK) and 10 µL proteinase K (10 mg/mL). The solution was incubated rotating at 56 °C for 5 hours, centrifuged at 14,100 ×*g* for 3 min and the DNA-containing supernatant placed in a fresh tube. DNA isolation was confirmed by amplification of a 438 bp fragment of the 23S rRNA coding region, using primers Uni_1870 and Uni_2308; *E. coli* isolates were identified using primers Eco_2083 and Eco_2795 (Riffon et al., 2001); and *M. haemolytica* isolates were identified using primers Mhaem_306 and Mhaem_449 (Guenther et al., 2008).

The identity of a random selection of 17 Gram-positive isolates was confirmed by partial sequencing of the 16S gene region. A 1,068 bp fragment was amplified with primers XB1 and XB4 (Moore et al., 2008) and sequenced using internal primers PSL and PSR (Moore et al., 2008) and BigDye chemistry on a 3130xl Genetic Analyzer (Applied Biosystems, Warrington, UK). Forward and reverse DNA sequences were aligned in BioEdit (Hall, 1999) to generate a consensus sequence for each isolate. This was compared to the GenBank database using the megablast function of nucleotide blast (http://blast.ncbi.nlm.nih.gov/), and sequence identity determined using a similarity of 97% or higher (Stackebrandt and Goebel, 1994).

Fifty of the 99 samples with a range of bacterial species were selected for culture at 4 and 8 weeks. All samples containing *S. aureus* and a representative subset of those containing *Streptococcus*-like, CNS, *M. haemolytica* and *E. coli* were selected to approximate the proportion of isolates that were

present in the full sample set; albeit with a slight reduction in CNS, but an increase in all other species.

The data were analyzed in 5 treatment-groups: time zero (0), 4 weeks frozen with glycerol (4G+), 4 weeks frozen without glycerol (4G-), 8 weeks frozen with glycerol (8G+) and 8 weeks frozen without glycerol (8G-), and categorized by the number of detected cfu/mL (0, ≤10, 11 to 100, and >100). We defined samples as positive if they yielded growth in any of the treatment-groups. The effect of treatment-group on bacterial recovery was tested using Fisher's exact tests by comparing the proportion of the 50 samples positive in each treatment-group with the proportion of samples positive in all other treatment-groups and overall.

Of the 99 samples, 2 were contaminated and 5 yielded no growth. In the remaining 92 samples, 23, 85, 9, 6, and 3 were positive for *Streptococcus*-like isolates, CNS, *S. aureus*, *M. haemolytica*, and *E. coli* respectively. Seventy-five samples had somatic cell counts (**SCC**) at time zero; of these, 4 were culture-negative, 42 yielded CNS with no other pathogen group, 5 yielded *S. aureus* (3 also contained CNS), 14 yielded *Streptococcus*-like isolates (11 also contained CNS), 2 contained *E. coli* (both contained CNS) and 1 yielded *M. haemolytica* in pure culture. The mean SCC of 1.2×10^6 cells/mL was comparable to those reported in dairy and meat sheep flocks (Gonzalez-Rodriguez et al., 1995, Gonzalo et al., 2002, Hariharan et al., 2004), and higher in samples containing >100 cfu/mL (Fig 1).

No sample contained isolates in more than 3 of the described pathogen groups, though 8.1% of samples yielded up to 4 isolates, mainly due to the phenotypic diversity of CNS. Although there were many pathogen species, none of the sheep had overt signs of mastitis and a large proportion of the samples contained fewer than 100 cfu/mL; it is likely that had we used a 10 µL inoculation volume many samples would have been classed as bacteriologically negative. Indeed, using a 1,000

