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REGULAR ARTICLE

Detection of viable *Salmonella* Typhi using three primer pairs specific to *invA*, *ivaB* and *fliC-d* genes

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ABSTRACT

The detection of viable (clinically significant) *Salmonella enterica* subsp. *enterica* serovar Typhi in food is essential from clinical, epidemiological, and infection control perspective. In this study, a method for detection of viable *S.* Typhi was developed based on reverse transcription-multiplex PCR (RT-MPCR). Three primer pairs used in RT-MPCR were *invA*-f/*invA*-r, *ivaB*-f/*ivaB*-r and *fliC-d*-f/*fliC-d*-r that were specific to *invA* gene, *ivaB* region and *fliC-d* gene, respectively. Of all *S. enterica* serovars, only *S.* Typhi had all target DNA that was able to be amplified by all 3 primer pairs. When RNA extracted from *S.* Typhi was subjected to RT-MPCR, 3 DNA products with the sizes of 284, 599 and 763 bp were observed. Moreover, RT-MPCR was shown to be able to detect *S.* Typhi and to discriminate between viable and nonviable cells in food.

Keywords: Multiplex PCR; Reverse transcription; *Salmonella* Typhi; Vi antigen

INTRODUCTION

Salmonella enterica subsp. *enterica* serovar Typhi (*S.* Typhi) is a gram negative bacillus belonging to the family *Enterobacteriaceae*. It is as a pathogen of human typhoid fever, one of the major health problems worldwide (Bhutta, 2006; Crump et al., 2004; Kothari et al., 2008; Polonsky et al., 2014). The transmission of *S.* Typhi is considered to be a direct contact transmission among human without any animal as an intermediate host. The potential detection of *S.* Typhi in food and drinking water is essential because they are the main sources of the pathogen.

The limitations of microbiological and serological methods of *S.* Typhi detection have encouraged the development of molecular detection methods based on the amplification of DNA. Many studies have reported on the detection of *S.* Typhi using PCR with the *S.* Typhi-specific primer pair (STY1599-f and STY1599-r) (Park et al., 2009) and multiplex PCR with combinations of primers specific to multiple genes such as the *invA* gene, the first gene in the *invABC* operon that is responsible for invasion of the intestinal epithelial cells (Galan et al., 1992), the *fliC-d* gene, the phase 1 flagellin gene encoding d antigen (or H1-d antigen), the *prt* gene (previously known as *rfbS*), the gene encoding paratose synthase, the *tyv* gene (previously known as *rfbE*), the gene encoding CDP-tyvelose epimerase, and the *ViaB* region, a chromosomal locus consisting of the structural genes specific for Vi (virulence) antigen expression (Banavandi et al., 2005; de Freitas et al., 2010; El-Sayed Ahmed et al., 2015; Kumar et al., 2006; Levy et al., 2008; Lim and Thong, 2009). However, the molecular methods used for *S.* Typhi detection suffer from the disadvantage that they may not distinguish viable (pathogenic) from nonviable cells. Therefore, a molecular method with discrimination power between viable and nonviable cells remains to be revealed.

In this paper, we proposed a molecular method for detecting *S.* Typhi based on reverse transcription-multiplex PCR (RT-MPCR) using primers specific to the *invA* gene, the *fliC-d* gene and the *viaB* region. This method allows not only the specific detection of *S.* Typhi but also the discrimination between viable and nonviable cells. In this study, the method was validated on both *S.* Typhi pure culture and a food model artificially contaminated with *S.* Typhi.

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MATERIALS AND METHODS

Bacterial RNA and DNA

A total of 18 serovars of *Salmonella enterica* subsp. *enterica* and 15 non-*Salmonella* strains were used in this study (Table 1). Their RNA and DNA were extracted from a single colony in 2 mL of BHI broth using protocols previously mentioned (Rattanachaikunsopon and Phumkhachorn 2012).

