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Evaluation of buffered peptone water (BPW) containing sodium thioglycolate as enrichment broth in combination with BPW as pre-enrichment broth for isolation of *Salmonella* from radish sprouts

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Abstract

The buffered peptone water (BPW) containing 0.5% sodium thioglycolate (BPWST) as enrichment broth in combination with BPW as pre-enrichment broth for strains of *Salmonella* from radish sprouts was evaluated. False-positive colonies were found on CHROMagar Salmonella agar used as an isolation medium in 28 out of 30 radish sprout samples (93%) on incubation using BPW, while such colonies were found in 3 samples (17%) using BPWST. The growth of non-starved and starved *Salmonella* strains in BPWST was found to range from 10^5 - 10^7 CFU/ml, while non-starved *Pseudomonas* which produces false-positive colonies was markedly inhibited, at <100 CFU/ml. All starved inoculated *Salmonella* strains were recovered from radish sprouts, and they were detected in small numbers on starved *Salmonella* inoculation using both BPW as pre-enrichment and BPWST as enrichment （BPW-BPWST method）. The use of the BPW-BPWST method is effective for the isolation of *Salmonella* from radish sprouts. It is thought that the findings obtained from this study can be applied for the isolation of *Salmonella* from environments and various hydroponically cultivated raw vegetables.

Key words: enrichment broth, pre-enrichment broth, buffered peptone water, detection, radish sprouts, *Salmonella*, sodium thioglycolate

Introduction

Raw vegetables are contaminated by many bacterial species of gram-negative facultative anaerobic rod (Fujisawa and Mori 1992). Gram-negative aerobic rods such as *Pseudomonas* are also usually present in water, air, soil, and on plants (Cousin 2000).

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In the isolation of food-poisoning-associated facultative anaerobic rods from raw vegetables, the presence of other gram-negative facultative anaerobes and aerobes hinders analysis, since most bacteria grow on selective agar media used for the isolation of these foodborne pathogenic bacteria, such as MacConkey agar, DHL agar, and CHROMagar Salmonella agar. Therefore, it is necessary to inhibit the growth of these bacteria during the isolation of foodborne pathogenic bacteria from raw vegetables. Although bacteria exhibiting different colonial morphologies from those of the target bacteria on selective agar may pose less of a problem, bacteria with a similar colonial morphology to that of the target bacteria are particularly problematic. CHROMagar Salmonella agar has been recommended as an effective selective agar medium (Gaillot et al. 1999; Maddocks et al. 2002). This agar

medium was developed for the isolation of *Salmonella* from various samples such as foods and clinical specimens. *Salmonella* developed mauve-colored colonies on this medium, and other *Enterobacteriaceae* yielded blue-colored or colorless colonies. Conventional media for the detection of *Salmonella* by virtue of its H_2S production show poor specificity, leading to a high rate of false-positives (*Citrobacter*, *Proteus*, etc.), and there are also H_2S-negative isolates. CHROMagar Salmonella agar can distinguish *Salmonella* from other bacteria by virtue of the colony color (Gaillot et al. 1999; Maddocks et al. 2002). However, *Pseudomonas aeruginosa* also produces false-positive colonies on this agar (Gaillot et al. 1999). Therefore, inhibition of the growth of this bacterium is necessary. On the other hand, it seems that food-poisoning bacteria which contaminate raw vegetables, especially those grown hydroponically, such as radish sprouts, are nutritionally starved (Sata et al. 2003). These bacteria are present in the water (Beuchat 1996) and seem to be injured (Terzieva and McFeters 1991). There is a possibility that these starved bacteria cannot grow in enrichment broth containing selective agents or at a high incubation temperature (Sata et al. 1999). It has been suggested that the sublethal injury of salmonellae may occur as a result of many food-related processes (Edel and Kampelmacher 1973; Wesche et al. 2009). Corry et al. (1969) reported that incubation at 43℃ in either selenite or tetrathionate medium is not suitable for heat-damaged salmonellae. Bacteria can survive stressful conditions such as limited nutrient availability, osmotic perturbation, large variations in temperature and pH, and predation by entering a viable but nonculturable (VBNC) state (Winfield and Groisman 2003). Based on these facts, the use of enrichment broth not containing selective agents for enrichment incubation may be essential for the isolation of foodborne bacteria from raw vegetable samples. It has been reported that buffered peptone water (BPW) containing sodium thioglycolate is effective for the isolation of *E. coli* O157:H7 from radish sprouts, since the growth of aerobic bacteria is inhibited and *E.coli* O157:H7 is starved in a water system (Sata et al. 2003). Sodium thioglycolate is often employed as a reducing agent in media used to support the growth of anaerobic bacteria. *Salmonella* has also been isolated from various raw vegetables (Garcia-Veillanova Ruiz et al. 1987; Beuchat 1996; Tauxe et al. 1997), and outbreaks of salmonellosis traced to the consumption of raw vegetables have been reported throughout the world (Beuchat 1996; Tauxe et al. 1997). In 2005, an outbreak of salmonellosis traced to the intake of radish sprouts occurred in Japan (Watanabe et al. 2006). Incidentally, the use of pre-enrichment medium is also recommended for food samples likely to contain low numbers of *Salmonella* that may have been stressed or injured (D'Aoust 1989). As pre-enrichment broth for the isolation of *Salmonella* from foods, non-selective media such as BPW are used widely. In this study, we evaluated the use of BPW as pre-enrichment broth and BPW containing sodium thioglycolate as enrichment broth for the isolation of starved *Salmonella* from radish sprouts.

