DETECTION OF *SALMONELLA INVA* GENE IN SHRIMP ENRICHMENT CULTURE BY POLYMERASE CHAIN REACTION

Bishnu Prasad Upadhyay[1], Fuangfa Utrarachkij[2], Jarinee Thongshoob[1], Yuvadee Mahakunkijcharoen[1], Niracha Wongchinda[3], Orasa Suthienkul[2] and Srisin Khusmith[1]

[1]Department of Microbiology and Immunology, Faculty of Tropical Medicine; [2]Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok; [3]Fishery Technical Development Division, Department of Fisheries, Kaset-Klang, Bangkok, Thailand

Abstract. Contamination of seafood with salmonellae is a major public health concern. Detection of *Salmonella* by standard culture methods is time consuming. In this study, an enrichment culture step prior to polymerase chain reaction (PCR) was applied to detect 284 bp fragment of *Salmonella invA* in comparison with the conventional culture method in 100 shrimp samples collected from four different shrimp farms and fresh food markets around Bangkok. Samples were pre-enriched in non-selective lactose broth (LB) and selective tetrathionate broth (TTB). PCR detection limit was 10 pg and 10^4 cfu/ml of viable salmonellae with 100% specificity. PCR assay detected 19 different *Salmonella* serovars belonging to 8 serogroups (B, C1, C2-C3, D1, E1, E4 and K) commonly found in clinical and environmental samples in Thailand. The detection rate of PCR following TTB enrichment (24%) was higher than conventional culture method (19%). PCR following TTB, but not in LB enrichment allowed salmonella detection with 84% sensitivity, 90% specificity and 89% accuracy. Shrimp samples collected from fresh food markets had higher levels of contaminated salmonellae than those from shrimp farms. The results indicated that incorporation of an enrichment step prior to PCR has the potential to be applied for detection of naturally contaminated salmonellae in food, environment and clinical samples.

Key words: *Salmonella*, shrimp, polymerase chain reaction, enrichment culture

INTRODUCTION

Salmonellae continue to be a major foodborne pathogen for animals and humans worldwide especially in developing and industrialized countries during the last few decades (Baird-Parker, 1990). Processing and handling of food as well as increased consumption of raw or slightly cooked food cause salmonellosis, but

Correspondence: Dr Srisin Khusmith, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2354 9100 ext 1594; Fax: 66 (0) 2354 9150
E-mail: tmskm@mahidol.ac.th
Co-correspondence: Dr Orasa Suthienkul, Faculty of Public Health, Mahidol University, 420/1 Ratchawithi Road, Bangkok 10400, Thailand.

human-to-human transmission and direct animal-to-human transmission can also occur (Prost and Rieman, 1967). The natural habitat of salmonellae is gastrointestinal tract and the organism finds its way into the river water, coastal and estuarine sediments through fecal contamination. Aquatic environments are the major reservoirs of salmonellae and aid its transmission between the hosts (Cherry *et al*, 1972). In shrimp processing industry, the principal sources of salmonellae contamination are culture ponds, coastal water used for handling and processing of seafood (Gopalakrishnan and Joseph, 1980).

Most of the ealier studies on the prevalence of salmonellae in tropical seafood were conducted using standard conventional culture method which is laborious and time consuming and requires a minimum of 5 days to complete analysis (Kumar *et al*, 2008). Moreover, one of the inherent difficulties in the detection of food pathogens is that they are generally present in a very low numbers (<100 cfu/g) in the mist of up to a million or more of other bacteria. These microbes may be lost among a background of indigeneous microflora, and substances in the foods themselves may hinder recovery (Sockett, 1991). ELISA, immunomagnetic, monoclonal antibody-based assay and DNA hybridization have been developed for detection of salmonellae in food samples. However, problems with sensitivity and specificity of these methods have limited their routine application (Garrett *et al*, 1993).