RT-PCR, RT-MPCR and MPCR

Reverse transcription-PCR (RT-PCR), reverse transcription-multiplex PCR (RT-MPCR) and multiplex PCR (MPCR) were performed as described previously (Rattanachaikunsopon and Phumkhachorn 2012) using three primer pairs including *invA*-f/*invA*-r, *fliC-d*-f/*fliC-d*-r and *ivaB*-f/*ivaB*-r. The sequences of the primers and sizes of the expected amplicons are shown in Table 2. All primers were designed from the nucleotide sequences encoding *S*. Typhi *invA* gene, *fliC-d* (or flagellin) gene and *viaB* region DNA for Vi antigen. The GenBank accession numbers of these genes are provided in Table 2. The products of RT-PCR, RT-MPCR and MPCR were analyzed on 2% agarose gel together with Fisher BioReagent s*ex* ACT G*ene* DNA ladder-100 bp DNA ladder (Fisher Scientific, Waltham, MA, USA)

Table 1: Bacteria used in this study

Bacteria	Source[a]	H1 antigen	Vi antigen[b]
Salmonella enterica subsp. *enterica*			
serovar Paratyphi A	ATCC 11511	a	-
serovar Paratyphi B	ATCC 8759	b	-
serovar Paratyphi C	ATCC 13428	c	+
serovar Typhi	ATCC 7251	d	+
serovar Typhi	ATCC 10749	d	+
serovar Typhi	ATCC 19939	d	+
serovar Typhi	DMST 1328	d	+
serovar Stanley	ATCC 7308	d	-
serovar Typhimurium	ATCC 29630	i	-
serovar Thompson	ATCC 8391	k	-
serovar Infantis	ATCC BAA-1675	r	-
serovar Bareilly	ATCC 9115	y	-
serovar Poona	ATCC BAA-1673	z	-
serovar Dublin	ATCC 15480	g,p	+
serovar Chester	ATCC 11997	e,h	-
serovar Agona	ATCC 51957	f,g,s	-
serovar Oranienburg	ATCC 9239	m,t	-
serovar Enteritidis	ATCC 13076	g,m	-
Non-*Salmonella*			
Aeromonas hydrophila	ATCC 7966		-
Bacillus cereus	ATCC 14579		-
Enterobacter aerogenes	ATCC 13048		-
Enterococcus faecalis	ATCC 12953		-
Escherichia coli	ATCC 35150		-
Klebsiella pneumoniae	ATCC 13884		-
Proteus mirabilis	ATCC 21100		-
Serratia marcescens	ATCC 13880		-
Shigella boydii	DMST 28180		-
Shigella dysenteriae	DMST 1511		-
Shigella flexneri	DMST 4423		-
Shigella sonnei	DMST 561		-
Listeria innocua	ATCC 51742		-
Listeria grayi	ATCC 700545		-
Yersinia enterocolitica	ATCC 9610		-

[a]ATCC=American type culture collection; DMST=Department of medical sciences thailand [b]+ = have Vi antigen, - = do not have Vi antigen

Distinguishing between viable and nonviable *S.* Typhi cells by RT-MPCR

RT-MPCR and MPCR were performed with all three primer pairs using RNA and DNA extracted from log phase cells of *S*. Typhi ATCC 7251 as templates, respectively. They were also performed using RNA and DNA extracted from heat killed (boiled at 100°C for 15 min) cells of *S*. Typhi ATCC 7251 as templates. The products from both experiments were compared on 2% agarose gel.

Detection of *S.* Typhi in a food model

Fresh ice-chilled seabass (*Lates calcarifer*) flesh was cut into 10 g slices with the thickness of 0.5 cm. Each sample were decontaminated by treating with ultraviolet light with the intensity of about 5 mW/cm^2 for 10 min. Each sample was then placed into a sterile plastic bag and inoculated with 0.1 mL live or heat killed *S*. Typhi ATCC 7251 to obtain the final concentration of 100 CFU/g of food. After the bacterial inoculated food samples were left at room temperature for 1 h, they were diluted with 10 mL of sterile phosphate buffer (pH7) and then homogenized for 1 min. Two mL of each food homogenate were collected for extracting RNA that was used in RT-MPCR. For a control, a food sample was inoculated with 0.1 mL of BHI broth instead of the bacterial culture and processed in the same way as the *S*. Typhi treated samples.

RESULTS AND DISCUSSION

Evaluation of each primer pair

Each primer pair was initially evaluated separately for specificity and functionality by RT-PCR using RNA extracted from all of the bacteria listed in Table 1. Primer pairs to be evaluated were *invA*-f/*invA*-r, *fliC-d*-f/*fliC-d*-r and *ivaB*-f/*ivaB*-r.

