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An Improved Pre-culture Procedure for the Detection of *Salmonella* in Frozen Chicken Meat by Duplex Polymerase Chain Reaction

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ABSTRACT

An improved 18-hour pre-culture procedure coupling with duplex polymerase chain reaction (PCR) method capable of detecting *Salmonella* in frozen chicken meat is herein reported. Two pairs of primers (TS5/TS11 and S18/S19) were used for the PCR method. Positive PCR results were obtained after the 18-hour preculture (6 h in lactose broth at 37°C followed by 12 h in Rappaport-Vassiliadis broth at 42°C) for spiked raw and irradiated meats with or without freezing treatment. The applicability of this improved procedure was tested in 50 retail frozen and 50 non-frozen chicken drumstick samples, and the results were compared with those obtained with the BAM standard culture method. No significant difference was found ($p \geqq 0.05$) between these two methods.

Key words: pre-culture, duplex PCR, *Salmonella*, frozen chicken meat

INTRODUCTION

Salmonella is a major cause of food poisoning(1). All *Salmonella* are considered pathogenic and should not be present in food. However, *Salmonella* is ubiquitous in nature, and can be found in the alimentary tracts of animals, poultry products, seafood, eggs and milk products. Raw chicken meat is known to be the major source for *Salmonella* food poisoning(2). Detection of *Salmonella* using BAM(3) (Bacteriological Analytical Manual of AOAC International) standard culture method is a labor-intensive and time-consuming process that requires 4 to 6 days to complete. Hence, the development of a rapid and effective detection method has become a global effort to minimize the health and economic impacts of *Salmonella* food poisoning(4).

Recently polymerase chain reaction (PCR) has been applied successfully, with appropriate preculture, for the rapid detection of *Salmonella*(5-7), *Shigella*(8), *Listeria*(9) and *Escherichia coli* O157(10). The use of multiplex PCR, with enhanced accuracy, to simultaneously amplify four genes, the florfenicol (flo_{st}), virulence (*spvC*), invasion (*invA*), and integron (*int*) of *Salmonella enterica* serovar Typhimurium DT104 has been developed(11). When used in combination with appropriate pre-culture and specific primers, PCR technique is a rapid and highly specific detection method(6). Food samples with high water activity, such as raw chicken meat, tend to have high and complex background microflora(2). Consequently, minimizing competition from other microorganisms is required in the pre-culture procedure(2,12,13). In addition, as *Salmonella* is easily injured during cold storage and prolonged recovery period, an initial period of enrichment could help shorten such period(14). For the detection of *Salmonella*, a modified conventional standard procedure including 48 h of pre-culture has been reported, including 24 h of non-selective enrichment in lactose broth (LB) for resuscitation purpose, followed by 24 h of selective enrichment in tetrathionate (TT), or selenite cystine (SC), or Rappaport-Vassiliadis (RV) broth(2,3,12,13). The purpose of this study was to improve and simplify the existing pre-culture methods prior to PCR, develop a quick pre-culture method within 24 hours, and most importantly, evaluate its application for frozen meat which has not been adequately addressed in the literature.

MATERIALS AND METHODS

I. *Salmonella and non-Salmonella Inocula*

All *Salmonella* and non-*Salmonella* inocula used for inoculation study were mixed cultures, consisting of five species each, mixed in equal ratio. The five serotypes for

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the *Salmonella enterica* inocula were *S. enterica* serovar Anatum NLFD JH006, *S. enterica* serovar Choleraesuis BCRC 10743, *S. enterica* serovar Derby BCRC 12435, *S. enterica* serovar Enteritidis NLFD JH004 and *S. enterica* serovar Typhimurium BCRC 10312 (Purchased from BCRC (Bioresource Collection and Research Center, Hsinchu, Taiwan) or isolated by BFDA (Bureau of Food and Drug Analysis, Department of Health, Executive Yuan, Taipei, Taiwan). *Citrobacter freundii* BFDA CF040, *Escherichia coli* BCRC 14824, *Proteus mirabilis* BFDA PM001, *Shigella dysenteriae* BFDA SYL1 and *Yersinia enterocolitica* BCRC 12986 were used as non-*Salmonella* cultures. Stock cultures were kept at -72°C. Frozen stock culture was activated in nutrient broth (NB) at 37°C for 24 h prior to streaking on trypticase soy agar (TSA) plates. Colonies were picked to prepare a water suspension and adjusted to roughly 10^8 CFU/mL within 30 min with the aid of a McFarland No. 2 filter in a Vitek colorimeter (DR 100 colorimeter, Hach Inc., Durham, NC, USA). Equal volumes of each strain or species were mixed to prepare the mixed inocula. Unless specified otherwise, all but one microbiological medium used in this study was obtained from DIFCO (distributed thru Voigt Global Distribution Inc., Lawrence, Kansas, USA).