The recent advent of quantitative PCR techniques and rapid real-time thermocyclers may provide means for substantially decreasing reaction time as well as improving quntitative detection of *Salmonella* contamination (Wittwer *et al*, 2001; Ellingson *et al*, 2004). The importance of PCR technique to detect all species of *Salmonella* has been documented in foods (Manzano *et al*, 1998; Lin and Tsen, 1999; Trkov *et al*, 1999; Cheung *et al*, 2004), poultry and poultry feeds (Schrank *et al*, 2001; Whyte *et al*, 2002; Eyigor and Carli, 2003), shellfish (Brasher *et al*, 1998), oyster (Bej *et al*, 1994; Vantarakis *et al*, 2000; Kumar *et al*, 2008) and shrimp (Kimura, 1999; Kumar *et al*, 2008). However, variations in limits of detection and accuracy have been observed with different primers (Malorny *et al*, 2003). Primers to amplify and detect a 102 bp fragment of *invA*, a highly conserved gene present in almost all salmonella serotypes (Galan *et al*, 1991, 1992) and *invA*-based primers F 139 and R 141 to amplify and detect a 284 bp product specific for *Salmonella* serovars (Rahn *et al*, 1992) have been reported. PCR can be extremely effective with pure preparations of nucleic acids, but its sensitivity may be reduced when it is directly applied to biological samples (Lantz *et al*, 2000) due to the inhibition caused by a number of compounds such as lipids, salts and proteins (Wilson, 1987). To achieve an efficient high-throughput PCR method suitable for routine analysis of food samples, a rapid and simple preparation method is required.

This study aimed to develop a PCR-based protocol to determine the possible presence of *Salmonella* contamination in shrimp by incorporation an enrichment step prior to PCR assay. The detection rate, sensitivity, specificity and predictive value of PCR were compared to the standard conventional culture detection method.

MATERIALS AND METHODS

Shrimp samples

A total of 100 shrimp samples comprising 79 black tiger shrimps (*Paeneus monodon*) and 21 white shrimps (*P. vannamei*) were

collected in sterilized plastic containers from four different shrimp farms and local fresh food markets around Bangkok. The samples were immediately placed on ice and transported to the laboratory for analysis.

Conventional culture method for isolation and identification of *Salmonella*

Conventional culture-based study of shrimp samples was performed as recommended by FDA Bacteriological Analytical Manual (FDA, 1992). In brief, 25 g of seafood were homogenized using Stomacher-400 (Seward Medicals, UK) for 2 minutes with 225 ml of lactose broth (LB) (Oxoid, England) and cultured at 37°C for 24 hours. One milliliter of culture broth was added in 9 ml of either tetrathionate broth (TTB) (Oxoid, England) or Rappaport-Vassiliadis broth (RV) (Oxoid, England) and incubated at 37°C and 42°C, respectively for 24 hours. Culture broths from TTB and RV were streaked onto Xylose Lysine Deoxycholate agar (XLD)(Oxoid, England), Bismuth Sulfite (BS) agar (Oxoid, England) and Brillient Green (BG) agar (Oxoid, England) plates and incubated for 24 hours at 37 °C. Pink colonies with or without black center on XLD agar, round colonies with black brown or metallic sheen on BS agar and round colonies with pink or red color on BG agar were subjected to standard biochemical tests and finally confirmed by slide agglutination with *Salmonella* polyvalent 'O' antiserum (S & R Reagent Lab, Bangkok, Thailand).

Shrimp sample preparation and DNA extraction for PCR

Shrimp samples were enriched in non-selective LB and selective TTB prior to PCR analysis. DNA was extracted from LB and TTB by boiling-Chelex method. In brief, one ml aliquots of LB and TTB were centrifuged at 20,000*g* for 5 minutes and the cell pellets were suspended in 0.5 ml of 0.6% Chelex-100 solution and incubated at 56 °C for 30 minutes. The cell suspensions were incubated at 95°C for 10 minutes, chilled in ice for 10 minutes and centrifuged at 13,000*g* for 3 minutes. The clear supernatant was collected and stored at -20 °C for PCR.

The DNA of reference strains (*Salmonella enteritidis* SH 2886 as positive control and *V. cholerae* O1 AQ 1002 as negative control) were extracted by phenol/chloroform and ethanol precipitation (Wilson, 1987). The concentration of DNA was determined from optical density (OD) at the wavelength of 260 nm (Spectronic, 3000 Array, Millton Roy, NY) and purity was evaluated by the ratio of OD at the wavelength of 260/280. The DNA were kept at -20°C until use.

