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Molecular detection of *Salmonella enterica* serovar Typhi by Vi-qPCR

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ABSTRACT

Aims: To develop a real-time polymerase chain reaction system Vi-qPCR in the detection of *Salmonella enterica* serovar Typhi (*S.* Typhi), targeting the *vexC* gene encoding for Vi antigen (capsular polysaccharide antigen) and to evaluate its sensitivity and specificity performance using pure cultures of *S.* Typhi and other enteric pathogens.
Methodology and results: Microbiological, biochemical and serotyping tests were conducted to determine the phenotypic characteristics of *S.* Typhi and other enteric pathogens in our collection. Primers were designed using Primer3 software and their *in-silico* specificity were analysed using Basic Local Alignment System Tool (BLAST). Optimisation of PCR annealing temperature was done prior to assessment of sensitivity and specificity performance against artificial serially diluted seeded stools. The primers were found to be 100% specific in the detection of *S.* Typhi towards 32 tested clinical strains. Verification of gene amplification by comparing the nucleotide sequences against reference genes in the GenBank database revealed high specificity to *S.* Typhi. Statistical analysis indicates that this method results in 100% sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Moreover, Vi-qPCR allows the detection of *S.* Typhi as low as 13±1.4 CFU/g of stool sample.
Conclusion, significance and impact of study: A rapid and sensitive method for detection of *Salmonella enterica* serovar Typhi (*S.* Typhi) is desired as a diagnostic tool to improve typhoid management. The Vi-qPCR represent a promising non-invasive diagnostic tool for medical microbiology laboratories as a method for the detection of *S.* Typhi in both pure culture and stool specimens especially in chronic asymptomatic carriers where shedding of *S.* Typhi is intermittent and sometimes occurs in low level.

Keywords: PCR, diagnostic, pathogen

INTRODUCTION

Typhoid fever is one of the most common foodborne illnesses caused by the Gram-negative bacterium, *Salmonella enterica* serovar Typhi (*S.* Typhi). The disease continues to be a global public health problem particularly in countries with poor sanitary conditions and hygiene standards (Crump *et al*., 2004; Brooks *et al*., 2005). It was estimated that this pathogen causes 21.6 million cases with 216,000 deaths each year worldwide (Crump *et al.*, 2010). The majority of cases and deaths are in Asia countries. The remaining cases occur mainly in Latin America and Africa. The incidence of typhoid fever is low in industrialised countries and commonly associated with travel history to endemic regions (Parry *et al*., 2011).

Salmonella enterica serovar Typhi can be transmitted via fecal-oral route. It is usually associated with consumption of contaminated water and food. After ingestion, colonisation of the small intestine will be initiated by invasion of the gastrointestinal mucosal surface. Subsequently, dissemination of the pathogen throughout the body in the reticulo endo-thelial system that includes the liver, spleen and bone marrow will take place. A person can be a long term chronic fecal shedding host due to relapse and *S.* Typhi carriage in gall bladder. Asymptomatic carriers play an important role in perpetuating the disease and are responsible for typhoid outbreaks. The signs and symptoms of typhoid may mimic other diseases including leptospirosis, malaria, dengue and rickettsioses. The unspecific signs and symptoms therefore complicate the clinical diagnosis (Feasey *et al.*, 2012; Wain *et al*., 2015).

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Currently, the gold standard method for typhoid diagnosis is the culture method. This method is time-consuming in view of requiring two to five days to produce results. Moreover, this method has poor sensitivity which depends to the type of samples used (30-60%). Detection of *S.* Typhi from bone marrow sample was reported to give the highest sensitivity compared to other types of samples (Wain *et al.*, 2001; WHO, 2003; Bhutta, 2006). Culture methods are highly depended on viability of targeted bacteria in the samples. In situations where the patient has received antibiotics prior to specimen collection, negative culture results will be obtained. This was reported to be one of the major obstacles in *S.* Typhi diagnosis (Tennant *et al.*, 2015; Wain *et al.*, 2015).