Materials and Methods

Bacterial growth in BPW and BPW containing sodium thioglycolate using radish sprouts. The thirty radish sprouts used in this study were obtained from a retail store in Japan. Ten grams of radish sprouts were incubated in 90 ml of BPW (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) or 90 ml of BPW containing 0.5% sodium thioglycolate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (BPWST), mixed using a stomacher (Pro-media, SH-001; ELMEX, Ltd., Tokyo, Japan) for 30 s, and incubated at 35℃ for 18 - 19 h. After incubation, the broth was serially diluted 10-fold in sterile saline. A total of 0.1 ml of the broth and diluted broth was spread onto each CHROMagar Salmonella agar, and incubated at 35℃ for 24±2 h. After incubation, three to ten mauve-colored colonies on CHROMagar Salmonella agar per one sample were picked up randomly and tested for fermentations of lactose, sucrose, and glucose as well as the production of H_2S using TSI agar medium (Nissui). Moreover, the lysine decarboxylase test using LIM medium (Nissui), cytochrome oxidase test using test paper (Nissui), and investigation of the *Salmonella invA* gene, the *Salmonella* invasion gene (Galán et al. 1992), using a *Salmonella invA* gene one-step PCR screening Kit (Takara Bio Inc., Shiga, Japan) were also conducted.

Isolates tested and preparation of starved cells. Nineteen serovars, comprising fifty strains of *Salmonella* listed in Table 1, and *P. aeruginosa* ATCC 35554, *P. putida* ATCC 31483, *P. fluoroecsen* ATCC 13525 and two strains of *P. aeruginosa* were used in this study. All *Salmonella* strains tested were from the caecal contents of healthy, recently slaughtered pigs, and two strains of *Ps. aeruginosa* were from human faeces. To prepare the starved cells, ten ml of isolates following overnight culture at 35℃ in BPW was washed three times using sterilized distilled water, and then the cell pellet was suspended in 30 ml of sterilized distilled water and kept at 25℃ for 21 days in a dark room employing the modification of a method described previously (Sata et al. 2003). Suspensions of starved cells were diluted using sterilized saline and spread onto nutrient agar (Nissui) to count bacterial numbers. Viable counts of the isolates starved in distilled water after treatment were 2.8×10^4 - 3.4×10^6 CFU/ml.

Growth of *Salmonella* and *Pseudomonas* in buffered peptone water containing sodium thioglycolate. The fifty strains of *Salmonella* and five strains of *Pseudomonas* incubated in BPW at 35℃ for 24 h, and five starved *Salmonella* isolates (one strain each of *S*. Anatum, *S*. Infantis, and *S*. Panama, and two of *S*. Typhimurium) were

diluted with sterile saline, and then 0.1 ml of diluted broth culture was spread onto nutrient agar to count bacterial numbers and also used to inoculate 10 ml of BPW and 10 ml of BPWST. They were then incubated at 35℃ for 18 - 19 h. After incubation, the broth was serially diluted 10-fold in sterile saline. Diluted broth (0.1 ml) was spread onto nutrient agar and commercially prepared CHROMagar Salmonella agar plates (Kanto Chemical Co., Inc., Tokyo, Japan), and incubated at 35℃ for 24±2 h. After incubation, the number of colonies was counted.