For 18 different *Salmonella* serovar strains used for testing the specificity of PCR, the organisms were harvested in 5 ml of Luria Bertani (LB) broth and incubated at 37 °C for 6 hours with rotation. One ml of log-phase culture was centrifuged at 20,000*g* for 5 minutes and the cell pellet was re-suspended with 200 µl of Tris-borate-EDTA buffer (pH 8.0), heated for 10 minutes at 95 °C and placed on ice immediately for 5 minutes. The clear supernatant was used as DNA template for PCR after centrifugation at 13,000*g* for 3 minutes.

Detection of *Salmonella invA* in shrimp samples by PCR

The specific primers for *invA* amplification were selected from those previously designed by Rahn *et al* (1992). The nucleotide sequence of forward primer F139 and the reverse primer R141 was 5'GTGAAA TTATCGCCACGTTCGGGCAA-3'and 5'TCATCGCACC GTCAAAGGAACC-3', respectively. These primers were used to amplify the 284 bp product of *invA* in

Salmonella genome located within 287 to 312 and 571 to 550 bp region. The oligonucleotide primers were synthesized by BioService Unit (National Center of Genetic Engineering and Biotechnology, Bangkok, Thailand). PCR amplification was performed using DNA Thermo Hybrid Px2 (Perkin Elmer Centus, USA). The 25 µl PCR mixture contained 0.4 µM of primers, 200 µM of each dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$ and 0.75 unit of *Taq* polymerase (DyNAzyme, Finland). The thermocycling conditions were 95ºC for 1 minute, followed by 38 cycles at 95ºC for 30 seconds, 64ºC for 30 seconds and 72ºC for 30 seconds, with a final extension at 72ºC for 4 minutes. The PCR products were separated by electrophoresis in 1.8% agarose gel (Research organics, USA) at 100 v for 1 hour, stained with ethidium bromide (0.5 µg/ml) (Merke, Darmstadt), visualized under UV transilluminator (Fotodyne, Hartland, WI) and photographed. DNA from reference strain *Salmonella enteritidis* SH 2886 and *V. cholerae* O1 AQ 1002 was used as positive and negative control, respectively.

Sensitivity and specificity of PCR

Sensitivity of PCR was evaluated by varying the concentration of genomic DNA and DNA extracted from various numbers of viable *S. enteritidis* reference strain. In brief, genomic DNA was diluted ten fold with deionized water from 100 ng to 1fg/µl and 1 µl of each solution was used. DNA from ten-fold dilutions of viable *Salmonella* cultured overnight varying from 10^7 to 1 colony forming unit/ml (cfu/ml) were also employed. DNA extracted from *S. enteritidis* and *V. cholerae* O1 was used as positive and negative control, respectively in each PCR run. PCR products were electrophoresed and documented as described above. The minimum concentration of DNA giving a positive signal was recorded.

Specificity of PCR was evaluated against 18 different *Salmonella* serovars (*S. anatum*, *S. cerro*, *S. paratyphi* B var. *java*, *S. emek*, *S. virchow*, *S. ohio*, *S. yoruba*, *S. mabandaka*, *S. montevideo*, *S. braenderup*, *S. senftenberg*, *S. rissen*, *S. weltevreden*, *S. bovismorbificans*, *S. meleagridis*, *S. derby*, *S. typhimurium*, *S. typhi*) and cross-tested against a reference panel of 13 non-*Salmonella* strains frequently contaminated in food comprising 9 gram-negative strains and 3 gram-positive strains (*S. bovis,* DMST 18567), *S. agalactiae (*DMST 16922), *B. cereus* (ATCC 11778), *E. faecalis* (ATCC 29212), *S. marcescens* (ATCC 8100), *Y. enterocolitica* (ATCC 27799), *P. mirabilis* (DMST 8212), *E. aerogenes* (ATCC 13048), *C. diversus* (DMST 15654), *V. parahaemolyticus* (AQ4613), *A. hydrophila* (DMST 15654), *V. cholerae* 01 (RIMD 1002) and *E. coli* (clinical isolate, BIDH)). Positive and negative control was genomic DNA of reference strain, *S. enteritidis* SH 2886 and *V. cholerae* O1 RIMD, respectively. These bacteria strains were kindly provided by Department of Microbiology, Faculty of Public Health, Mahidol University. Ten clinical *Salmonella* isolates kindly provided by National Institute of Health (NIH), Thailand were also included.

