Evaluation of Universal Preenrichment Broth for the Recovery of Foodborne Pathogens from Milk and Cheese

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ABSTRACT

The use of universal preenrichment broth for the recovery of verotoxigenic *Escherichia coli*, *Salmonella* spp., and *Listeria monocytogenes* from milk and cheese was examined. Universal preenrichment broth supported the growth of low inoculum levels (10 cfu/ml) of these organisms in pure cultures and in mixed cultures containing higher levels of other pathogens or bacterial flora from raw milk. This medium also supported the recovery and growth of heat-injured *Salmonella* spp., *L. monocytogenes*, and verotoxigenic *E. coli* at inoculum levels of 10^2 cfu/ml to yield cell levels of 10^8 cfu/ml in pure cultures and at least 10^5 cfu/ml in the presence of high levels of known competitive pathogens or microflora of cheese samples after 24 h of incubation. Universal preenrichment broth performed better than *Listeria* enrichment broth in supporting the recovery and growth of heat-injured *L. monocytogenes* and equally as well as buffered peptone water or trypticase soy broth in supporting the growth of uninjured *L. monocytogenes*, *Salmonella* spp., and verotoxigenic *E. coli*. Coenrichment of these pathogens in universal preenrichment broth reduced the quantity of milk or cheese samples that were required for analysis and also reduced the cost and labor involved in preparing and processing separate preenrichment media.

(**Key words**: universal preenrichment broth, foodborne pathogens, milk, cheese)

Abbreviation key: **BHI** = brain-heart infusion, **BPW** = buffered peptone water, **LEB** = *Listeria* enrichment broth, **NAL** = nalidixic acid, **NB** = nutrient broth, **TSB** = trypticase soy broth, **UPB** = universal preenrichment broth, **VTEC** = verotoxigenic *E. coli*.

INTRODUCTION

The recovery of bacterial pathogens from foods, including raw milk and dairy products, is often complicated by the presence of high numbers of indigenous microflora and other pathogens and because the pathogens of interest may be sublethally injured at the time of testing (5, 12). Indigenous microflora in food can interfere with the isolation of pathogens by multiplying exponentially, causing a decrease in the pH of isolation media, which may inhibit the growth and recovery of pathogens (1, 2, 10). Sequential enrichment in nonselective (preenrichment) and selective media is usually required for the detection and identification of foodborne pathogens. Currently, the commonly used preenrichment media include buffered peptone water (**BPW**), trypticase soy broth (**TSB**), and nutrient broth (**NB**) (3, 10, 11). The recovery of *Listeria* spp. from foods, as currently performed, requires the use of selective enrichment media, such as *Listeria* enrichment broth (**LEB**), containing antibiotics that are inhibitory to competitive microorganisms (1, 6). However, these antibiotics may inhibit the recovery of sublethally injured *Listeria* spp. (9). A nonselective medium, which allows simultaneous enrichment of these pathogens from foods, including milk and dairy products, would offer savings in media, processing time, incubator space, and sample size required for analysis.

Universal preenrichment broth (**UPB**) was recently developed for use as a common preenrichment step for both *Listeria monocytogenes* and *Salmonella* spp. from foods (1). This medium is strongly buffered to prevent the decrease in the pH of the medium that can occur as a result of heavy microbial growth. A reduction in pH has been shown to interfere with the recovery of *L. monocytogenes* from foods (1), and media such as LEB do not contain a sufficient amount of buffers to prevent a rapid decline in the pH of the broth (7). A study on the recovery of *L. monocytogenes* and *Salmonella* spp. from chicken, hot

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dogs, and Brie cheese showed that UPB can be used as an effective preenrichment medium for these organisms in foods, despite high levels of competing microorganisms (1). Universal preenrichment broth supplemented with Oxyrase has been shown to support rapid growth of pure cultures of *Salmonella* spp. and *L. monocytogenes* (8). The present study examined the ability of UPB to support the growth of healthy and heat-injured strains of *L. monocytogenes*, *Salmonella* spp., and verotoxigenic *Escherichia coli* (**VTEC**) in pure cultures and in samples of raw milk and cheese containing competing pathogens or microflora. The effects of the competitive microflora from raw milk and cheese and of the presence of high levels of other known pathogens on the growth of these organisms in UPB were also examined.

