Cryopreservation of *Salmonella enterica* in porcine fecal samples

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Abstract

Fecal samples are normally tested for *Salmonella* soon after collection because storage at any temperature, including refrigeration or freezing, can reduce detection. To evaluate several cryopreservation techniques, autoclaved porcine feces with and without additives were inoculated with 10^3 CFU S. Derby (UW -9)/g with autoclaved feces prior to freezing. The mixtures and % of the CFU inoculum that was recovered was as follows: Feces only, 11%; 50% feces plus 50% glycerol, 45%; 25% feces, 50% glycerol, 25% tetrathionate broth, 63%; 25% feces, 50% glycerol, 25% buffered peptone water (BPW), 66%; 50% feces, 50% glycycerol/Tris buffer, 58%; 50 % feces, 50% BPW, 30%. When fresh (not autoclaved) feces were used, inoculated with a nalidixic acid resistant S. Typhimurium (WI-73), 4% of the inoculum was recovered from undiluted frozen feces while the addition of 50% BPW before freezing increased recovery to 27%.

In a 2x2x2 fully randomized block factorial study, fresh fecal samples were inoculated with 10^3 CFU UW-73 /g feces. These samples were processed undiluted or with the addition of glycerol and/or BPW before freezing. The addition of 20%-40% of each compound simultaneously resulted in increased recovery of UW-73 when compared with undiluted fecal samples ($p<0.05$). The addition of 40% BPW and 20% glycerol in combination resulted in the highest recovery (83%). It is concluded that the addition of 20-40% glycerol and 20-40% BPW before freezing may be effective cryopreservatives for *Salmonella* in porcine fecal samples, allowing for simplified and more economical laboratory enumeration.

Introduction

Freezing of bacteria can result in cell death due to increased osmotic pressure and the formation of ice crystals. A cryoprotective agent (e.g. glycerol or dimethyl sulfoxide) can depress the temperature at which this occurs (Gherna, R., 1994). Effective and economical cryopreservation of fecal samples would simplify application of conventional culture techniques, permitting sampling and laboratory work to be conducted on asynchronous schedules. Further, costs of large scale quality assurance evaluations may be reduced due to lower transportation costs for overnight delivery.

While freezing of ileocolic lymph nodes resulted in no detected change in sensitivity of qualitative microbial culture of *Salmonella* in a study using a double enrichment system (Bahnson et al., 2006), no effective systems for cryopreservation of fecal samples have been reported. Anecdotal evidence suggests that freezing reduces survival. For example, from pigs with known exposure to Salmonella, the organism was detected in 19 of 20 non-frozen samples, but in only 5 of 20 paired frozen fecal samples. The mean log CFU/g was 2.0 in non-frozen and 0.5 in frozen feces (unpublished observations).

Our objective was to test whether the addition of cryopreservatives protected salmonellae in frozen (-70°C) fecal samples. Initial trials indicated that the addition of 20% volume / weight glycerol improved survival. When autoclaved porcine fecal samples diluted with 50% glycerol were inoculated with approximately 10^3 CFU S. Derby / g fecal weight before freezing, the Log CFU / g was 4.49 and 3.87 in the unfrozen and frozen samples, respectively (unpublished observations).

Following up on these preliminary observations, we designed two studies to identify possible cryoprotective storage protocols. In study I, we evaluated combinations of glycerol, buffered

peptone water (BPW), tetrathionate broth, and TRIS-HCl (MgSO4 buffer ("TRIS buffer"). In study II, we determined which concentrations of the two most effective cryoprotective agents from Study I would result in a higher % recovery in frozen samples.

Material and Methods

Study 1. Differing combinations of glycerol, buffered peptone water (BPW), tetrathionate broth (TT), and Tris buffer (ACTUAL CONCENTRATION INGREDIENTS) were added to either autoclaved or fresh porcine feces as follows: A, 10 g feces only; B, 5 g feces plus 5 ml glycerol; C, 2.5 g feces plus 2.5 ml TT and 5 ml glycerol; D, 5 g feces plus 2.5 ml BPW and 5 ml glycerol; E, 5 g feces plus 5 ml TRIS buffer; and F, 2.5 g feces plus 7.5 ml BPW. S. Derby WI-9 was mixed with autoclaved feces from growing pigs while S. Typhimurium WI-73, a naladixic acid resistant strain that was isolated from ileocolic lymph node tissue from a normal slaughtered pig, was added to fresh feces (Table 1). The inoculum for each sample was adjusted in BPW prior to addition to the feces so that each sample would contain approximately 10^3 CFU/g of the final fecal/cryopreservative mixture. The size of the inoculum was determined by triplicate plate counts on blood agar for autoclaved feces and on XLT-4 w/ 25 µg/mL naladixic acid. For mixing the feces with the various additions and the inoculum, the samples were placed in a paddle blender (Stomacher 80, Seward), for 2 minutes on the high setting. Immediately after mixing, 100 µL of each sample was plated in triplicate to determine the number of organisms present. The remainder of the sample was immediately frozen at -70C for at least 24 hours. The frozen samples were thawed at 37C in a water bath and then plated as for the unfrozen samples. The autoclaved feces samples were plated on Blood Agar, while the fresh feces samples were plated on XLT-4 agar with 25 µg naladixic acid/ml to select for S. Typhimurium WI-73. Results are reported as CFU observed / CFU expected.