Data analysis

Detection rate of *Salmonella* in shrimp samples was expressed as percentage. The accuracy, sensitivity, specificity, positive predictive value and negative predictive value of the test were determined as previously described (Galen, 1979).

RESULTS

Detection limit of PCR assay

The detection limit of PCR using the primer set of F-139 and R-141 targeting

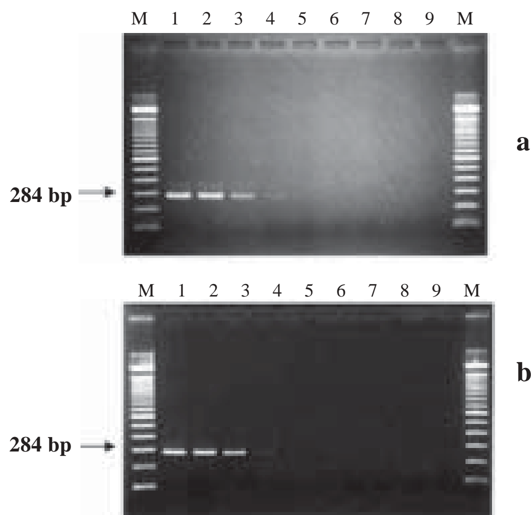

Fig 1–Detection limit of PCR for *Salmonella* detection. PCR amplifications were performed as described in Materials and Methods. a) genomic DNA: lanes 1 to 8: *S. enteritidis* DNA at 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg, respectively; lane 9, *V. cholerae* O1 DNA as negative control; lane M, 100 bp DNA ladder. b) Salmonella: lanes 1 to 8: *S. enteritidis* starting from 10^7 to 1 cfu/ml, respectively; lane 9, *V. cholerae* O1 DNA as negative control; lane M, 100 bp DNA ladder.

invA gene of *Salmonella* is shown in Fig 1. When genomic DNA from *S. enteritidis* was tested, the specific band of 284 bp could be observed at the lowest concentration of 10 pg. When DNA extracted from *Salmonella* was tested, the detection limit was equal to 10^4 cfu/ml. DNA from negative control strain, *V. cholerae* O1 did not give any amplicon.

Specificity of PCR assay

The PCR assay was able to produce a single band corresponding to 284 bp for all 18 most prevalent *Salmonella* serovars (Fig 2) and 10 clinical *Salmonella* isolates (data not shown). No amplification was observed for 13 non-*Salmonella* strains