There are several serological methods for typhoid diagnosis such as Widal test, Typhi-Dot, Tubex (Naheed *et al.*, 2008) and lateral flow (Nakhla *et al.*, 2011). These immunological methods were designed based on the types of *S.* Typhi O and H antigens. Despite their rapidity and simplicity, the tests are not suitable for typhoid early prevention. In addition, these assays also are relatively low in sensitivity. They are prone to be influenced by the phases of the disease. The interpretation of the immunological reaction depends on the antibody production and its titre. The antibodies are normally induced at least one week after *S*. Typhi infection. Thus, there is an urgent need for the development of a rapid and reliable test to detect this food-borne pathogen for prevention and effective management of typhoid especially during outbreaks.

Polymerase chain reaction (PCR) has been successfully used to detect bacterial pathogens in clinical samples, aquatic environments and food products (Moganedi *et al.*, 2007; Elizaquivel and Azhar, 2008; Park *et al.*, 2009; Kumar *et al.*, 2010; Fan *et al.*, 2015). In conventional PCR, amplicon is detected by agarose gel electrophoresis whilst in real-time PCR, the PCR product is quantified by measuring the fluorescent signal. Real-time PCR has several advantages over conventional PCR which are more rapid and robust. These criteria contribute in less variability in the assay as well as the interpretation. The real-time PCR method was reported to have advantages over the culture method for detection of *S.* Typhi in sensitivity as well as specificity and require shorter time for analysis (Wang *et al.*, 1997; Ambati *et al.*, 2007; Levy *et al.*, 2008; Ali *et al.*, 2009; Germini *et al.*, 2009; Zhang *et al.*, 2014). In this study, a real-time PCR assay targeting the *S*. Typhi *vexC* gene that encodes the *S*. Typhi Vi antigen (capsular polysaccharide antigen) was developed. The Vi antigen was previously suggested as a specific target for the identification of *S*. Typhi due to its association with virulence (Hashimoto *et al.*, 1995; Sharma *et al.*, 1995; Hirose *et al.*, 2002; Farrell *et al.*, 2005). The antigen modulates several pro-inflammatory signalling pathways of macrophages, dendritic cells and microfold cells (Schadich *et al.*, 2016). Hence, this study aims to develop Vi-qPCR and evaluate the performance using pure cultures of *S*. Typhi and other enteric pathogens. In addition, the sensitivity of the optimised methods was further evaluated using artificially seeded stool samples with similar strains of bacteria that also included Vi-possessed bacteria, *Citrobacter freundii* (Kolyva *et al.*, 1992).

MATERIALS AND METHODS

Bacterial strains

A total of 32 bacterial strains were used in this study as listed in Table 1. All strains were obtained from National Public Health Laboratory (NPHL), Ministry of Health Malaysia's culture bank. A *Salmonella enterica* serovar Typhi TP279 (Bionumber 0015610540144200) was used as positive control throughout the experiments. Identifications of positive control strain and other strains used in this study were carried out by means of standard microbiological and biochemical methods using Vitek®2 compact system (bioMérieux, France). Serotyping of the strains was performed by slide agglutination following the manufacturer's instructions (Staten Serum Institute, Denmark).

Bacterial DNA extraction

All bacterial strains were grown overnight (18-24 h) on blood agar (BA) medium (Thermo Fisher Scientific, Malaysia) at 35 °C±2 °C. Genomic DNA of the strains were extracted using QIAmp® DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. DNA concentrations and purity were determined spectrophotometrically using a biophotometer (Eppendorf, Germany). The presence and quality of intact DNA was visualised on 0.8% agarose gel stained with GelRed™ (Biotium®, USA). The DNA was stored in ─20 °C until further use.

Primer design

Primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) based on the *vexC* gene of *S*. Typhi (GenBank Accession Number AL513382). Both forward and reverse primers were respectively named as SViF and SViR. These primers were expected to generate a 300 bp nucleotide product. *In silico* specificity of the primers were analysed using Basic Local Alignment System Tool (BLAST) from the GenBank at http://blast.ncbi.nlm.nih.gov. Details of the primers are as in Table 2.