MATERIALS AND METHODS

Broth Media

Universal preenrichment broth (Difco, Detroit, MI), LEB (BBL, Cockeysville, MD), BPW (Oxoid, Hampshire, England), TSB (Difco), and NB (BBL, Cockeysville, MD) media were prepared according to the instructions of the manufacturer and were stored at 4°C until use.

Bacterial Strains

Four strains each of *Salmonella* spp., *L. monocytogenes*, and VTEC were selected for this study. Two strains per pathogen were obtained from the American Type Culture Collection (Rockville, MD). These strains included *L. monocytogenes* ATCC 19115 and ATCC 19116, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* ATCC 14028, and *E. coli* O157:H7 ATCC 43889 and ATCC 43894. The other two strains were isolates that were obtained from raw milk in this laboratory. Two nalidixic acid (**NAL**)-resistant strains of *Salmonella* spp. (provided by the Health of Animals Laboratory, Health Canada, Guelph, ON, Canada) were used for inoculations of raw milk and cheese to allow differentiation of test strains of *Salmonella* spp. from microflora in milk and cheese. The cultures were transferred from frozen storage tubes to brain-heart infusion (**BHI**) broth (BBL) and were incubated at 35°C for 18 to 24 h. A loop of broth culture was inoculated onto BHI agar. The agar was incubated at 35°C for 18 to 24 h, and the resulting cultures were kept at 4°C for use in individual experiments.

Preparation of Bacterial Cell Suspension

Selected strains were grown on BHI agar for 18 h at 35°C. The cultures were harvested with a cotton swab and were suspended in 0.85% physiological saline to obtain a cell suspension with an optical density reading of 0.1 using a turbidity meter (MicroScan™; Baxter Diagnostics Inc., Deerfield, IL). The bacterial concentration of this cell suspension in saline solution was approximately 10^8 cfu/ml. The colony-forming units per milliliter of the cell suspension were confirmed by plating an aliquot of the suspension on BHI agar and incubating the agar for 18 h at 35°C.

Heat Treatment of Bacterial Strains in Pure Culture

One milliliter (10^8 cfu) of the culture suspension in saline, prepared as described previously, was added to a 99-ml bottle of preheated TSB (Difco) and transferred to a circulating water bath for heat treatments. *Salmonella* spp. were heated at 48°C for 30 min (1), and strains of *L. monocytogenes* and VTEC were heated at 55°C for 20 min (1, 12). After the heat treatment was complete, the bottle was removed from the water bath and was immediately cooled in an ice water bath to prevent additional injury to the bacteria. The proportion of the bacterial cells that were injured by the heat treatment was determined by the difference in growth of the cells on BHI agar and on brilliant green sulfa agar plus 2% NaCl for *Salmonella* spp., or BHI agar plus 4% NaCl for *L. monocytogenes*, or BHI agar plus 2% NaCl for VTEC.

Growth in the Presence of High Levels of Competing Pathogenic Bacteria in Pure Culture

Healthy cells suspended in saline solution (10^8 cfu/ml) and heat-treated cells suspended in TSB were prepared as described previously and were inoculated into 2% UHT-sterilized milk (Beatrice Foods Inc., Toronto, ON) at 10^2 cells/ml. Two-log higher concentrations of one of the other pathogens included in this study were added to the UHT milk as competing organisms. One milliliter of each UHT milk sample containing competing pathogens was inoculated into tubes containing 9 ml of the different preenrichment medium or enrichment broth medium to final concentrations of 10 cells/ml of the test strains and 10^3 cells/ml of the competing bacteria. Aliquots of the inoculated preenrichment medium or enrichment broth medium were spiral plated on selective media before

TABLE 1. Growth of unstressed organisms in pure cultures in different broth media incubated at 35°C for 18 and 24 h.

Organism	Inoculum	UPB[1]		BPW[2]		TSB		NB	
		18 h	24 h	18 h	24 h	18 h	24 h	18 h	24 h
	(cfu/ml)								
Salmonella spp.	10^1	10^8	10^8	10^8	10^8	10^9	10^9	10^8	10^8
Salmonella spp.	10^3	10^8	10^8	10^8	10^8	10^9	10^9	10^8	10^8
Listeria monocytogenes	10^1	10^8	10^8	10^5	10^7	10^8	10^9	10^7	10^7
L. monocytogenes	10^3	10^8	10^8	10^7	10^7	10^9	10^9	10^7	10^7
VTEC[3]	10^1	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8
VTEC	10^3	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8

[1]UPB = Universal preenrichment broth, BPW = buffered peptone water, TSB = trypticase soy broth, and NB = nutrient broth.

