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Short Communication

Isolation of *Salmonella* grown poorly at 42°C from radish sprouts

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Abstract

Incubation conditions using tetrathionate broth (TT) and Rappaport-Vassiliadis broth (RV), which are typically employed as enrichment broths, were investigated for the isolation of *Salmonella* Brandenburg grown poorly at 42°C from radish sprouts. A small number (10^2 CFU/ml of broth) of *Salmonella* with *Pseudomonas aeruginosa* were injected into TT and RV; however, *Salmonella* colonies were absent when these broths were incubated at 42°C. No differences were observed in the detection rates of colonies on either TT or RV incubated anaerobically at 35°C and aerobically at 35°C, with the exception of a small number of *Salmonella* with *P. aeruginosa* on RV. In case of mixed culture of *Salmonella* Brandenburg and *Escherichia coli* in TT and RV at 42°C, there were samples that *Salmonella* colonies were not detected. In the mixed culture of *Salmonella* Brandenburg and radish sprouts in TT and RV, the detection rate of *Salmonella* was better on incubation at 35°C than that at 42°C when a large number of *Salmonella* Brandenburg was injected (10^5 or 10^6 CFU/ml of broth). Moreover, the detection rate at colonies was better at 35°Cthan at 42°C in the case of the injection (10^2 CFU/ml of broth) into RV. On the other hand, no remarkable differences were observed in the detection rates of colonies on either TT or RV incubated anaerobically at 35°C and aerobically at 35°C. These results demonstrated that the isolation of *Salmonella* grown poorly at 42°C from radish sprouts using a high temperature incubation was not favorable.

Introduction

The official analytical method used in Japan to isolate *Salmonella* involves an enrichment incubation conducted at 42.0±0.5°C for its isolation from egg pulp, and 43.0±1.0°C or 35.0±1.0°C for its isolation from processed meat products. There are currently no official analytical methods for other foods, and 42.0±0.5°C has been shown by the National Institute of Health Sciences Japan in the Methods for the Microbiological Examination of Foods as the incubation temperature for tetrathionate broth (TT) and Rappaport-Vassiliadis broth (RV) as enrichement broths. Previous studies reported that some isolates of *Salmonella* grew poorly on enrichment media such as RV and Hajna-tetrathionate broth at 42°C (Osumi *et al.*, 2003), while *Pseudomonas aeruginosa* grew on several enrichment broths containing MK-tetrathionate broth at 41.5°C (Patil and Pahad, 1986). The use of TT with an incubation at 35°C and RV with an incubation at 42°C has previously been recommended for the recovery of *Salmonella* from various food samples with a low microbial load (Hammack *et al.*, 1999).

The specificity of selective agar plates conventionally used for the isolation of *Salmonella* based on the production of H_2S is poor because there are H_2S- producing bacteria other than *Salmonella*, such as *Citrobacter* and *Proteus*, as well as H_2S-negative *Salmonella* isolates. CHROMagar™ *Salmonella* medium (CAS) can distinguish *Salmonella* from other bacteria by color independent of the production of H_2S. Previous studies demonstrated that the efficiency of this medium was superior to other selective and differential media, and recommended it as an effective selective agar medium (Gaillot *et al*., 1999; Maddocks *et al.,* 2002). While animal meat products, eggs, and dairy products are the most commonly implicated sources in salmonellosis outbreaks, a relationship has also been reported with the consumption of fruits and vegetables (Gayler *et al*., 1955; O´Mahony *et al*., 1990; Hedberg *et al*., 1994; Tauxe *et al.*, 1997). In 2005, an outbreak of salmonellosis in Japan was