II. *Preparation of Chicken Meat*

Ground chicken meat for inoculation and preculture studies was prepared from boneless chicken breast purchased at local supermarket, cut into cubes, grounded, bagged (25g each, in sterile stomacher bags), and stored at -18°C for 2 days. Approximately one half of the bags were saved and the other half was sterilized by γ-ray irradiation at the Nuclear Science and Technology Development Center of the National Tsing Hua University (Hsinchu, Taiwan). Irradiation was carried out at 2.5 kGy/h for 4 h (at 23.5 cm from the γ-ray source). Bagged raw meat and irradiated meat used for this study were stored at -18°C before experimentation. Ten irradiated bags were randomly selected and checked for sterility by total plate count method. The bags were also checked for the absence of *Salmonella*[15] using procedures detailed in part III *Detection of Salmonella*.

Prior to each experiment, bags were removed from the freezer, thawed for 1 h at room temperature, and then inoculated with 1 mL of *Salmonella* and/or non-*Salmonella* cultures. For the freezing treatment study, inoculated bags were allowed to stand for an additional 3-5 min at room temperature, and then subjected to freezing treatment at -18°C.

For the evaluation study, one hundred chicken drumstick samples were used which included 50 frozen and 50 non-frozen (room temperature handling) samples purchased at local markets. Each sample suspension was collected according to the rinse method described[16]. Each sample containing 2 drumsticks was placed in a double-layered stomacher bag, and then 270 mL of 0.1% Peptone Water (PW) was added and shaken for 15 sec. The fluid was transferred into a sterilized centrifuge tube and centrifuged for 15 min at 12,000×g in a refrigerated centrifuge. The pellet was saved and resuspended in 10 mL of 0.1% PW, from which 1 mL was used for the pre-culture evaluation study.

III. *Detection of Salmonella*

A modified BAM method based on Andrews *et al.*[3] was used which involved the use of automated biochemical identification thus reduced the total required time from 6 to 4 days. 225 mL of LB was added to each bag of 25g thawed ground meats. After incubation for 24 h at 37°C, 1 mL of this LB culture suspension was removed and used as inoculum for subsequent growth in 9 mL of selective enrichment broth, TT (tetrathionate) and SC (selenite cystine). Prior to inoculation, TT broth was activated by the addition of 0.1 mL of Brilliant Green and 0.2 mL of iodine solution. Incubation of culture was carried out at 42°C for TT and at 37°C for SC. One loop of culture fluid was removed at 24 h, and streaked onto XLD (xylose lysine dexoxycholate) HE (Hektoen enteric) and BS (bismuth sulfite) agar plates. Typical colonies were picked for stab-and-streak culture on TSI (triple sugar iron) and LIA (lysine iron) agar slants. In addition, one TSA plate was densely streaked. All colonies were then incubated at 37°C for 24 h. Colonies with characteristic color formation were further tested by polyvalent antisomatic antiserum (purchased from Denka, Seiken, Co., Ltd., Tokyo Japan). Only agglutination positive colonies were selected and subjected to automated biochemical identification. Fresh colonies (24 h culture) from TSA plate were used to prepare a McFarland 1.0 equivalent suspension in 0.45% saline (~3×10^8 CFU/mL) for automated identifications with AMS VITEK system (BioMérieux Vitek, Inc., Marcy L'Etoile, France) and Gram negative identification card system (GNI, Vitek, Marcy L' Etoile, France).

With a few exceptions, most of the pre-culture fluid for PCR analyses was examined on a routine basis to check for the presence of *Salmonella* using a verification test. Pre-culture fluid was used to make a streak plate on XLD and after 24h growth at 37°C typical colonies were picked and tested for the presence of *Salmonella* with antiserum agglutination test.

IV. *Detection of Indicator Organisms*

To determine microbial loads of ground meat and irradiated ground meat used in this study, total plate counts (TPC) was performed following the procedures reported by Maturin and Peeler[17]. In addition, the most probable number (MPN/g) method[18] was carried out for materials with very low bacterial count. Coliform counts were obtained following the procedures detailed by Hitchins *et al.*[19].