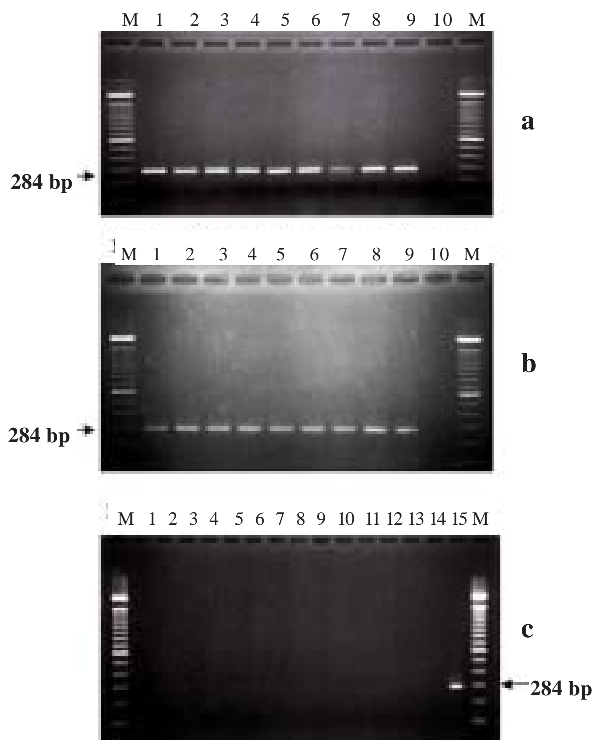

Fig 2–Specificity of polymerase chain reaction evaluated with 18 different *Salmonella* serovar strains and non-salmonella bacterial strains. a) lanes 1 to 9: *S. cerro*, *S. paratyphi* III B, *S. emek*, *S. virchow*, *S. ohio*, *S. yoruba*, *S. mabandaka*, *S. paratyphi* I B and *S. montevideo*, respectively; lane 10, *V. cholerae* O1 as negative control; lane M, 100 bp DNA ladder. b) lanes 1 to 9: *S. braenderup*, *S. senftenberg*, *S. rissen*, *S. weltevreden*, *S. bovis*, *S. meleagridis*, *S. derby*, *S. typhimurium* 1980051 and *S. typhi* 1404, respectively; lane 10, *V. cholerae* O1 as negative control; lane M, 100 bp DNA ladder. c) lanes 1 to 13: *S. bovis*, *S. agalactiae*, *B. cereus*, *E. faecalis*, *S. marcescens*, *Y. enterocolitica*, *P. mirabilis*, *E. aerogenes*, *C. diversus*, *V. parahaemolyticus*, *V. cholerae*, *E. coli*, *A. hydrophila*, respectively; lane 14, *V. cholerae* O1 as negative control; lane 15, *S. enteritidis* as positive control and lane M, 100 bp DNA ladder. PCR was conducted as described in legend of Fig 1.

Table 1
Detection rate of salmonellae in shrimp enrichment cultures by PCR and conventional culture method.

Shrimp samples	No. of samples tested	% positive (No. of positive samples/total)		
		CCM	PCR (TTB)	PCR LB)
Black tiger shrimp	79	14(11/79)	18(14/79)	0(0/79)
White shrimp	21	38(8/21)	48(10/21)	0(0/21)
Total	100	19	24	0

CCM, conventional culture method; TTB, tetrathionate broth; LB, lactose broth

Table 2
Detection rate of salmonellae in shrimp enrichment tetrathionate broth by PCR in comparison to conventional culture method.

PCR	Conventional culture method		Total
	Positive	Negative	
Positive	16	8	24
Negative	3	73	76
Total	19	81	100

usually contaminating seafood, indicating 100% specificity of PCR.

Detection of Salmonellae by PCR and conventional culture method

The detection rates of *Salmonella* by PCR in shrimp enrichment cultures in comparison with the conventional culture method (CCM) are summarized in Table 1. PCR could detect *Salmonella* in enrichment selective tetrathionate broth (TTB) (24%) at a higher rate than CCM (19%). However, PCR could not detect *Salmonella* in enrichment lactose broth (LB). It is worth noting that 16 and 73 shrimp samples were positive and negative, respectively by both CCM and PCR in enrichment TTB culture, while 8 shrimp samples were negative by CCM, but positive by PCR in enrichment TTB culture. In contrast, 3 shrimp samples were positive by CCM but negative by PCR in enrichment TTB culture (Table 2).

Among 79 black tiger shrimp samples, *Salmonella* could be detected in 14 (18%) and 11 samples (14%) by PCR in enrichment TTB and CCM, respectively, while among 21 white shrimp samples, 10 (48%) and 8 (38%) harbored *Salmonella* as detected by PCR in enrichment TTB and CCM respectively (Table 1). In this study, the shrimp samples from fresh food markets (8/16) had higher contaminated *Salmonella* than those from four different shrimp farms (12/84) as detected by both methods.

DISCUSSION

Salmonella continues to be a serious threat to consumer health due not only to its pathogenicity, but also to its ability in adapting to many different environments and its broad range of transmission routes. In this study, the efficiency of different enrichment culture prior to PCR assay was compared with the conventional culture method in shrimp samples collected from four different shrimp farms and fresh food markets around Bangkok. Primers targeting *inv*A gene of *Salmonella* used to

amplify 284 bp product could detect as low as 10 pg of genomic DNA and 10^4 cfu/ml of *Salmonella*. This was similar to the result of 27 pg of genomic DNA from the previous report using the same primers (Rahn *et al*, 1992). However, the detection limit of 10^4 cfu/ml of *Salmonella* was higher than $3x10^2$ cells/ml reported by the same group, but was similar to the recent observation of 10^4 cfu/ml by using *invA* gene-based primers (Vazquez-Novelle *et al*, 2005).

The specificity of PCR assay was 100% based on the tests using *S. enteritidis* and 18 most prevalent *Salmonella* serovars and 13 non-*Salmonella* strains usually contaminating seafood. Hence, *invA* gene based primers F-139 and R-141 was specific for detection of *Salmonella* serovars. This corresponded to the previous report in *C. diversus*, *E. aerogenes* and *E. coli* (Rahn *et al*, 1992).