By using the primer pair *invA*-f/*invA*-r, RT-PCR products with a size of 284 bp were obtained from all *Salmonella* strains, but not from any of non-*Salmonella* strains. This is because this prime pair is specific to the *invA* gene of the bacterial genus *Salmonella*. This result is consistent with other previous reports mentioning that the primer pair was

Salmonella specific. Many previous works have used the *invA* gene to specifically detect *Salmonella* (Amini et al., 2015, Chiu and Ou, 1996; Malorny et al., 2003; Krascsenicsova et al., 2008; Paiao et al., 2013; Rahn et al., 1992; Wolffs et al., 2006; Zhai et al., 2014). Apart from the *invA* gene, other *Salmonella* genes have been used for the specific detection of *Salmonella* such as the *fimA* (Cohen et al., 1996), *fimY* (Yeh et al., 2002), *stdA* (Chuang et al., 2008) and *ompC* (Ngan et al., 2010) genes.

By using the primer pair *fliC-d*-f/*fliC-d*-r, RT-PCR products with a size of 763 bp were obtained only from *Salmonella* serovars possessing H1:d antigen (*S*. Typhi and *S*. Stanley), but not from the rest of bacteria used in this study. The primer pair *fliC-d*-f/*fliC-d*-r can also detect the *fliC-j* gene which is an alternate phase of serovar Typhi H1 antigen (H1:j antigen) genes. The *fliC-j* gene is a 261 bp deletion derivative of the *fliC-d* gene (Levy et al., 2008, Lim and Thong, 2009). Therefore, this primer pair can be used in PCR to differentiate *S*. Typhi having the H1:d antigen from that having the H1:j antigen by producing a 763 bp amplicon for the former one and a 502 bp amplicon for the latter one. We did not include *S*. Typhi having the *fliC-j* gene in this study; therefore, the production of the 502 bp amplicon was not presented here. However, such result was reported by Levy et al. (2008) with *S*. Typhi starins CDC 06-0418, CDC 01-0274, CDC 2433 and CDC 95-0344.

By using the primer pair *ivaB*-f/*ivaB*-r, RT-PCR products with a size of 599 bp were obtained only from *S*. Typhi and *S*. Paratyphi C, serovars possessing Vi antigen, but not from the rest of bacteria used in this study. The primer pair *ivaB*-f/*ivaB*-r was designed to be specific to the *viaB* regions of *S*. Typhi and *S*. Paratyphi C, but not to those of *S*. Dublin and *Citrobacter freundii* (Hashimoto et al., 1995). Therefore, it is not capable of amplifying the *viaB* regions from Vi positive *S*. Dublin and *C. freundii*. As a result, no RT-PCR product was observed in the case of Vi positive *S*. Dublin ATCC 15480 when the primer pair *ivaB*-f/*ivaB*-r was used in RT-PCR.

Specificity of RT-MPCR

To develop a RT-MPCR assay, all primers were evaluated for functionality and specificity when used simultaneously. For each RNA template, RT-MPCR was performed using all three primer pairs. The results from RT-MPCR were in agreement with those obtained from RT-PCR using the primer pairs separately. There were 5 patterns of the RT-MPCR results. The first pattern (no RT-MPCR product) was produced by all of non-*Salmonella* bacteria (Fig. 1). The second pattern (one RT-MPCR product of 284 bp) was produced by *Salmonella* serovars having no H1:d and Vi antigens (Fig. 1). The third pattern (two RT-MPCR products of 284 and 763 bp) was produced by *Salmonella* serovar having H1:d antigen with no Vi antigen (*S*. Stanley) (Fig. 1). The fourth pattern (two RT-MPCR products of 284 and 599 bp) was produced by *Salmonella* serovars having Vi antigen with no H1:d antigen (*S*. Paratyphi C) (Fig. 1). RT-MPCR of RNA extracted from *S*. Dublin ATCC 15480 produced only one RT-MPCR product of 284 bp (the second pattern) despite the fact that this strain has Vi antigen (data not shown). This is because the primer pair *ivaB*-f/*ivaB*-r cannot detect the *viaB* region of *S*. Dublin (Hashimoto et al. 1995). The last pattern (three RT-MPCR products of 284, 599 and 763 bp) was produced only by S. Typhi because this serovar had both H1:d and Vi antigens (Fig. 1). These results suggest that all primers maintain their functionality and specificity in the RT-MPCR and that they can be used for the specific detection of *S*. Typhi.