Study II. Since in Study I the two additives with highest % recovery following freezing were glycerol and BPW (see below), we designed a randomized incomplete block study to test the hypothesis that certain combinations of these two compounds would result in higher % CFU recovery after freezing than others. Fresh feces from ~50 kg growing pigs were collected <6 h before inoculation. Four final concentrations (0%, 20%, 40%, and 60%) of each additive were evaluated.

The inoculum was prepared and enumerated for each test condition as described for Study I and was kept on ice or in a cold room at all times during before mixing with the feces and cryopreservative agents. The final concentration of organisms in each fecal mixture contained ~10^3 CFU / g feces, to approximate what might occur in fresh animal feces from *Salmonella* shedding commercial swine. The fecal mixtures were immediately frozen after 100 µL of the fecal mixture was plated on XLT-4 agar containing 25 µg/ml naladixic acid. Results are reported as the % of expected *Salmonella* CFU per g fecal sample. Statistical analysis was by SAS PROC MIXED, with levels of treatments treated as nominal variables. Pairwise comparisons used the Tukey-Kramer adjustment to guard against inflated experiment-wise error rate.

Results

Study I. For autoclaved feces, the highest number of CFU / mL mixed solution were detected before freezing in samples comprised of 2.5 g feces and 7.5 g BPW. After freezing the highest CFU / g was detected in samples comprised of 5 g feces, 2.5 mL glycerol and 2.5 mL BPW (Table 1).

Table 1. The % recovery of *Salmonella* Typhimurium from porcine feces (autoclaved or fresh) with or without the addition of glycerol, tetrathionate broth (TTB) or buffered peptone water (BPW) after freezing. Treatment groups A-F were plated on blood agar and treatment groups (G-H) were plated on XLT-4 agar containing 25 µg naladixic acid / mL. Results are reported as the % of expected *Salmonella* CFU per g fecal sample.

Treatment Group	Cryopreservative Agent	Autoclaved Feces	Fresh Feces	*Salmonella* strain	% of expected CFU recovered
A	-	+	-	Derby WI-9	11%
B	Glycerol	+	-	Derby WI-9	45%
C	Glycerol, TTB	+	-	Derby WI-9	63%
D	Glycerol, BPW	+	-	Derby WI-9	66%
E	Glycerol, Tris buffer	+	-	Derby WI-9	58%
F	BPW	+	-	Derby WI-9	30%
G	-	-	+	Typhimurium WI-73	4%
H	BPW	-	+	Typhimurium WI-73	27%

Study II.

The average percentage recovery varied from 11% for samples frozen without BPW or glycerol to 83% for samples frozen with BPW (40% final concentration) and glycerol (20% final concentration. The addition of any of tested glycerol ($p = 0.01$) or BPW concentrations ($p < 0.01$) to the fecal mixture before freezing resulted in a higher recovery of *Salmonella* after freezing. When compared to other combinations of glycerol and BPW, 40% BPW plus 20% glycerol resulted in the highest recovery of *Salmonella* from frozen feces. This combination, when compared with all specific treatment combinations higher recover than samples frozen with feces only ($p < 0.01$), 20% BPW and 20% glycerol ($p = 0.02$), 40% BPW and 0% glycerol ($p = 0.01$), and 0% BPW and 20% glycerol ($p = 0.07$).

Discussion

Freezing of fresh or autoclaved feces spiked with Salmonella without the addition of a cryopreservative agent resulted in poor recovery of *Salmonella*. We failed to recover 89% of the inoculum in autoclaved feces and 96% of the inoculum in fresh feces (Table 1). These results are in agreement with a prior report in which approximately 85% of *Salmonella* were non-culturable from fresh bovine feces inoculated with *S.* Typhimurium after freezing at -70 or -20C (Daniels).

Study I results suggest that the addition of glycerol immediately prior to freezing may protect *Salmonella* from the deleterious effects of freezing and that this protection is greatest when TTB or BPW, both of which contain peptones, are also added. BPW has been commonly used to increase sensitivity of conventional culture for *Salmonella* after freezing. TTB is commonly used as a first step in *Salmonella* enrichment bacterial culture.

Study II confirmed that glycerol and BPW are both protective during freezing, used either alone or in combination. 20-40% final concentration of each compound added in combination resulted in the highest recovery. This observation suggests that the protective effects of glycerol and BPW are additive.

Conclusions

The addition of glycerol and BPW may result in an increased rate of recovery of *Salmonella* from frozen fecal samples. Thus, these or other cryopreservatives may be useful to improve the logistics and to reduce the costs of epidemiologic studies. Since porcine fecal samples are expected to contain low numbers of *Salmonella* and because available quantitative techniques are cumbersome and/or costly, it may be advantageous to screen fecal samples qualitatively first, freeze paired samples, then thaw and enumerate only those which test positive. This may reduce

the cost of epidemiologic studies and enable *Salmonella* quantification as a practical outcome for quality assurance or food safety programs.

References

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