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V. *Pre-culture Methods*

For the preliminary study, spiked irradiated meat was used and three pre-culture methods were examined: A 24-hour LB method (named 24 h LB), a magnetic immunobead enrichment method (named 24 h LB-MIPA) and a tandem culture method with a 24-hour selective enrichment in TT/SC following 24 h LB (named 24 h LB-24 h TT/SC). The 24-hour LB method was used to determine the applicability of PCR technique in *Salmonella* detection. To each bag of 25g of spiked meat, 225 mL LB was added and incubated for 24 h. In the 24 h LB-MIPA method, 2 mL of this 24-hour LB pre-culture fluid was used to mix with immunobeads and proceed with the recommended procedures of the manufacturer (Vicam, Watertown, MA, USA). In the 24-hour LB-24 h TT/SC method, 1 mL of this 24-hour LB culture fluid was used to inoculate TT and SC broth and incubated for an additional 24 h as described earlier.

To shorten the pre-culture time using spiked, frozen irradiated meat samples was carried out as follows: 1) reducing the LB pre-culture period to 4-6 h; and 2) reducing selective enrichment period to 18-20 h. Samples of culture fluid were removed every two hours, examined by PCR and XLD streak plate in order to determine the minimal required time for culturing. Three selective enrichment media, RV (Rappaport-Vassiliadis, Merck, Darmstadt, Germany) TT and SC were tested in order to develop a quick preculture method.

Finally a simplified pre-culture method was adopted and evaluated using frozen ground meats and frozen retail drumstick samples. 9 mL LB was added to 1 mL of drumstick rinse suspension and incubated at 37°C for 6 h, and then 0.1 mL of this culture fluid was removed and transferred to a tube with 9 mL of RV and incubated at 42°C for 12 h.

VI. *PCR Procedures*

DNA extract was prepared from 1 mL of preculture fluid. After centrifugation for 2 min at 12,000×g, the supernatant was discarded and the pellet was washed by resuspension in 1 mL of sterile distilled water and centrifuged again. This washing step was repeated two more times to eliminate inhibitory substances such as proteins or fat[20]. The pellet was resuspended in 0.5 mL of sterile distilled water, heated at 100°C for 15 min[21] and then centrifuged. The supernatant containing DNA extract, to be used as DNA template in the PCR reaction, was transferred to a new tube and stored at -18°C[22]. The *S. enterica* serovar Typhimurium BCRC 10312 (10^8 CFU/mL) culture was used as the positive control for PCR technique.

DNA extract (20 μL) was added to a reaction mixture (80μL) consisting of dNTP mixture (final concentration 0.2 mM for each of 4 dNTPs, ProTech Technologies, Inc., Taipei, Taiwan), DNA polymerase (final 0.02 unit, Finnzyme, Riihtontuntie, Finland), DNA duplex primers (final concentration 0.1μM for each of 4 primers, TS11/TS5[7] and S18/S19[5], TIB MOLBIOL Syntheselabor, Berlin, Germany) in 0.15mM $MgCl_2$ buffered to pH 8.4 at room temperature (ProTech Technologies, Inc., Taipei, Taiwan). The DNA duplex primers, TS11/TS5 and S18/S19, consist of the following sequences:

TS11 (5' GTCACGGAAGAAGAGAAATCCGTACG 3')
TS5 (5' GGGAGTCCAGGTTGACGGAAAATTT 3')
S18 (5' ACCGCTAACGCTCGCCTGTAT 3')
S19 (5' AGAGGTGGACGGGTTGCTGCCGTT 3').

Amplification was performed on a programmable thermal controller PTC-100-60 (MJ. Research Inc.,Waltham, MA, USA). Samples were denatured at 94°C for 4 min, 35 cycles of amplification at 94°C for 1 min, 60°C for 2 min and 72°C for 2 min. Aliquots of amplification products were separated on 2% agarose gel (Amersco, Solon, Ohio, USA) in 0.5X TBE buffer at 75 volts for 40-45 min and visualized by ethidium bromide staining and UV transillumination. A 100-bp DNA ladder (ProTech Technologies, Inc., Taipei, Taiwan) was used as a molecular size marker. The presence of both 159 bp and 375 bp indicated the presence of *Salmonella*.

VII. *Evaluation of the Improved Rapid Preculture Method in Retail Chicken Drumsticks*

In order to validate the applicability of the improved rapid pre-culture method developed in this study, all PCR results were compared with those obtained directly with BAM method and analyzed for relative specificity, sensitivity and agreement tests according to the following formulas[6] :

a. Relative specificity = {number of double-negative results (N) } / {N + A (PCR positive only results)}× 100%
b. Relative sensitivity = {number of double-positive results (P) } / {P + B (BAM positive only results)}×100%
c. Agreement = {(P + N) / total sample size}×100%

For significance test, these data were analyzed by the following formula[23]:

$$\chi^2 = (|A - B| - 1)^2 / (A + B)$$

Significant difference between the results of BAM and PCR methods can be concluded if $\chi^2 > 3.84$ ($p \leq 0.05$).