Ability of RT-MPCR to discriminate between viable and nonviable cells

When DNA extracted from viable and heat killed cells of *S*. Typhi ATCC 7251 were subjected for MPCR using

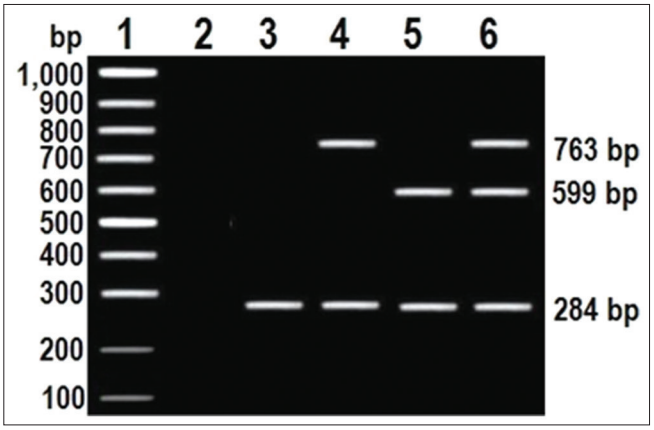

Fig 1. Products from RT-MPCR using three primer pairs (*invA*-f/*invA*-r, *fliC-d*-f/*fliC-d*-r, *ivaB*-f/*ivaB*-r) simultaneously. Lane 1, 100 bp DNA ladder; lane 2, *E. coli* (*invA*$^-$, H1:d$^-$, Vi$^-$); lane 3, *S.* Typhimurium (*invA*$^+$, H1:d$^-$, Vi$^-$); lane 4, *S.* Stanley (*invA*$^+$, H1:d$^+$, Vi$^-$); lane 5, *S.* Paratyphi C (*invA*$^+$, H1:d$^-$, Vi$^+$); lane 6, *S.* Typhi ATCC 7251 (*invA*$^+$, H1:d$^+$, Vi$^+$).

Table 2: Primer sequences, Sizes of expected amplicons and sources of primers

Primer pair	Sequence	Size of amplicon	Accession number
invA-f/*invA*-r	5'gtgaaattatcgccacgttcgggcaa3' and 5'tcatcgcaccgtcaaaggaacc3'	284 bp	M90846
fliC-d-f/*fliC-d*-r	5'actcaggcttcccgtaacgc3' and 5'ggctagtattgtccttatcgg3'	763 bp	L21912
ivaB-f/*ivaB*-r	5'gttatttcagcataaggag3' and 5'acttgtccgtgttttactc3'	599 bp	D14156

the primer pairs (*invA*-f/*invA*-r, *fliC-d*-f/*fliC-d*-r, *ivaB*-f/*ivaB*-r) simultaneously, 3 bands of 284, 559 and 763 kb were observed in both cases (Fig. 2). However, when RNA extracted from viable and heat killed cells of *S*. Typhi ATCC 7251 were subjected for RT-MPCR using the same primer pairs, only RNA of the viable cells gave positive results showing 3 bands of 284, 559 and 763 kb (Fig. 2). This may be due to the rapid loss of RNA in the heat killed cells (Deutscher et al., 2006). These results, therefore, suggest that RT-MPCR is able to discriminate viable *S*. Typhi cells form nonviable *S*. Typhi cells. Several methods based on detection of RNA have been used to discriminate live *S*. Typhi cells from dead *S*. Typhi cells. Fan et al. (2015) detect live *S*. Typhi cells by using reverse transcription loop-mediated isothermal amplification (RT-LAMP) to detect mRNA of the specific gene marker STY1607. Morin et al. (2004) used reverse transcription in conjunction with polymerase chain reaction to amplify mRNA of the *tyv* gene in order to detect live *S*. Typhi cells.