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traced to the intake of radish sprouts (Watanabe *et al*., 2006). Plants can be contaminated with obligate aerobic rods such as *Pseudomonas* (Cousin, 2000). CAS is often used with the addition of cefsulodin to inhibit *Pseudomonas*, which produces a mauve-colored colony similar to that of *Salmonella* on agar. However, the addition of cefsulodin to this medium may not be recommended in the future following the identification of cefsulodin-reistant *P.aeruginosa* (Mogi *et al*., 1996; Gaillot *et al*., 1999). The growth of these aerobic bacteria needs to be inhibited during the isolation of *Salmonella* from raw vegetables without the use of antibiotics. Therefore, another method is needed to inhibit the growth of aerobic bacteria with a similar colonial color to that of *Salmonella* on CAS when isolating *Salmonella* from raw vegetables. The use of buffered peptone water (BPW) containing 0.5% sodium thioglycolate was previously attempted for the isolation of *Salmonella* from radish sprouts (Fujisawa *et al*., 2010). Sodium thioglycolate is often used as a reducing agent in media to support the growth of anaerobic bacteria.

We currently have a few isolates of *Salmonella* grown poorly at high temperatures. In the present study, the conditions used for the enrichment incubation to isolate *Salmonella* grown poorly at high temperatures from radish sprouts were investigated while considering several obstacles such as the presence of aerobes and incubation temperatures.

Materials and Methods

Growth of bacterial isolates under various conditions

We examined the incubation temperatures of TT and RV, which are typically employed as enrichment broths, for the isolation of *Salmonella* grown poorly at 42°C from radish sprouts. Nineteen serovars, comprising twenty isolates of *Salmonella* (one isolate each of *S.* Anatum, *S.* Blockley, *S*. Brandenburg, *S.* Cerro, *S*. Derby, *S*. Friedrichsfelde, *S*. Give, *S.* Havana, *S.* Heidelberg, *S.* Infantis, *S*. Johannesburg, *S*. Kiambu, *S*. Litchfield, *S*. London, *S*. Mbandaka, *S*. Senftenberg, *S*. Typhimurium, *S*. Yaounde, and two isolates of *S*. Panama) and three isolates of *P. aeruginosa*, were used in the experiment to measure growth under various conditions. The *Salmonella* isolates tested were collected from the cecal contents of healthy pigs while *P. aeruginosa* was isolated from radish sprouts. These isolates were incubated in BPW (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 35.0±1.0°C for 22±2 h. After being incubate, the broth was serially diluted 10-fold in sterile saline. A total of 0.1 ml of diluted broth was spread onto each nutrient agar (Nissui), followed by a further incubation at 35.0±1.0°C for 22±2 h. The number of colonies grown on the agar plate was then counted. One milliliter of each BPW culture was used to inoculate 10 ml of TT (Nissui), and 0.1 ml of each BPW culture was used to inoculate 10 ml of RV (Nissui). TT and RV were then incubated aerobically and anaerobically using AnaeroPack® (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 35.0±1.0°C or 42.0±0.5°C for 22±2 h. After being incubated, the broth was serially diluted 10-fold in sterile saline. A total of 0.1 ml of the diluted broth was spread onto CAS (Kanto Chemical Co., Inc., Tokyo, Japan) and incubated at 35.0±1.0°C for 24 h. The number of colonies on the plate was then counted.

Growth of Salmonella *in the presence of competing bacteria*

To investigate the growth of *Salmonella* in the presence of competing bacteria, six isolates each of *P. aeruginosa* from radish sprouts and *Escherichia coli* from healthy human feces as competing bacteria and *S*. Brandenburg grown weakly at 42°C were used in this study. The *Salmonella* isolate and competing bacterium incubated in BPW at 35.0±1.0°C for 22±2 h were diluted with sterile saline. A total of 0.5 ml of broth or the diluted broth culture of *Salmonella* and 0.5 ml of broth or diluted broth culture of the competing bacterium were then added to 10 ml of TT. Moreover, 0.05 ml of broth or diluted broth culture of *Salmonella* and the same volume of broth or the diluted broth culture of competing bacterium were also added to 10 ml of RV. They were then incubated aerobically and anaerobically with the method described above at 35.0±1.0°C or 42.0±0.5°C. After being incubated, one loopful of the broth was streaked onto CAS, and incubated at 35.0±1.0°C for 24 h. The detection of *Salmonella* colonies was then conducted. The number of bacteria injected was measured using nutrient agar.