RESULTS AND DISCUSSION

I. *Examination of Preculture Methods for Salmonella Detection in Spiked Ground Meat*

Ground meat before irradiation sterilization had a total aerobic plate count of 10^6 CFU/g and a coliform count of 380 MPN/g, but contained no detectable level of Salmonella. In the irradiated meat, no bacteria was detected by the TPC method, but the estimate was 23 MPN/g based on MPN method.

Using 24-hour LB pre-culture method, positive PCR results were obtained for irradiated meats spiked with a low dose (10^1 CFU/25g) of *Salmonella*, and negative results were obtained in irradiated meats spiked with a wide range of non-*Salmonella* cultures (Table 1). These results indicated high specificity of the PCR method for *Salmonella* detection, with little or no cross reaction with 5 enteric bacteria selected for these experiments. However, for 24 h LB method, negative PCR results were obtained from meats spiked with a combination of *Salmonella* and non-*Salmonella* cultures, while no *Salmonella* colony was detected in the verification test (Table 2). `As *Salmonella* was present in the initial inoculated meat (10^1 CFU/25g meat), strong competition favoring the growth of the enteric bacteria was likely the cause of this double negative results. It has been reported that using non-selective medium, as opposed to using selective media, reduced the chances of *Salmonella* detection by PCR technique[24]. Presumably *Salmonella* was present in small number which was beyond detection by both PCR and XLD streak plate. This point will become clear when results of 24 h LB are compared side by side with those of 24 h LB-24 h TT/SC. Upon further culturing in selective media TT and SC, the small number of *Salmonella* presumably present in the 24-hour LB can grow to a population size that yield positive PCR results and *Salmonella* could be found in the verification test (Table 2). In our estimation, this verification test requires a minimal *Salmonella* density of 10^3 - 10^4 CFU/mL in order to show typical colonies on XLD streak plate. Double negative results were obtained for the 24-hour LB-MIPA method in ground meats spiked with *Salmonella* (Table 2). Failure of the 24 h LB-MIPA method could be due to: 1) large number of enteric bacteria in LB pre-culture reduced the capacity of immunobeads to recover *Salmonella* [25]; 2) the presence of lipid in meat samples interfered and reduced the specificity of immunobeads[26]; and 3) possible cross reaction of enteric bacteria to immuno-

Table 1. Comparison of the PCR and BAM method on the detection of *Salmonella* in spiked irradiated ground meat with 24 h of lactose broth pre-culture

Detection Method	Inoculated Cultures (CFU/25g irradiated meat)					
	Salmonella[a]		non–*Salmonella*[a]			Control[b]
	10^1	10^3	10^3	10^5	10^8	0
24 h LB-PCR[c]	+*	+	–	–	–*	–*
BAM[d]	+*	+*	–*	–*	–*	–*

[a] *Salmonella* or non–*Salmonella* cultures were detailed in the materials and methods section.
[b] 1 mL of sterile water was added instead.
[c] Lactose broth pre-culture for 24 h prior to PCR.
[d] Bacteriological analytical manual. +: positive result; –: negative result.
* Results were obtained from two or more sets of experiments.

Table 2. Effects of pre-culture methods on the PCR and BAM results for *Salmonella* detection in spiked irradiated and ground meats

Method of detection	Mixed inoculation of *Salmonella* (10^1/25g) and varying doses of non-*Salmonella* (CFU/25g)							
	Irradiated meat						Non-irradiated meat	
	10^1	10^3	10^5	10^7	10^8	Control[a]	10^7	Control
PCR pre-culture method								
24 h LB[b]	–(–)*	–(–)	–(–)	–(–)	–(–)	–	–(–)	–
24 h LB-MIPA[c]	–(–)	–(–)	–(–)	–(–)	–(–)	–	–(–)	–
24 h LB-24 h TT/SC[d]	ND[f]	+(+)	+(+)	+(+)	+(+)	–	+(+)	–
BAM method[e]	+	+	+	+	+	–	+	–

[a] 1 mL of Sterile water was added instead.
[b] Lactose broth pre-culture for 24 h prior to PCR.
[c] Magnetic imunobead enrichment following 24 h lactose broth preculture prior to PCR.
[d] 24 h lactose broth preculture followed by tetrathionate/selenite cystine broth for 24 h prior to PCR.
[e] Bacteriological Analytical Manual.
*All results were obtained from two or more sets of experiment (results from verification test of culture fluid are in parentheses). +: positive result; –: negative result.
[f] ND: Not determined.