[2]The *Listeria* enrichment broth, was used in place of BPW for *L. monocytogenes* strains only.

[3]Verotoxigenic *Escherichia coli*.

and after incubation at 35°C for 24 h to determine the increase in colony-forming units per milliliter of the pathogens of interest. The selective media used were bismuth sulfite agar for *Salmonella* spp., Oxford agar for *L. monocytogenes*, and sorbitol-MacConkey's agar for VTEC.

Growth of Unstressed Bacterial Pathogens in the Presence of Raw Milk Flora

Saline cell suspensions (10^8 cfu/ml) of healthy untreated cells of NAL-resistant *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157:H7 were prepared as described in previous sections and were inoculated into raw milk at 10^2 cells/ml. The raw milk used in these experiments was pooled milk of test aliquots; milk was 2 to 3 d old and was obtained from Laboratory Services Division (University of Guelph, Guelph, ON, Canada). One milliliter of raw milk was inoculated with competing pathogens and was inoculated into preenrichment broth, enrichment broth, or both media to final concentrations of 10 cells/ml of the test strains. The concentration of the microflora in raw milk was determined by spiral plating of an aliquot of the raw milk on BHI agar followed by incubation at 35°C for 18 h. Aliquots of the inoculated preenrichment broth, enrichment broth, or both media were spiral plated on selective media before and after incubation at 35°C for 24 h. The selective media used were brilliant green sulfa agar plus 200 ppm of NAL for NAL-resistant *Salmonella* spp. and modified Oxford agar for *L. monocytogenes*. The Petrifilm Test Kit-HEC (3M Health Care, St. Paul, MN) was used to detect and count *E. coli* O157:H7.

Growth of Heat-Injured Pathogenic Bacteria in Cheese Samples

Cheese samples (sliced extra aged Cheddar cheese and double cream Brie cheese) were purchased from a local grocery store. Test strains of NAL-resistant *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157:H7 were suspended in saline solution and heat treated as described in previous sections. Heat-injured cultures were inoculated into different broth media in stomacher bags containing cheese slurries (1 part cheese to 9 parts broth) at a final concentration of 10^2 cfu/ml of each test pathogen. Total aerobic plate counts of the cheese samples were determined. An aliquot of the cheese slurries was plated and was incubated for 18 h at 35°C. The selective media used for quantifying the growth and recovery of heat-injured organisms from cheese were the same as those used for raw milk.

RESULTS AND DISCUSSION

Growth of Pure Cultures of Test Strains in Different Broth Media

Ultra-high temperature milk that had been inoculated with competing pathogens at concentrations of 10^2 and 10^4 cfu/ml of each pathogen was used to inoculate UPB, BPW, TSB, and NB (LEB in place of BPW for *L. monocytogenes*) media to initial concentrations of 10^1 and 10^3 cfu/ml. The growth results for *Salmonella* spp., *L. monocytogenes* and VTEC strains after 18 and 24 h of incubation at 35°C are summarized in Table 1.

The *Salmonella* strains multiplied to at least 10^8 cfu/ml after 18 h of preenrichment at 35°C. Among

TABLE 2. Growth of pure cultures of heat injured organisms in different broth media incubated at 35°C for 24 h.

Organism	Inoculum	UPB[1]	BPW[2]	TSB
	(cfu/ml)			
Salmonella spp.	10^2	10^8	10^8	10^8
Listeria monocytogenes	10^2	10^8	10^5	10^8
VTEC[3]	10^2	10^9	10^9	10^9

[1]UPB = Universal preenrichment broth, BPW = buffered peptone water, and TSB = trypticase soy broth.

[2]The *Listeria* enrichment broth, was used in place of BPW for *L. monocytogenes* strains only.

[3]Verotoxigenic *Escherichia coli*.

the four different broth media, TSB best supported the growth of all four *Salmonella* strains; cell concentrations reached 10^9 cfu/ml after 18 or 24 h incubation. The UPB, BPW, and NB had similar abilities to support the growth of *Salmonella* pure cultures. No growth difference was observed between the 18- and 24-h incubations.

All VTEC strains multiplied to 10^8 cfu/ml after 18 h of preenrichment at 35°C in each of the four broth media. There was no difference between the colony-forming units per milliliter at 18 and 24 h of incubation.