Recovery of Salmonella *from the enrichment broth added to cultures of* Salmonella *and radish sprouts*

S. Brandenburg grown weakly at 42°C was used in the experiment to recover *Salmonella* from the enrichment broth added to the cultures of *Salmonella* and radish sprouts. The radish sprouts samples used in this study were purchased from a retail store in Japan, and obtained from three Japanese makers. Ten grams of radish sprouts were added to 90 ml of BPW, mixed using a stomacher (Pro-media, SH-001; ELMEX Ltd., Tokyo, Japan) for 30 sec, and incubated at 35.0±1.0°C for 22±2 h. *S*. Brandenburg was also incubated at 35.0±1.0°C for 22±2 h using BPW. After the incubation, 0.5 ml of the *Salmonella*

Table 1. Growth of bacteria

Enrichment broth	Bacteria	No. of isolate injected (Log CFU/ml of broth)	No. of colonies on CHROMAgar™ Salmonella (Mean ± SD of log CFU/ml of broth)		
			Aerobic incubation at		Anaerobic incubation at
			35°C	42°C	35°C
Tetrathionate	*S.* Brandenburg*	7	8.2±0.3	7.6±0.1	7.9±0.1
		3	8.0±0.2	2.1±0.6	8.0±0.2
	S. Derby*	7	7.5±0.1	6.6±0.1	7.5±0.1
		3	7.7±0.1	7.1±0.2	7.7±0.1
	S. Senftenberg*	7	8.2±0.2	7.4±0.1	8.2±0.1
		3	8.1±0.1	7.6±0.1	8.1±0.1
	20 isolates of *Salmonella*	7	8.3±0.3	Not tested	8.2±0.3
		3	8.1±0.2	Not tested	8.1±0.2
	3 isolates of *P. aeruginosa*	7	8.2±0.6	Not tested	7.0±0.1
		3	7.3±0.1	Not tested	6.7±0.1
Rappaport-Vassiliadis	*S.* Brandenburg*	6	7.7±0.3	5.3±0.5	7.4±0.1
		3	7.6±0.3	3.1±1.5	7.4±0.3
	S. Derby*	6	7.9±0.2	7.0±0.3	7.5±0.1
		3	7.8±0.3	5.5±0.4	7.5±0.1
	S. Senftenberg*	6	8.2±0.2	7.0±0.1	7.6±0.2
		3	8.1±0.1	6.5±0.7	8.1±0.2
	20 isolates of *Salmonella*	6	7.7±0.3	Not tested	7.6±0.4
		3	7.9±0.4	Not tested	7.8±0.4
	3 isoaltes of *P. aeruginosa*	6	7.8±0.2	Not tested	6.7±0.2
		3	5.7±0.5	Not tested	3.7±0.9

*Data were obtained from three trials

culture or diluted culture and 0.5 ml of radish sprout culture were added to 10 ml of TT, while 0.05 ml of *Salmonella* culture or diluted culture and 0.05 ml of the radish sprouts culture were also added to 10 ml of RV. Each broth was then incubated aerobically and anaerobically with the method described above at 35.0±1.0°C or 42.0±0.5°C for 22±2 h. The numbers of *Salmonella* added to both enrichment broths (10^2 to 10^6CFU/ml of broth) were adjusted on the assumption that *Salmonella* grew in BPW as pre-enrichment broth. Sterilized saline was used as a control to represent the non-addition of *Salmonella* instead of a *Salmonella* culture. After being incubated, one loopful of the broth was streaked onto CAS and then incubated at 35.0±1.0°C for 24 h. After the incubation, three to ten mauve-colored colonies per one sample were picked up randomly and tested for the fermentations of lactose, sucrose, and glucose as well as the production of H_2S using TSI agar medium (Nissui). Moreover, the lysine decarboxylase test and indole test using LIM medium (Nissui), cytochrome oxidase test using test paper (Nissui), and investigation of the *Salmonella invA* gene, the *Salmonella* invasion gene, using a *Salmonella invA* gene one-step PCR screening Kit (Takara Bio Inc., Shiga, Japan) for some isolates, were also conducted.