Conventional PCR optimisation

The initial part of PCR optimisation was performed using conventional PCR. The *S.* Typhi TP279 DNA was used as template throughout the optimisation. Each reaction in a total of 25 µL contained 12.5 µL PCR Master Mix (Promega, USA), 1 µL each of the forward and reverse primers, 5 µL of DNA template and 5.5 µL molecular grade water. The primer concentration used in the reaction was kept constant at 10 µM. Amplification was performed in Mastercycler Nexus (Eppendorf,

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Germany). Annealing temperature optimisation was performed at 12 points using the following condition; initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C denaturation for 1 min, annealing at 49.8 °C to 65.1 °C for 1 min and extension, 72 °C for 1 min. PCR products (5 µL) were electrophoresed on 2% agarose gel in a 0.5× TBE buffer at 85 V for 60 min. The gel was stained with GelRed™ (Biotium®, USA). A 100 bp DNA ladder (Fermentas, USA) was included as the molecular weight marker in every electrophoresis run. The PCR products were visualised under ultraviolet (UV) illumination using gel image documentation system (GelDoc 1000 system, Bio-Rad, USA). Optimised parameters (annealing temperature) by conventional PCR were used in subsequent experiments for real-time PCR.

Real-time PCR (Vi-qPCR)

Real-time PCR was carried out in LightCycler® 480 (Roche, USA). The reaction mix consisted of 10 µL of LightCycler® 480 SYBR Green I Master (Roche, USA), 1.0 µL of each forward and reverse primers (10 µM), 5 µL of DNA template and 3 µL molecular grade water. The thermal cycling protocol of the assay consisted of 5 min pre-incubation at 95 °C, 40 cycles of PCR amplification (95 °C for 10 sec, 60 °C for 10 sec, and 72 °C for 10 sec), post-amplification melting (95 °C for 5 sec, 65 °C for 1 min) and a cooling step (40 °C for 30 sec).

Verification of *vexC* amplification

Verification of primer amplification was performed by sequencing the PCR product of *S.* Typhi TP279 (1st Base Sdn. Bhd., Malaysia). The sequence was then analysed using Basic Local Alignment Search Tool (BLAST) nucleotide database (http://blast.ncbi.nlm.nih.gov).

Vi-qPCR specificity assessment

Specificity of SViF and SViR primers were assessed towards known *S*. Typhi, other enteric pathogens strains and gastrointestinal normal flora (Table 1) by employing an optimised Vi-qPCR conditions. The experiment was repeated three times to ensure reproducibility.

Evaluation of Vi-qPCR detection limit

Detection limit of the assay was evaluated against extracted DNA from a serially diluted seeded stool of *S*. Typhi TP279 culture (in selenite F) as described by Fan *et al.* (2015). The detection limit of Vi-qPCR was defined as the lowest CFU of *S*. Typhi that could be detected. Briefly, a mid-log phase culture of *S*. Typhi TP279 [approximately 1×10^9 CFU/mL, OD_{600} 0.8) in tryptone soy broth (TSB) (Difco Laboratories, Detroit, USA) was ten-fold serially diluted (10^8 to 10^0 CFU/mL) in sterile phosphate-buffered saline (PBS) pH 7.3]. A total of 200 µL of each dilution culture was then mixed with 0.2 g stool of a healthy person. Thus, 1×10^8 to 1×10^0 CFU/g of *S.* Typhi seeded stool samples were obtained. Five millilitre of selenite F broth was added to each of the spiked stool sample, mixed well and incubated overnight at 35 °C±2. Non spiked stool with sterile PBS pH 7.3 served as negative control. Quantification of actual CFU used to the spike stool samples was determined by performing viable counts. The assay and viability count tests were performed in duplicate to achieve the optimum fidelity. Pre-treatment of the sample was done prior to the procedures. The selenite F broth culture was centrifuged at 4 °C, 6000×*g* for 15 min. Collected pellet was washed with 1 mL of sterile phosphate buffer saline (PBS) pH 7.3 and centrifuged for 2 min. Template DNA was then extracted from the harvested pellet by using QIAmp® DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Statistical analysis

Specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) of the developed assay were statistically analysed using the formula as described by Parikh *et al.* (2008) which is also available at the MedCalc.net website (https://www.medcalc.net/tests/diagnostic_test.php).

RESULTS

Optimisation of PCR

Primer set SVi was used to amplify *S*. Typhi TP279 DNA template by targeting the *vexC* gene. The annealing temperature at 60 ºC provided the highest intensity single 300 bp amplicon in conventional PCR (Figure 1). This annealing temperature was chosen for subsequent DNA amplification in real-time PCR. Sequencing analyses of the positive control PCR product led to the highest predicted specificity (99%) to *S*. Typhi Vi antigen genes (*vexC*), GenBank Accession Numbers CP012151, CP012091, AL513382, CP003278, CP002099, AE014613 and D14156.