Strains of *L. monocytogenes* were able to reach concentrations of at least 10^7 cfu/ml in UPB, TSB, and NB after 18 h of incubation at 35°C from initial inoculum levels of 10 cfu/ml. The resulting concentrations of *L. monocytogenes* strains were lower in LEB than in the other three broth media, particularly at the low initial level (10^1 cfu/ml) and short incubation time (18 h) (Table 1). Inhibitory agents in the LEB probably interfered with the growth of *L. monocytogenes*. In addition, the bacterial counts after 24 h of incubation in LEB were higher than after 18 h of incubation for most *L. monocytogenes* strains. *Listeria monocytogenes* exhibited slower growth in LEB than in UPB or TSB, especially at the inoculum concentration of 10^1 cfu/ml.

Recovery of Heat-Injured Pure Cultures in Different Broth Media

The UHT milk that was contaminated with heat-injured pathogens was inoculated into three different types of media, UPB, BPW, or TSB (LEB in place of BPW for *L. monocytogenes*) at inoculum concentrations of 10^2 cfu/ml. The bacterial counts for all three pathogens after 24 h of incubation at 35°C are summarized in Table 2.

Pure cultures of heat-injured *Salmonella* spp. and VTEC multiplied to at least 10^8 cfu/ml in the UPB, BPW, and TSB media. Pure cultures of heat-injured *L. monocytogenes* multiplied to 10^5 cfu/ml in LEB and to 10^8 cfu/ml in UPB or TSB. Inhibitors in LEB may have affected the recovery of the heat-injured cells of *L. monocytogenes*.

Effect of Raw Milk Microflora on the Growth of Unstressed Test Pathogens

The growth of pure cultures of all three test organisms in the presence of raw milk flora are summarized in Table 3. Two cultures of NAL-resistant *Salmonella* spp. that were unstressed were used to evaluate the effect of the competitive microflora in raw milk on the growth of *Salmonella* spp. Cultures were plated on media containing NAL to distinguish the test strains from raw milk flora. The total aerobic plate count of 3-d old samples of raw milk was 8.3×10^2 cfu/ml. Both NAL-resistant *Salmonella* strains reached a concentration of 10^8 cfu/ml in all three broth media within the 24-h incubation, showing that the growth of *Salmonella* spp. was not inhibited by the microflora of raw milk.

The Petrifilm kit that was specific to *E. coli* O157:H7 was used to test the effect of the flora of raw milk on VTEC strains. *Escherichia coli* O157:H7 strains ATCC 43889 and 43894 grew to a concentration of 10^8 cfu/ml in all three broth media from an initial level of 10 cfu/ml in the presence of raw milk flora. Therefore, the flora of raw milk did not affect the growth of VTEC cultures.

Unlike *Salmonella* spp. and VTEC, the growth of *L. monocytogenes* was inhibited by the flora of raw milk in all preenrichment or enrichment broths.

TABLE 3. Growth of unstressed organisms in the presence of raw milk microflora in different broth media incubated at 35°C for 24 h.

Organism	Inoculum	UPB[1]	BPW[2]	TSB
	(cfu/ml)			
Salmonella spp.	10^1	10^8	10^8	10^8
Listeria monocytogenes	10^1	10^4	10^6	10^4
VTEC[3]	10^1	10^8	10^8	10^8

[1]UPB = Universal preenrichment broth, BPW = buffered peptone water, and TSB = trypticase soy broth.

[2]The *Listeria* enrichment broth, was used in place of BPW for *L. monocytogenes* strains only.

[3]Verotoxigenic *Escherichia coli*.

TABLE 4. Growth of heat injured organisms in the presence of cheese microflora in different broth media incubated at 35°C for 24 h.

Organism	Inoculum	UPB[1]	BPW[2]	TSB
	(cfu/ml)			
Salmonella spp.	10^2	10^8	10^8	10^8
Listeria monocytogenes	10^2	10^5	10^3	10^4
VTEC[3]	10^2	10^8	10^8	10^8

[1]UPB = Universal preenrichment broth, BPW = buffered peptone water, and TSB = trypticase soy broth.

[2]The *Listeria* enrichment broth, was used in place of BPW for *L. monocytogenes* strains only.

[3]Verotoxigenic *Escherichia coli*.

Listeria monocytogenes attained 10^6 cfu/ml in LEB but only 10^4 cfu/ml in UPB and TSB in the presence of raw milk flora. The growth of raw milk flora appeared to be inhibited in LEB, allowing the growth of *L. monocytogenes* to reach a level that was 2 logs higher in the LEB broth than in the UPB and TSB broths.