148 cfu/mL cutoff as indicative of infection, as employed by Watkins et al. (1991), results in 16 (16.2%)
149 samples (udder halves) being classed as positive, all in pure culture. This is comparable to the 13.2%
150 of glands that were bacteriologically positive in the earlier study (Watkins et al., 1991).
151
152 Coagulase-negative staphylococci were virtually ubiquitous, being present in 92.4% of samples, and
153 100% of samples with 2 or 3 pathogen-groups (n=22 and n=6 respectively). Gram-positive isolates
154 were more likely to be present with increasing numbers of pathogen-groups. *E. coli* was detected
155 only in a mixed culture with CNS (n=2), and *M. haemolytica* was detected both in pure culture (n=2)
156 and with 2 other bacterial groups (n=4). This relationship was also influenced by bacterial load;
157 there was a non-significant tendency for plates with more colonies to contain fewer pathogen
158 groups (1 / 16 plates where >100 cfu were counted had a mixed infection). This may be due to
159 operator error because when more colonies were on a plate, it was more difficult to identify
160 colonies that were phenotypically different. Alternatively if high cfu were linked to high SCC, the
161 udder might have been protected against a second bacterial species infecting the udder, as reported
162 in dairy cows (Nickerson and Boddie, 1994, Schukken et al., 1999).
163
164 The 50 samples selected for further analysis included 2 contaminated samples, 4 bacteriologically
165 negative samples and 15, 38, 9, 5, and 2 where *Streptococcus*-like isolates, CNS, *S. aureus*, *M.*
166 *haemolytica*, and *E. coli* were cultured at time zero (Table 1). Eight CNS, 5 *S. aureus*, and 2
167 streptococci were confirmed by sequencing; a further 2 *Streptococcus*-like isolates were identified
168 as *Aerococcus* spp. The potential for misclassification of catalase-negative Gram-positive cocci
169 from milk samples has been described elsewhere (Fortin et al., 2003) however, the characterization
170 of Gram-positive, catalase-negative isolates as *Streptococcus*-like was sufficient for the current
171 study. All resultant DNA sequences have been deposited in GenBank under accession numbers
172 HM003050 to HM003066.
173

Using the results of culture at time zero as the gold standard, freezing with and without glycerol had a negative effect on the proportion of culture-positive samples for all species. Samples <100 cfu/mL were more likely to be negative in later treatment-groups than those above this threshold (Fig. 2). Only samples containing >100 cfu/mL *S. aureus* at time zero (n=4) were detectable at this concentration throughout the study. The concentration of *Streptococcus*-like isolates and CNS although high initially in some samples was lower after freezing. For all Gram-positive isolates, samples that were culture-negative at time zero were more likely to yield detectable growth after 4 rather than 8 weeks storage, but the addition of glycerol had inconsistent effects (Fig. 2).

Of the 4 samples that were culture-negative at time zero, 1 remained negative throughout, 2 were culture-positive in one treatment-group each (4G+ and 4G-) and the remaining sample was positive in all 4 subsequent treatment-groups. Only CNS were detected in these samples, and the treatment-groups without glycerol yielded slightly higher colony counts (56 vs 20 cfu/mL). This suggests that if there had been higher numbers of culture-negative samples at time zero, the chance of finding a positive effect of freezing may have been increased. Further analysis of bacteriologically-negative samples is required to see if this is the case.

The low prevalence of Gram-negative isolates in the original samples and throughout the study is likely to be representative of the culturable pathogen distribution of the samples because they originated from a single farm. There were 2 *E. coli*, and 5 *M. haemolytica* detected at time zero. *E. coli* was never detected again in these 2 samples, however a further 4 were detected later; all 4 were detected in glycerol supplemented samples, and 1 also in milk frozen without a cryopreservant. Of the 5 samples positive for *M. haemolytica* at time zero, 2 with 11 to 100 cfu/mL were undetectable thereafter; 2 that initially contained >100 cfu/mL were detected in 3 and 4 of the 4 subsequent treatment-groups. Glycerol supplementation increased isolation of Gram-negative pathogens, both