Results

Growth of Salmonella *and* P. aeruginosa *isolates under various conditions*

The growth of the bacterial isolates tested was shown in Table 1. The growth of *S.* Brandenburg on both TT and RV at 42°C was markedly inferior to that of *S.* Derby or *S.* Senftenberg, especially when small (10^3 CFU /ml of broth) numbers were added to the enrichment broths. No marked differences were observed in bacterial numbers between broth incubated anaerobically and that incubated aerobically when large (10^7 CFU/ml or 10^6 CFU/ml of broth) and small (10^3 CFU/ml of broth) numbers were added to TT and RV. The growth of 20 isolates of *Salmonella* on both TT and RV media at 35°C was similar under both the anaerobic and aerobic conditions. The growth of *P. aeruginosa* under the anaerobic condition was lower than that under the aerobic condition, in general.

Detection of Salmonella *incubated with competing bacteria*

The detection of *Salmonella* colonies incubated with competing bacteria on CAS is shown in Table 2. *Salmonella* colonies were not detected on TT and RV incubated at 42°C following the injection of small number (10^2 CFU/ml of broth) of *Salmonella* with *P. aeruginosa* into both broths. No differences were

Table 2. Recovery of *Salmonella* Brandenburg incubated with competing bacteria

Enrichment broth	Number of each isolate injected (Log CFU /ml of broth)			Detection of *Salmonella* colonies on CHROMagar™ Salmonella		
				Aerobic incubation at		Anaerobic incubation at
	S. Branbenburg	*P. aeruginosa*	*E. coli*	35°C	42°C	35°C
Tetrathionate	6	+ 7		6/6 (100)*	6/6 (100)	6/6 (100)
	2	+ 7		6/6 (100)	0/6 (0)	6/6 (100)
Rappaport-Vassiliadis	5	+ 6		6/6 (100)	2/6 (33)	6/6 (100)
	2	+ 6		4/6 (67)	0/6 (0)	6/6 (100)
Tetrathionate	6	+	6	6/6 (100)	6/6 (100)	Not tested
	2	+	6	6/6 (100)	0/6 (0)	Not tested
Rappaport-Vassiliadis	5	+	5	6/6 (100)	4/6 (67)	Not tested
	2	+	5	6/6 (100)	2/6 (33)	Not tested

*No. of cultures detected / No. of cultures tested (%)

observed in the detection rate of colonies on TT and RV incubated anaerobically at 35°C or aerobically at 35°C, with the exception of a small number of *Salmonella* with *P. aeruginosa* on RV. In the mixed culture of *S.* Brandenburg and *E. coli* in TT and RV incubated at 42°C, there were samples that *Salmonella* colonies were not detected.

Recovery of Salmonella *incubated with the culture of radish sprouts in enrichment broths*

The recovery of *Salmonella* from TT and RV with added cultures of *S.* Brandenburg with radish sprouts is shown in Table 3. In TT and RV, the detection rates at 35°C were better than those at 42°C when a large number (10^5 or 10^6 CFU/ml of broth) injected. Moreover, the detection rate in RV was better at 35°C than at 42°C when a small number (10^2 CFU/ml of broth) was injected. The detection rate was relatively high (77%) at 42°C when a large number was injected into TT. However, no marked differences were observed in the detection rate on both TT and RV under the anaerobic and aerobic conditions at 35°C.