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beads occurred. However, the main reason for the failure of the 24-hour LB-MIPA method was strong competition and the concomitant presence of enteric bacteria, as in the case of the 24-hour LB method. The 24 h LB-24 h TT/SC method yielded positive PCR results and *Salmonella* could be found on XLD streak plate of the culture fluid. Similar with previous findings, this is a more effective pre-culture method than MIPA for *Salmonella* detection[25]. The initial pre-culture phase of the BAM method was actually identical to this 24 h LB-24 h TT/SC method. In the BAM method, this is then followed by extensive plating on several selective media, isolation of single colony in order to proceed with biochemical and antiserum tests for identification which take another 2 to 4 days to complete. An advantage of the PCR technique is that it can be applied directly using the culture fluid to detect *Salmonella* (in the presence of large population of other microorganisms) thus bypasses these 2 to 4 days of time-consuming steps.

II. *Improved Pre-culture Method for Salmonella Detection in Spiked Meat with or without Freezing Treatment*

Improvement study was carried out in spiked meat without freezing treatment at first. Irradiated meats spiked with a high dose of non-*Salmonella* cultures (10^7 CFU/25g) mixed with a low dose of *Salmonella* cultures (10^1 CFU /25g) were used. These combination inoculums were chosen to simulate the condition of microbial load in retail raw meat. Of the three types of selective enrichment, RV appeared to be the most effective, detecting *Salmonella* as early as 8 h (Table 3). The presence of *Salmonella* in culture fluid was also verified on the XLD plate. *Salmonella* could be detected after 16 h with TT, and 18 h with SC culture. This was in agreement with that reported in the literature[27]. In all of the above experiments, the PCR results were substantiated by the results of verification test.

Further studies were carried out in spiked irradiated meat with freezing treatment. Resuscitation for 6 h in LB appeared to be sufficient for *Salmonella* recovery and growth, and positive results were obtained for RV culture after 12 h. Negative PCR results were obtained for TT and SC, even after 18 h incubation, and no *Salmonella* was detected in TT and SC culture fluids in the verification tests (Table 3). In comparison with the results of RV selective enrichment without or with freezing treatment, it was apparent that there was a delay of about 4 hours in the *Salmonella* detection by PCR method in meat samples subjected to freezing treatment. *Salmonella* could be detected as early as 8 hours in RV for non-frozen meat, whereas a minimum of 12 hours was required for frozen meat. Differences in the selectivity of the media appeared to play a major role, as only RV yielded positive results for PCR and in the verification tests (Table 3). This finding is highly significant for the establishment of a pre-culture method that is to be applied for *Salmonella* detection in raw, retail chicken meats that often contain high numbers of complex flora.

Finally, this pre-culture method (6 h LB-12h RV) was adopted and tested with raw meat spiked with a low dose of *Salmonella* cultures (10^1 CFU/25g) and subjected to freezing treatment. Positive PCR results were obtained (Figure 1), and *Salmonella* were detected in the RV fluid in the verification tests. The results indicated that 6 h of LB-12h RV pre-culture could be the method of choice for *Salmonella* detection for retail raw chicken meats, particularly when the meats had been stored in freezer or refrigerator.

III. *Applicability of the 6-hour LB-12h RV Pre-culture Method for Salmonella Detection in Retail Chicken Drumstick Samples*

In this evaluation study, the simplified pre-culture-PCR method was tested, and the results were compared to those obtained using the BAM method (Table 4). When retail chicken drumsticks were examined with the BAM method, fewer frozen samples appeared to be contaminated (20%) compared to that of the non-frozen ones (34%). All BAM positive results are PCR positive in the non-frozen drumsticks, whereas only 3 samples (6%) were BAM positive in frozen drumsticks. Future improvement of the rapid pre-culture-PCR method could increase the pre-culture period for frozen food samples as evident in the results in Table 3.