199 culture-positive and the number of cfu detected, however, the identification of *E. coli* and *M.*
200 *haemolytica* positive samples was low and these results should be confirmed in a larger study.
201
202 Culture at time zero was the most sensitive method for detection of all bacteria, with a sensitivity
203 that ranged from 33.3% (*E. coli*) to 100.0% (*M. haemolytica*; Table 1). There was no significant
204 difference in the sensitivity of detection of any bacterial species at weeks 4 and 8 compared with
205 time zero. However, there were significantly fewer ($p<0.05$) culture-negative samples at time zero
206 compared with weeks 4 and 8; and, when compared to the total number of samples ever positive,
207 significantly fewer *Streptococcus*-like isolates and CNS ($p<0.05$) were detected in weeks 4 and 8.
208 For all isolates, a higher sensitivity was achieved by combining the results of fresh sample culture
209 with subsequent treatment-groups (Table 2). The combination of time zero plus 4 weeks with
210 glycerol increased the sensitivity of detection to above 85% for all species except *E. coli*.
211
212 Our original hypothesis was that the addition of 10% (vol/vol) glycerol, a commonly used
213 cryoprotectant, to ovine milk before freezing would result in increased preservation of viable
214 bacteria compared with unsupplemented samples, as demonstrated in cow milk (Bradley et al.,
215 2002). It was surprising therefore that glycerol supplementation had no significant positive effect
216 on pathogen isolation rates, although it did produce a small non-significant benefit when samples
217 were frozen for 4 weeks compared with 8. This was noticeable even though the addition of glycerol
218 reduced the volume of milk plated by 10%, and is similar to the effects of glycerol supplementation
219 on *Mycoplasma* spp. recovery from frozen bovine milk (Boonyayatra et al., 2010).
220
221 There are differences, however, between our study and the investigation into the effects of glycerol
222 supplementation on the recovery of mastitis pathogens from frozen milk samples by Bradley et al.
223 (2002). The most obvious being the source of milk samples, we used sheep rather than cow with
224 double the percent solids; and our samples were naturally infected, rather than manually inoculated

225 culture-negative milk samples. There are also differences in the concentration of minerals in ewes'
226 and cows' milk (Polychroniadou and Vafopoulou, 1985, Rodriguez Rodriguez et al., 2001) and
227 these are likely to vary during an intra-mammary infection. The effects of milk composition on
228 bacterial viability after a freeze-thaw cycle are unknown. One possible mechanism, postulated
229 previously (Schukken et al., 1989) which may explain why some species grow better and others
230 worse after freezing is the response to the potential increase in mineral composition of milk, due to
231 freezing extracellular water. This would expose bacterial cells to ice crystals and an osmotic
232 gradient, which might lead to cell shrinkage and possibly cause membrane lesions.
233
234 The increased isolation rate of *S. aureus* (Sol et al., 2002, Villanueva et al., 1991) and CNS
235 (Schukken et al., 1989) from bovine milk after freezing is believed to be due to the disruption of
236 bacterial cell aggregates, and damage to phagocytic leukocytes during freezing and thawing, leading
237 to the release of intracellular staphylococci (Schukken et al., 1989, Villanueva et al., 1991). It is
238 possible in the present study that glycerol preserved host phagocytic cells, preventing release of
239 intracellular bacteria, and thus negated any improvement in the detection of CNS or *S. aureus*.
240 However, samples without cryopreservant did not yield increased numbers of Gram-positive
241 bacteria.
242
243 Combining the results from treatment-groups increased detection sensitivity for some species, as
244 has been demonstrated with *S. aureus* and streptococci in cow milk (Sol et al., 2002). However,
245 augmented culture (Dinsmore et al., 1992, Sol et al., 2002) and molecular techniques (Gillespie and
246 Oliver, 2005, Koskinen et al., 2009) might represent a more effective method for detection of
247 pathogens.
248
249 In conclusion, the supplementation of ovine milk samples with 10% sterile glycerol had no
250 significant negative effect on the culture of milk stored frozen for 4 and 8 weeks compared with

unsupplemented samples, and may offer some protection to Gram-negative organisms. Repeat analysis of samples improved the sensitivity of detection of all bacterial species. Further studies are required to fully determine the effects of freezing and cryopreservants on the culture of bacteria from meat sheep milk.