Discussion

Sata *et al.* (1999) reported that starved bacteria could not grow in enrichment broth containing selective agents and at a high incubation temperature. A previous study suggested that food-poisoning bacteria that contaminate raw vegetables grown hydroponically, such as radish sprouts, were nutritionally starved (Sata *et al*., 2003). Bacteria can be exposed to starvation stress in water (Wesche *et al*., 2009). However, bacteria can survive stressful conditions such as limited nutrient availability by entering a viable, but non-culturable state (Winfield and Groisman, 2003). The use of non-selective enrichment broth for the isolation of *E. coli* O157:H7 from radish sprouts instead of enrichment broth containing selective agents is recommended (Sata *et al.*, 2003). A previous study also suggested that the use of BPW as a pre-enrichment broth increased the rate of recovery of *Salmonella* from environmental samples (Thomason *et al*., 1977). Incidentally, the use of pre-enrichment medium is recommended for the isolation of stressed or injured *Salmonella* from food samples (D´Aoust, 1989). Pre-enrichment broth and/or enrichment broth not containing selective agents such as antibiotics may be desirable for an enrichment incubation to isolate food-borne bacteria from raw vegetable samples. However, the use of non-selective broth has been associated with the growth of many kinds of bacteria including *Pseudomonas*.

No marked differences were observed in the detection rates under anaerobic and aerobic conditions (Table 3). This was consistent with the results obtained

Table 3. Recovery of *Salmonella* incubated with radish sprouts

Enrichment broth	No. of isolates injected (Log CFU/ml of broth)	Incubation condition		
		Aerobic at 35°C	Aerobic at 42°C	Anaerobic at 35°C
Tetrathionate	6	26/26 (100)*	20/26 (77)	26/26 (100)
	2	2/26 (8)	8/26 (31)	2/26 (8)
	Not injected	0/26 (0)	0/26 (0)	0/26 (0)
Rappaport-Vassiliadis	5	19/23 (83)	9/23 (39)	20/23 (87)
	2	9/20 (45)	2/20 (10)	8/20 (40)
	Not injected	0/23 (0)	0/23 (0)	0/23 (0)

*No. of samples recovered / No. of samples tested (%)

for the inoculation of *Salmonella* with competing bacteria, as shown in Table 2. Although the bacterial count of *P. aeruginosa* incubated aerobically was higher than that incubated anaerobically, the count under the anaerobic condition was not less than that injected (Table 1). Aerobes have been suggested to suppress growth, but may also survive in enrichment broth under anaerobic incubation conditions.

There is a possibility of fecal contamination in food samples in which *Salmonella* is detected. Regarding the incubation of *Salmonella* poorly grown at 42°C with *E.coli*, which is an index of fecal contamination, the detection rate of *Salmonella* colonies incubated at 42°C was lower than that at 35°C.

The results of the present study demonstrated that incubating the enrichment broths at 35°C was effective for the detection of *Salmonella* grown poorly at 42°C from radish sprouts using isolation methods with CAS. The detection rate in TT was better at 42°C than at 35°C when a small number (10^2 CFU/ml of broth) of *Salmonella* was injected as shown in Table 3. However, the reason is not clear. In the present study, *S.* Brandenburg was H_2S-positive and produces black-color colonies on DHL agar. DHL agar (Nissui) was also used for several samples in the recovery test, and no differences were observed in the detection rates between DHL agar and CAS. Furthermore, the importance of the pre-enrichment incubation was confirmed. Sufficient growth in a pre-enrichment medium is essential for the isolation of *Salmonella* grown poorly at 42°C form radish sprouts using the method of high temperature enrichment incubation.

Although the incubation of RV at high temperatures has been associated with high detection rates of *Salmonella* in meat products (Vassiliadis *et al*., 1981; Vassiliadis, 1983), the incubation of RV at high temperature may not be suitable for isolation of the *Salmonella* grown poorly at 42°C from radish sprouts samples in the present study. *Salmonella* contaminates various kinds of raw vegetable (Beuchat, 1996; Tauxe *et al.*, 1997). It will be of interest to determine whether an enrichment incubation at 35°C can effectively isolate *Salmonella* grown poorly at high temperatures from several kinds of foods including raw vegetables other than radish sprouts.

Conclusion

The isolation of *Salmonella* grown poorly at 42°C from radish sprouts using a high temperature incubation was not favorable. There are currently no official analytical methods for many kinds of foods. Regarding official methods for the future detection of *Salmonella* from foods, it may be necessary to consider the presence of *Salmonella* grown poorly at high temperatures, even if its abundance rate is low.

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