It is interesting that relatively small number of samples (4%) showed PCR positive results for both frozen

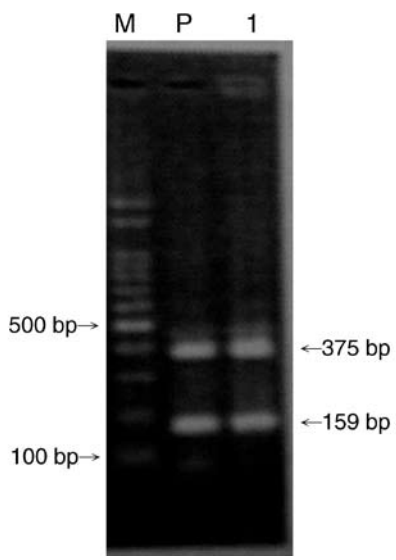

Figure 1. Analysis of raw meat spiked with *Salmonella* (10^1 CFU/25g) and subjected to freezing treatment by a 6 h LB-12 h RV-PCR procedure. Two-step pre-culture, 6 h lactose broth at 37°C follow by 12 h in Rappaport-Vassiliadis broth at 42°C, was adopted prior to PCR (6 h LB-12 h RV-PCR). Result is shown in Lane 1. M: 100 bp ladder markers; P: positive control with S. enterica serovar Typhimurium BCRC 10312.

and non-frozen drumsticks. Unlike the BAM method which detects only live *Salmonella*, the PCR method can detect both live and dead microorganism as it is based on the presence of DNA materials of the target organism. In this regard, PCR positive results may still be taken as an indication of the presence of *Salmonella* in food. In other words, food samples may have been contaminated with high level of *Salmonella* but the *Salmonella* was mostly non-viable, killed or damaged beyond repair (such as by antibiotics). As the results, no *Salmonella* can be cultured by the BAM method, but the presence of DNA left on the food would yield PCR positive result. Taking all these findings into consideration, *Salmonella* contamination could be underestimated if BAM is used as the only method of detection. Hence, the actual percentage of "contaminated" samples could be 38% (instead of 34%) for non-frozen, and 24% (instead of 20%) for frozen drumsticks when results of both tests are considered.

Three indicator tests were applied to determine the efficacy of this pre-culture-PCR method in comparison with the BAM method. The results of relative specificity, sensitivity and agreement tests were 95, 70 and 90% respectively for the frozen samples; while the results for the non-frozen samples were 94, 100 and 96% respectively. It would be difficult to judge the significance of a slightly lower sensitivity (70%) for frozen samples as the number of *Salmonella* positive samples was very small. Possible reasons for lower sensitivity could be differential recovery of *Salmonella* spp. to cold damage[28], slow growth of *Salmonella* spp. in RV as reported for *S. enterica* serovar Typhi[29], and/or insufficient incubation time such that the resultant culture did not reach the minimally required population size for detection by PCR method. A positive PCR result required roughly a culture density of 10^6-10^7 CFU/mL in our estimate, based on using *S. enterica* serovar Enteritidis BFDA JH004 culture as standard. This would mean the selected pre-culture method has to support the growth of *Salmonella* spp. to reach a density of at least 10^6-10^7 CFU/mL in order to obtain PCR positive result. Using BPW and in combination with TT-H (tetrathionate-Hajna) for selective enrichment, the relative sensitivity of PCR results could be increased significantly, yet still not comparable to the BAM method[6]. The use of TT-H may improve the sensitivity of the PCR results[6], but the use of TT did not yield PCR positive results in frozen irradiated meats in our study (Table 3). Based on the most recent update (Dec. 2007) on the BAM website[12], the number one recommended medium for *Salmonella* is RV, followed by TT as the second. Our results would also support such recommendation.

In order to show that this pre-culture-PCR method is applicable for *Salmonella* detection, test data were subjected to significance tests. As no significant difference was found ($p \geqq 0.05$) between the results of the two methods in frozen and non-frozen retail samples, it is concluded that the pre-culture-PCR method is statistically as good as the BAM method for *Salmonella* detection. Note that improvement on pre-culture is still needed for frozen food. This pre-culture-PCR method is not and should not be used as a replacement of the traditional BAM method. However, when time is critical, the method of choice becomes obvious considering the pre-culture-PCR method requires less than 1 day vs. 4-6 days needed by the BAM method in making the final judgment call.

Table 3. Detection of *Salmonella* in spiked irradiated meat by PCR method following a 6 h of lactose broth enrichment and different second step selective enrichment

Selective enrichment	Incubation time, hours						
	6	8	10	12	14	16	18
Without freezing treatment*							
RV [a]	– (–) [d]	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
TT [b]	– (–)	– (–)	– (–)	– (–)	– (–)	+ (+)	+ (+)
SC [c]	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	+ (+)
With freezing treatment*							
RV	ND[e]	– (–)	– (–)	+ (+)	+ (+)	+ (+)	+ (+)
TT	ND	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
SC	ND	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)

* The meat samples were spiked with *Salmonella* (10^1 CFU/25g) and non-*Salmonella* cultures (10^7 CFU/25g).

[a] RV: Rappaport-Vassiliadis broth prior to PCR.