Recovery of *Salmonella* from BPWST used in the incubation of starved *Salmonella* cells with radish sprouts. A total of 0.1 ml of different numbers of starved cells from eighteen starved strains (one strain each of *S.* Blockley, *S.* Brandenburg, *S.* Cerro, *S.* Derby, *S.* Fridrichsfelde, *S.* Give, *S.* Havana, *S.* Heidelberg, *S.* Infantis, *S.* Johannesburg, *S.* Kiambu, *S.* Litchfield, *S.* London, *S.* Mbandaka, *S.* Panama, *S.* Senftenberg, *S.* Typhimurium, and *S.* Yaounde) was incubated with 10 g of radish sprouts in 90 ml of BPW at 35℃ for 24 h. One strain of *Salmonella* was inoculated per one sample of radish sprouts. Before incubation, all prepared samples were mixed using a stomacher for 30 s. After incubation, 0.01 ml of each broth was used to inoculate 10 ml of BPWST and incubated at 35℃ for 18 - 19 h (BPW-BPWST method). Incubated BPWST broths were diluted with sterilized saline, 0.1 ml of broth and diluted broth were spread onto each CHROMagar Salmonella agar and incubated at 35℃ for 24 ± 2 h, and three to ten mauve-colored colonies grown on an agar plate per one sample were picked up and identified as described above.

Statistical analysis. The significance of the frequency of mauve-colored non-*Salmonella* colonies in BPW and BPWST using radish sprouts was assessed employing MacNemar's test. Furthermore, a comparison of the data on *Salmonella* and *Pseudomonas* counts grown in BPW and BPWST shown in Table 2 was carried out using the paired *t* - test. Data were converted to log CFU/ml for statistical analysis. *P* - values of < 0.05 revealed a significant difference.

Table 1. *Salmonella* strains tested.

Serovar	Number of strains	Serovar	Number of strains
Salmonella Anatum	3	*Salmonella* Johannesburg	4
Salmonella Blockley	1	*Salmonella* Kiambu	1
Salmonella Brandenburg	2	*Salmonella* Litchfield	1
Salmonella Cerro	1	*Salmonella* London	2
Salmonella Derby	10	*Salmonella* Mbandaka	1
Salmonella Fridrichsfelde	1	*Salmonella* Panama	4
Salmonella Give	1	*Salmonella* Senftenberg	1
Salmonella Havana	1	*Salmonella* Typhimurium	7
Salmonella Heidelberg	2	*Salmonella* Yaounde	1
Salmonella Infantis	6		
		Total	50

Results

The frequency of mauve-colored colonies, similar to the color of *Salmonella* colonies, in the two enrichment broths (BPW and BPWST) incubated with radish sprouts, was significantly lower ($p < 0.05$) on incubation using BPWST

(17%) compared with BPW (93%). Namely, mauve-colored colonies occurred in 5 out of 30 samples on incubation using BPWST, while they occurred in 28 out of 30 samples on incubation using BPW. Furthermore, no mauve-colored.

colonies picked up and tested in these experiments were identified as *Salmonella*.

The growth of *Salmonella* and *Pseudomonas* strains in BPWST was lower than that in BPW, as shown in Table 2.

Similarly, the growth of starved cells of *Salmonella* was also significantly lower ($p < 0.05$) as compared with that in BPW. The growth of *Salmonella* was found to range from 10^5 - 10^7 CFU/ml of BPWST broth, while *Pseudomonas* was found to be < 100 CFU/ml. The colony color of all *Pseudomonas* strains tested on CHROMagar *Salmonella* agar was mauve.

All *Salmonella* strains tested were recovered from radish sprouts, and they were detected in small numbers on starved *Salmonella* inoculation using the BPW-BPWST method (Table3

Table 2. Growth of *Salmonella* and *Pseudomonas* incubated in buffered peptonewater and buffered peptone water containing 0.5% sodium thioglycolate at 35℃ for 18 - 19h.

Bacteria	Number of strains tested	Mean number of log CFU±SD/ml of broth (Range)			
		Nutrient agar		CHROMagar Salmonella agar	
		BPW[a]	BPWST[b]	BPW	BPWST
Salmonella (Non-starved cells[c])	50	8.16±0.27 (7.60 - 8.72)	7.16±0.59[e] (5.00 - 7.90)	8.05±0.31 (6.88 - 8.58)	7.08±0.64[e] (5.00 - 7.66)
Salmonella (Starved cells[d])	5	7.95±0.98 (6.20 - 8.58)	6.60±0.93[e] (5.60 - 7.41)	7.81±0.97 (6.08 - 8.30)	6.64±0.80[e] (5.78 - 7.34)
Pseudomonas (Non-starved cells[d])	5	7.48±0.80 (6.26 - 8.20)	< 2	7.19±1.12 (5.30 - 8.04)	< 2

[a]BPW, buffered peptone water.
[b]BPWST, buffered peptone water containing 0.5% sodium thioglycolate.
[c]Bacterial cell count for inoculation per sample was 1 - 9 CFU/ml of broth.
[d]Bacterial cell count for inoculation per sample was 10 - 60 CFU/ml of broth.
[e]The value is significantly different ($p < 0.05$) from that of BPW on the same agar plate (Paired *t*-test).