Effect of Microflora from Cheese Samples on the Recovery of Heat-Injured Test Pathogens

Heat-injured *Salmonella* spp. and VTEC strains attained levels of 10^8 cfu/ml in the presence of normal flora from both Brie Cheese and extra aged Cheddar cheese. The recovery of heat-injured *Salmonella* spp. and VTEC strains was not affected by the microflora of the cheese sample, and the three different broth media that were tested showed a similar ability to support the growth and recovery of heat-injured *Salmonella* spp. and VTEC (Table 4).

The recovery of heat-injured *L. monocytogenes* was strongly inhibited by the microflora in the cheese samples. The *L. monocytogenes* reached a level of 10^5 cfu/ml in UPB and 10^4 cfu/ml in TSB, but we recovered substantially less *L. monocytogenes* from the LEB (Table 4). Because cheese manufacturing usually involves thermal processing, exposure to this kind of sublethal environmental stress may result in the loss of the ability by microorganisms to grow under conditions that are satisfactory for unstressed cells (4). Selective agents in a medium, such as LEB, have been shown to inhibit the growth of sublethally damaged microorganisms compared with growth of cells that have not been exposed to environmental stress (9). The UPB broth medium, however, is a nonselective preenrichment broth that does not contain inhibiting agents. In addition, UPB is strongly buffered to prevent the drop in media pH that occurs as a result of heavy microbial growth and that can interfere with the recovery of *L. monocytogenes* cells.

Growth of Pathogens in Broth Media in the Presence of High Levels of Other Competing Pathogens

Unstressed strains of *Salmonella* spp. in pure cultures multiplied to 10^8 cfu/ml in all three broth media within 24 h in the presence of 2 log higher initial inoculum levels of *L. monocytogenes* (Table 5), suggesting that the growth of *Salmonella* spp. was not affected by competing *L. monocytogenes*. In the presence of a high level of competitive VTEC, however, the level of *Salmonella* spp. attained was only 10^6 cfu/ml in BPW and TSB and 10^7 cfu/ml in UPB under the same conditions, a reduction of about 1 to 2 log caused by the presence of VTEC.

The growth of *L. monocytogenes* in broth media was inhibited by the presence of either *Salmonella* spp. or VTEC for UPB and TSB (10^6 cfu/ml) compared with results for LEB (10^8 cfu/ml) (Table 5). The inhibitory agents in LEB appear to limit the growth of these competing pathogens.

The growth of VTEC in enrichment or preenrichment media was not significantly affected by the presence of *Salmonella* spp. or *L. monocytogenes*. A pure culture of VTEC multiplied to 10^8 cfu/ml in UPB and 10^7 cfu/ml in BPW or TSB in the presence of high levels of *Salmonella* spp. In the presence of *L. monocytogenes*, VTEC reached 10^8 cfu/ml in UPB and TSB and 10^7 cfu/ml in BPW (Table 5).

TABLE 5. Growth of unstressed organisms in the presence of high levels of other competing pathogens in different broth media incubated at 35°C for 24 h.

Organism	Inoculum	UPB[1]	BPW[2]	TSB
	(cfu/ml)			
Salmonella spp. with *Listeria monocytogenes*	10^1	10^8	10^8	10^8
Salmonella spp. with VTEC[3]	10^1	10^7	10^6	10^6
L. monocytogenes with *Salmonella* spp.	10^1	10^6	10^8	10^6
L. monocytogenes with VTEC	10^1	10^6	10^8	10^6
VTEC with *Salmonella* spp.	10^1	10^8	10^7	10^7
VTEC with *L. monocytogenes*	10^1	10^8	10^7	10^8

[1]UPB = Universal preenrichment broth, BPW = buffered peptone water, TSB = trypticase soy broth.

[2]The *Listeria* enrichment broth, was used in place of BPW for *L. monocytogenes* strains only.

[3]Verotoxigenic *Escherichia coli*.

CONCLUSIONS

Universal preenrichment broth can be an effective preenrichment medium for the recovery of *Salmonella*, *L. monocytogenes*, or VTEC from samples of raw milk or cheese. The UPB performed better than did LEB for recovery of heat-injured *L. monocytogenes* in the presence of cheese microflora. Therefore, UPB might offer an advantage when heat injury is a factor. However, LEB is better when a high background of flora is expected and the target organisms are not injured.

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