[b] TT: Tetrathionate broth prior to PCR.

[c] SC: Selenite cystine broth prior to PCR.

[d] All results were obtained from two or more sets of experiment (results from verification test of culture fluid are in parentheses). +: positive result; –: negative result.

[e] ND: Not determined.

Table 4. Evaluation of the 18-hour pre-culture-PCR method for detecting *Salmonella* in frozen and non-frozen drumsticks

Non-frozen drumsticks			
	PCR+	**PCR–**	**BAM subtotal**
BAM+	17	0	17 (34%)
BAM–	2	31	33 (66%)
PCR subtotal	19 (38%)	31 (62%)	50 (100%)
Frozen drumsticks			
	PCR+	**PCR–**	**BAM subtotal**
BAM+	7	3	10 (20%)
BAM–	2	38	40 (80%)
PCR subtotal	9 (18%)	41 (82%)	50 (100%)

Fifty samples of frozen and fifty samples of non-frozen drumsticks were examined. Prior to PCR, the samples were enriched by a 6-hour lactose broth at 37°C followed by 12 h in Rappaport-Vassiliadis broth at 42°C.

CONCLUSIONS

The use of a 2-step pre-culture procedure (6 h of LB then 12 h of RV) in conjunction with duplex PCR technique (TS11/TS5 and S18/S19 primers) could be used to detect *Salmonella* in chicken meat spiked with low dose of *Salmonella* cultures then subjected to freezing treatment. No significant difference was found between the results of this pre-culture-PCR method and those of the BAM method when tested in retail frozen and non-frozen chicken drumsticks. Pre-culture has been reduced from 48 h to 18 h. Combination with PCR technique makes a quick and simplified method for detecting low level of *Salmonella* in frozen raw chicken meat.

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REFERENCES

1. Wallace, D. J., Van Gilder, T., Shallow, S., Fiorentino, T., Segler, S. D., Smith, K. E., Shiferaw, B., Etzel, R. and Garthright, W. E. 2000. Incidence of foodborne illnesses reported by the foodborne diseases active surveillance network (FoodNet)-1997. FoodNet working group. J. Food Prot. 63: 807-809.
2. Jay, J. M. 1996. Fresh meats and poultry. In“ Modern Food Microbiology ”. 5th ed. chapter 4. p. 69-96. Jay, J. M. ed. International Thomson Publishing. New York, U.S.A.
3. Andrews, W. H., Bruce, V. R., June, G. A., Sherrod, P. S., Hammack, T. S., and Amaguana, R. M. 1995. Salmonella. In“ Bacteriological Analytical Manual ”. 8th ed. Ch. 5. P.5.01-5.20. AOAC International Gaithersburg MD. U.S.A.
4. Chen, C. J., Liu, P. R. and Shih, Y. C. 1998. Rapid detection of *Salmonella* spp. in chicken samples. Food Sci. 25: 222-233.
5. Kwang, J., Littledike, E. T. and Keen, J. E. 1996. Using of the polymerase chain reaction for *Salmonella* detection. Lett. Appl. Microbiol. 22: 46-51.
6. Myint, M. S., Johnson, Y. J., Tablante, N. L. and Heckert, R. A. 2006. The effect of pre-enrichment protocol on the sensitivity and specificity of PCR for detection of naturally contaminated *Salmonella* in raw poultry compared to conventional culture. Food Microbiol. 23: 599-604.
7. Tsen, H. Y., Liou, J. W. and Lin, C. K. 1994. Possible use of a polymerase chain reaction method for specific detection of *Salmonella* in beef. J. Ferment. Bioeng. 77: 137-143.
8. Farshad, S., Sheikhi, R., Japoni, A., Basiri, E. and Alborzi, A. 2006. Characterization of *Shigella* strains in Iran by plasmid profile analysis and PCR amplification of *ipa* gene. J. Clin. Microbiol. 44: 2879-2883.
9. Huang, B., Eglezos, S., Heron, B. A., Smith, H., Graham, T., Bates, J. and Savill, J. 2007. Comparison of multiplex PCR with conventional biochemical methods for the identification of *Listeria* spp. isolates from food and clinical samples in Queensland, Australia. J. food Prot. 70: 1874-1880.
10. Hsu, C. F., Tasi, T. and Pan, T. M. 2005. Use of duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. J. Clin. Microbiol. 43: 2668-2673.
11. Khan, A. A., Nawaz, M. S., Khan, S. A. and Cerniglia, C. E. 2000. Detection of multidrug-resistant *Salmonella typhimurium* DT 104 by multiplex polymerase chain reation. FEMS Microbiol. Lett. 182: 355-360.
12. Andrews, W. H. and Hammack, T. S. 2007. Salmonella. In “Bacteriological Analytical Manual Online”. 8th ed. Chapter 5. (Available online at the US-FDA website: http://www.cfsan.fda.gov/~ebam/bam-5.html).
13. AOAC. 1998. In “Official Methods of Analysis”. 17th ed. sec. 989.13. Association of Official Analytical Chemists. Washington, D. C., U.S.A.
14. Mackey, B. M. and Derrick, C. M. 1982. The effect of sublethal injury by heating, freezing, drying and gamma-radiation on the duration of the lag phase of *Salmonella typhimurium*. J. Appl. Bacteriol. 53: 243-251.
15. Fluit, A. C., Widjojoatmodjo, M. N., Box, T. A. and Torensma, V. J. V. 1993. Rapid detection of *Salmonella* in poultry with the magnetic immuno-polymerase chain reaction assay. Appl. Environ. Microbiol. 59: 1342-1346.
16. Hunt, J. M., Abeyta, C. and Tran, T. 1998. Campylo-