Table 3. Recovery of starved *Salmonella* incubated with radish sprouts in the enrichment broth.

Method of enrichment	Number of strains tested[a]	Range of bacterial counts of starved cells for inoculation per broth (CFU/ml)	Number of samples Recovered (%)
BPW[b]-BPWST[c] method	18[d]	Not inoculated	0 (0)
	18	1- 9	13 (72)
	18	10 - 94	18 (100)

[a] One strain of *Salmonella* was inoculated per one sample of radish sprouts.
[b]BPW, buffered peptone water.
[c]BPWST, buffered peptone water containing 0.5% sodium thioglycolate.
[d]Number of trials.

Discussion

In the present study, most isolates of mauve-colored colonies on CHROMagar Salmonella agar from radish sprouts were oxidase-positive. *Pseudomonas* is present in the environment (Cousin 2000), and so its growth inhibition is important in order to isolate *Salmonella* from food samples. Incidentally, cefsulodin-resistant *P. aeruginosa* has been detected (Mogi et al. 1996; Gaillot et al. 1999). For this reason, the use of antibiotics may not be recommended in the future.

Several studies have shown that the difference in the results of *Salmonella* recovery depend on the enrichment culture (Patil and Parhad 1986; Bager and Petersen 1991; Blivet et al. 1997; Davies et al. 2000; Harvey *et al*. 2001; Voogt et al. 2001; Osumi et al. 2003). Enrichment media also play an important role in the isolation of *Salmonella* from water, because this represents a nutrient-poor environment, and so *Salmonella* is usually in a state of stress or injury. Patil and Parhad (1986) reported that stressed strains of *Salmonella* are inhibited by selenite-F, MK-tetrathionate, and Rappaport-10 broths. Sata et al. (2003) reported that the use of non-selective enrichment broth should be performed as a routine practice for the optimal recovery of *E. coli* O157:H7 from water systems, and recommended the use of BPW for the isolation of *E. coli* O157:H7 from radish sprout-derived samples instead of modified *E. coli* broth supplemented with novobiocin. It has been suggested that BPW is preferable to isolate freeze-injured *Salmonella* from frozen vegetables (Sadovski 1977). Thomason et al. (1977) also reported that the use of BPW increased the rate of recovery of *Salmonella* from environmental samples.

The growth of starved *Salmonella* cells in BPWST was significantly lower ($p < 0.05$) compared with that in BPW, as shown in Table 2. It seems that the growth of *Salmonella* is somewhat influenced by the presence of sodium thioglycolate, and the use of a non-selective medium as a pre-enrichment medium is desirable to increase the detection rate. Pre-enrichment is recommended for materials such as food and environmental samples, likely to contain low numbers of *Salmonella* that may have been stressed or injured by factors such as thermal or osmotic shock, or freezing and thawing (D'Aoust 1989). Generally, the use of non-selective media such as BPW as a pre-enrichment medium for the isolation of *Salmonella* from food samples is recommended. In the BPW-BPWST method, BPW was used as a pre-enrichment medium, and it was shown that high-level recovery of *Salmonella* could be achieved employing this method. The frequency of mauve-colored non-*Salmonella* colonies, similar to the color of *Salmonella* colonies, in the two enrichment broths (BPW and BPWST) incubated with radish sprouts, was significantly lowered ($p < 0.05$) by incubation using BPWST (17%) compared with BPW (93%). Moreover, the growth of *Pseudomonas* was inhibited markedly in BPWST. These results indicate that the addition of sodium thioglycolate to non-selective enrichment broth is effective for the detection of *Salmonella* from radish sprouts in the isolation method using CHROMagar Salmonella agar, in agreement with the results of Sata *et al.* (2003). The BPW-BPWST method seems to be suitable to isolate starved *Salmonella* from raw radish sprouts. Further studies on the BPW-BPWST method using various strains are necessary to confirm its effectiveness.

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