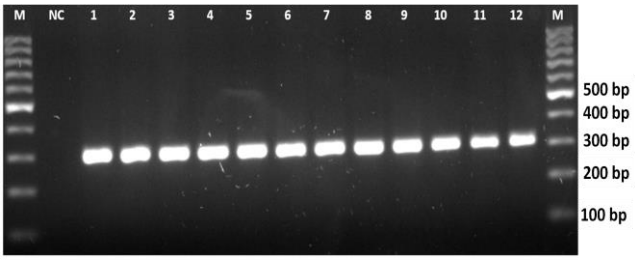

Figure 1: Agarose gel electrophoresis of Vi-PCR amplicons of *S*. Typhi TP279 at different annealing temperatures.
M: 100 bp molecular weight marker; NC: negative control; lane 1: 49.8 °C; lane 2: 50.2 °C; lane 3: 51.1 °C; lane 4: 52.5 °C; lane 5: 54.3 °C; lane 6: 56.2 °C; lane 7: 58.3 °C; lane 8: 60.2 °C; lane 9: 62.0 °C; lane 10: 63.5 °C; lane 11: 64.6 °C; lane 12: 65.1 °C.

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No amplification was observed from PCR of the negative control stool sample. The results proved that SViF and SViR primers are specific for *S*. Typhi detection. The Vi-qPCR results were defined as positive or detected for a C_p value less than or equal to 35 with a T_m (melting curve) value of 85.0±0.5 (Figure 2).

Analytical specificity

Vi-qPCR assay performed on known genomic DNAs of *S*. Typhi and non *S.* Typhi strains resulted in 100% specificity. Genomic DNA from all *S*. Typhi strains yielded positive results. Negative results were observed from PCR of other 27 non *S*. Typhi isolates. Therefore, the sensitivity as well as NPV and PPV of the assay are 100% respectively. The results indicate specific detection of *S.* Typhi.

Vi-qPCR detection limit

Positive Vi-qPCR results were observed for genomic DNAs from 1×10^9 CFU/g to 1×10^1 CFU/g seeded stool of *S*. Typhi TP279 (Table 3). There were also good concordances between the actual CFU in the serially diluted samples and the viable count results. No amplification was detected from the negative control stool sample (Figure 2).

DISCUSSION

Typhoid by *S.* Typhi remains as an important health problem especially in developing countries including Malaysia. The emergence and spread of multi-drug resistant (MDR) *S.* Typhi worsened the situation, contributing to morbidity and mortality (Kumar *et al*., 2008; Zaki *et al.*, 2011). The spread of MDR strains may also introduce increment in treatment cost due to the need of powerful and expensive antibiotics.

Rapid and reliable method for *S*. Typhi detection is crucial to detect *S.* Typhi in patients and to identify the carrier. Detection of *S*. Typhi in the carrier will break the infection cycle by inhibiting the pathogen transmission and help in early typhoid prevention. A good diagnostic tool is important for effective typhoid treatment. Due to varied presentations of the disease and flaws of culture method as well as serological method, there is a delay in typhoid diagnosis and there is a possibility for few cases to be remained under diagnosed. Polymerase chain reaction is a rapid, reproducible, specific and sensitive assay. Several PCR methods have been established for *S*. Typhi detection targeting a number of genes such as the flagella genes (Song *et al*., 1993; Massi *et al.*, 2003; Hatta *et al.*, 2007; Fan *et al*., 2015; Chaudhry *et al.*, 2010), *tyv*, *prt*, *groEL*, *(*Ali *et al*., 2009), Vi capsular gene *viaB (*Nizami *et al*., 2006), the 16sRNA (Zhu *et al.*, 1996) and 23S rRNA genes (Pui *et al.*, 2011).

Nested-PCR was utilised to increase the sensitivity. Unfortunately, this technique is complicated, very sensitive and usually introduces inherent problems with unspecific amplification and contamination. In addition, the use of conventional PCR in a number of studies require additional step for post-amplification agarose gel analysis. Loop-mediated isothermal amplification (LAMP) technique by Abdullah *et al*. (2014) was reported to have higher sensitivity and specificity for detection of *S.* Typhi in comparison to conventional PCR assay. The test is able to detect up to 20 CFU/reaction of *S.* Typhi. However, this method is only recommended for screening purpose. Thus, further confirmation with gold standard methods such as culture method and PCR are required.