bacter. In "FDA Bacteriological Analytical Manual." 8th ed. Ch. 7. AOAC International. Gaithersburg, MD., U.S.A.

17. Maturin, L. J. and Peeler, J. T. 2001. Aerobic plate count. Available at http://www.cfsan.fda.gov/~ebam/bam-3.html. Accessed 29 October 2007.
18. Wallace, E. G. 1998. Most probable number from seriel dilutions. In "Bacteriological Analytical Manual" appendix 2. 8th ed.(Revision A). AOAC International. Gaithersburg, MD., U.S.A.
19. Hitchin, A. D., Feng, P., Watkins, W. D., Rippey, S. R. and Chandler, L. A. 1998. *Escherichia coli* and the coliform bacteria. In " Bacteriological Analytical Manual". Chapter 4. 8th ed.(Revision A). P.4.01-4.29. AOAC International. Gaithersburg, MD., U.S.A.
20. Rossen, L., Nφrskov, P. and Holmstrφm, K. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int. J. Food Microbiol. 17: 37-45.
21. Soumet, C., Ermel, G., Rose, V., Rose, N., Drouin, P., Salvat, G. and Colin, P. 1999. Identification by a multiplex PCR-Based assay of *Salmonella typhimurium* and *Salmonella enteritidis* strains from environmental swabs of poultry houses. Lett. Appl. Microbiol. 1999: 1-6.
22. Gibson, J. B. and McKee, R. A. 1993. PCR products generated from unpurified *Salmonella* DNA are degraded by thermostable nuclease activity. Lett. Appl. Microbiol. 16: 59-61.
23. Curiale, M. S., Gangar, V. and Gravens, C. 1997. VIDAS enzyme-linked fluorescent immunoassay for detection of *Salmonella* in foods: collaborative study. J. AOAC Int. 80: 491-504.
24. Oliveira, S. D., Rodenbusch, C. R., Cé, M. C., Rocha, S. L. S. and Canal, C. W. 2003. Evaluation of selective and non-selective enrichment PCR procedures for *Salmonella* detection. Lett. Appl. Microbiol. 36: 217-221.
25. Giancarlo, R., Lucia, M. and Grasso, G. M. 1999. Evaluation of immunomagnetic separation and plating media for recovery of *Salmonella* from meat. J. Food Prot. 62: 198-201.
26. Vermunt, A. E. M., Franken, A. A. J. M. and Beumer, R. R. 1992. Isolation of *Salmonella* by immunomagnetic separation. J. Appl. Bacteriol. 72: 112-118.
27. June, G. A., Sherrod, P. S., Hammack, T. S., Amaguana, R. M. and Andrews, W. H. 1995. Relative effectiveness of selenite cystine broth, tetrathionate broth, and Rappaport-Vassiliadis medium for the recovery of *Salmonella* from raw flesh and other highly contaminated foods: precollaborative study. J. AOAC Int. 78: 375-380.
28. Farkas, J. 1997. Physical methods of food preservation. In "Food Microbiology. Fundamentals and Frontiers". Doyle, M. P., Beuchat, L. R. and Montville, T. J. eds. pp. 497-519. ASM Press. Washington, D.C., U.S.A.
29. Moriñigo, M. A., Muňoz, M., Martinez-Mazanares, E., Sànchez, J. M. and Borrego, J. J. 1993. Laboratory study of several enrichment broths for the detection of *Salmonella* spp. particularly in relation to water samples. J. Appl. Bacteriol. 74: 330-335.