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Table 1. Number (%) of samples culture-positive by bacterial group or species in each treatment-group with the number of samples ever positive as the denominator

Treatment -group	*Streptococcus*-like	CNS[1]	*S. aureus*	*M. haemolytica*	*E. coli*	NGD[2]	Cont.[3]
0	15 (65.2^{ab})	38 (84.4^{ab})	9 (75.0)	5 (100.0)	2 (33.3^{ab})	4 (25.0^{a})	2 (100.0)
4G+[4]	10 (43.5^{a})	34 (75.6^{a})	7 (58.3)	3 (60.0)	2 (33.3^{ab})	9 (56.3^{ab})	0 (0.0)
4G-[5]	8 (34.8^{a})	34 (75.6^{a})	6 (50.0)	2 (40.0)	0 (0.0^{a})	9 (56.3^{ab})	0 (0.0)
8G+[4]	7 (30.4^{a})	33 (73.3^{a})	6 (50.0)	3 (60.0)	2 (33.3^{ab})	10 (62.5^{ab})	0 (0.0)
8G-[5]	7 (30.4^{a})	33 (73.3^{a})	8 (66.7)	2 (40.0)	1 (16.7^{ab})	11 (68.8^{ab})	0 (0.0)
All[6]	23 (100.0^{b})	45 (100.0^{b})	12 (100.0)	5 (100.0)	6 (100.0^{b})	16 (100.0^{b})	2 (100.0)

Detection sensitivities within a column with different superscripts differ ($p<0.05$).

[1]Coagulase-negative staphylococci.

[2]No growth detected.

[3]Contaminated samples.

[4]G+: Samples with 10% (vol/vol) glycerol added before freezing.

[5]G-: Samples with no glycerol added.

[6]Sum of the positive results of the 50 samples from all treatment-groups.

346 **Table 2**. Sensitivity of combinations of treatment-groups for all bacterial groups or species with the

347 number of samples ever positive as the denominator

Treatment-group(s)	*Streptococcus*-like	CNS[1]	*S. aureus*	*M. haemolytica*	*E. coli*
0	65.2	84.4	75.0	100.0	33.3
0 and 4G+[2]	87.0	93.3	91.7	100.0	66.7
0 and 8G+	69.6	93.3	83.3	100.0	66.7
0 and 4G+ and 8G+	91.3	95.6	100.0	100.0	100.0
0 and 4G-[3]	73.9	95.6	83.3	100.0	33.3
0 and 8G-	73.9	91.1	83.3	100.0	50.0
0 and 4G- and 8G-	82.6	95.6	83.3	100.0	50.0

348 [1]Coagulase-negative staphylococci.

349 [2]G+: Samples with 10% (vol/vol) glycerol added before freezing.

350 [3]G-: Samples with no glycerol added.

351

Figure 1. Somatic cell count by bacterial concentration of ewe milk samples that were culture-negative (n=4; squares), contained *Streptococcus*-like isolates (n=14; circles), *S. aureus* (n=5; triangles), coagulase-negative staphylococci (n=42; diagonal crosses), *E. coli* (n=2; horizontal crosses) or *M. haemolytica* (n=1; diamond) at time zero. The log bacterial concentration +1 allows inclusion of culture-negative samples. The 6 samples with the highest bacterial concentration yielded growth that was too numerous to count, and have been plotted at 250 cfu/mL.

Figure 2. The proportion of samples containing Gram-positive pathogens that were culture-negative (white bars), yielded ≤10 cfu/mL (diagonal lines), 11 to 100 cfu/mL (grey bars) or >100 cfu/mL (black bars) from those originally containing >100 cfu/mL (row A), 11 to 100 cfu/mL (row B), ≤10 cfu/mL (row C) or that were culture-negative for that pathogen-group (row D), in all treatment-groups (0: time zero; 4G+: frozen for 4 weeks with glycerol; 4G-: frozen for 4 weeks without glycerol; 8G+: frozen for 8 weeks with glycerol; 8G-: frozen for 8 weeks without glycerol). Sample numbers (row A, B, C, D): *Streptococcus*-like isolates (4,6,5,8); CNS (22,13,3,7); *S. aureus* (4, 0, 5, 3).

[1]Coagulase-negative staphylococci