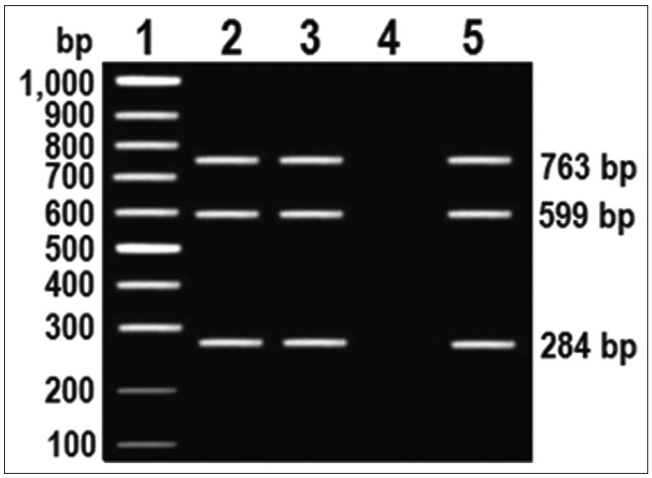

Fig 2. Products from RT-MPCR and MPCR of RNA and DNA extracted from *S.* Typhi ATCC 7251, respectively. Lane 1, 100 bp DNA ladder; lane 2, RT-MPCR with RNA of viable cells; lane 3, MPCR with DNA of viable cells; lane 4, RT-MPCR with RNA of heat killed cells; lane 5, MPCR with DNA of heat killed cells.

Detection of *S. Typhi* in a food model

The ability to detect *S*. Typhi in a food model by the proposed method was examined. When RNA extracted from *S*. Typhi ATCC 7251 artificially inoculated onto a seabass slice was amplified by RT-MPCR with primers specific to *S*. Typhi, the expected pattern of RT-MPCR product was observed (Fig. 3). The food sample that was inoculated with heat treated *S*. Typhi ATCC 7251 culture and that was used as a control gave the negative result. No RT-MPCR product was observed in both cases (Fig. 3). This result suggests that the method developed in this study may provide a means for detection of *S*. Typhi and discrimination between viable and nonviable cells in food. Hosseinzadeh et al. (2014) used RT-PCR with a pair of primers specific to the *invA* gene to detect live cells of *Salmonella* spp. in milk. Unlike our proposed method, their method could not differentiate *S*. Typhi from other species of *Salmonella*. Although MPCR developed by Kumar et al. (2006) was able to specifically detect *S*. Typhi in food, it could not distinguish between viable and nonviable cells. With some further experiments, our proposed method may also be used to detect live *S*. Typhi in clinical samples as a more convenient alternative to RT-LAMP suffering from the complication and difficulty of primer design (Fan et al., 2015).

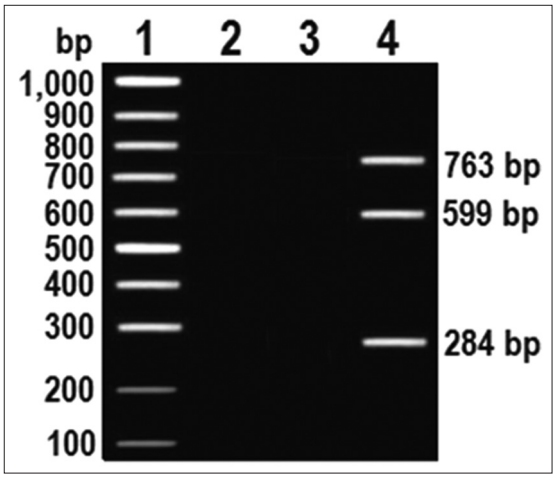

Fig 3. Products from RT-MPCR of RNA extracted from *S.* Typhi ATCC 7251 isolated from artificially contaminated seabass. Lane 1, 100 bp DNA ladder; lane 2, untreated seabass; lane 3, seabass treated with nonviable *S.* Typhi; lane 4, seabass treated with viable *S.* Typhi.

CONCLUSIONS

This is the first report of the successful use of RT-MPCR with 3 primer pairs, *invA*-f/*invA*-r, *fliC-d*-f/*fliC-d*-r and *ivaB*-f/*ivaB*-r, to specifically detect *S*. Typhi. This method overcomes the disadvantage of PCR based detection methods that usually detect dead (non clinically significant) *S*. Typhi. Our developed method was able to specifically detect *S*. Typhi and discriminate between viable and nonvialble *S*. Typhi cells in both pure culture and in artificially contaminated food. Experiments are underway on the valuation of this method's ability to detect *S*. Typhi in naturally contaminated foods and environment samples in which many background microflora reside.

Author's contributions

Parichat Phumkhachorn and Pongsak Rattanachaikunsopon have equal contribution in designing the research, conducting the experiments, analyzing the data and preparing the manuscript. Both authors are aware of manuscript submission for publication and take public responsibility for the content, while declaring no conflict of interest associated with any aspect of this manuscript.

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