It is important to note that PCR after enrichment in selective TTB prior to *invA* gene - based PCR gave higher detection rate than conventional culture, while PCR detection after enrichment in non-selective LB did not. These results are similar to the previous study in poultry, animal and clinical samples in that PCR after enrichment detected more *Salmonella* compared to conventional culture (Eyigor and Carli, 2003; Marciorowski *et al*, 2005). In general, the addition of a requisite enrichment step in selective culture medium substantially increases cell number and subsequent DNA and mRNA accordingly. However, the negative results after enrichment in LB was unexpected and its explanation could be possibly due to the high amounts of inhibitor, interference and growth inhibition by other competing bacteria as previously reported (Blais *et al*, 1997). It is also possible that chromosomal DNA of *Salmonella* may be degraded by the metabolic products of other competing microorganism or DNA *Taq* polymerase may be inhibited by high amount of proteinous substances contained in the sample. Moreover, the efficiency of the broths may also be related to the source of samples previously demonstrated in seafood samples (Shabarinath *et al*, 2007). Therefore, it seems that enrichment in non-selective LB prior to PCR might not be appropriate. However, the effect of this negative result needs to be validated.

The results of 8 samples being negative for *Salmonella* by conventional culture but positive by PCR indicated that salmonellae might be in a viable but nonculturable state (Guo *et al*, 2000). However, 3 samples were positive only by conventional culture. A possible reason is that the culture method was able to detect only living cells of *Salmonella*, which should be present in sufficient number and could be resuscitated in the pre-enrichment and selective enrichment steps. Thus, the positive result by the culture method is based on the chance of selectivity whereas PCR detection depends on the amount of DNA from either viable or nonviable cells. The main drawback with the PCR assay is the potential detection of nonviable cells as DNA can persist in a sample long after the target organism has died, potentially leading to the production of false-positive results (Drabovska *et al*, 2001). In addition, high DNA concentration and number of cells other than the target organism have been shown to affect both sensitivity and specificity of PCR (Wang *et al,* 1992). The reliability of PCR assay also depends on the presence of inhibitory substances from the seafood matrix. However, a recent study in seafood concluded that incorporation of enrichment cultures prior to PCR rules out any possibility of detecting dead cells causing false positive results in naturally contaminated seafood (Kumar *et al,* 2008).

In this study, PCR using the primer set of F-139 and R-141 targeting *invA* gene of *Salmonella* allowed detection of 19 different *Salmonella* serovars belonging to 8 serogroups of B, C1, C2-C3, D1, E1, E4 and K, which are commonly found in clinical and environmental samples in Thailand (Bangtrakulnonth *et al*, 2004) including *S. anatum*, *S. cerro*, *S. paratyphi* B var. *java*, *S. emek*, *S. virchow*, *S. ohio*, *S. yoruba*, *S. mabandaka*, *S. montevideo*, *S. braenderup*, *S. senftenberg*, *S. rissen*, *S. weltevreden*, *S. bovismorbificans*, *S. meleagridis*, *S. derby*, *S. typhimurium*, *S. typhi* and *S. stanley*. The results are inagreement with the previous report showing that application of *invA* (284 bp amplicon) allowed detection of almost all *Salmonella* serovars without any nonspecific product from *Salmonella*–related strains (Kumar *et al*, 2008). A high possibility of *Salmonella* contamination in shrimp samples from fresh food markets was found when compared to those from four farms and white shrimps were more contaminated with salmonellae than black tiger shrimps. However, further studies should include a larger sample size from different areas in order to draw a definite conclusion.

In summary, the results demonstrated that enrichment in selective medium prior to PCR using *inv*A gene-based primers provided an essential preliminary step in the application of the assay and could be an alternative approach for detection of *Salmonella* in seafood samples or it could be applied for non-culturable but viable cells and sub-lethally destroyed cells in food, environment and clinical samples. Although the method described herein takes 24-48 hour enrichment period prior to PCR, it dramatically decreased the time and effort required in standard microbiological testing. In order for the method to be applied extensively, a study in larger sample size should be conducted to assess the accuracy and reproducibility of the assay. Development of PCR using primers targeting other genes should be also considered.

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