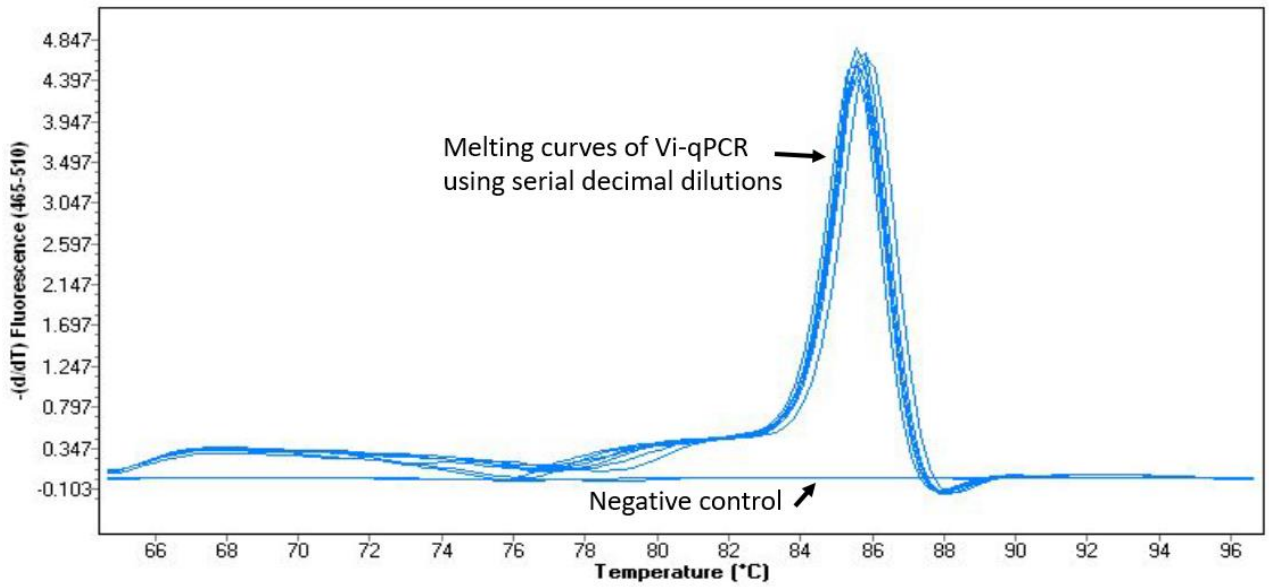

Figure 2: Melting curves of Vi-qPCR assay using serial decimal dilutions (1×10^9 CFU/g to 1×10^1 CFU/g seeded stool of *S*. Typhi TP279).

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Table 1: Bacterial strains used for the Vi-qPCR assessment.

No.	Strains	Vi-qPCR result[a]
1	*Aeromonas hydrophila* ATCC[b] 7965	-
2	*Escherichia coli* ATCC 25922	-
3	*Escherichia* O157	-
4	*Escherichia coli* EPEC	-
5	*Escherichia coli* 0104	-
6	*Klebsiella pneumoniae*	-
7	*Proteus mirabilis*	-
8	*Salmonella typhimurium* ATCC 14028	-
9	*Salmonella enterica* serovar Weltevreden 1	-
10	*Salmonella enterica* serovar Weltevreden 2	-
11	*Salmonella enterica* serovar Weltevreden 3	-
12	*Salmonella enterica* serovar Weltevreden 4	-
13	*Salmonella enterica* serovar Weltevreden 5	-
14	*Salmonella enterica* serovar Weltevreden 6	-
15	*Salmonella enterica* serovar Weltevreden 7	-
16	*Salmonella enterica* serovar Typhi TP279	+
17	*Salmonella enterica* serovar Typhi 1	+
18	*Salmonella enterica* serovar Typhi 2	+
19	*Salmonella enterica* serovar Typhi 3	+
20	*Salmonella enterica* serovar Typhi 4	+
21	*Shigella sonnei*	-
22	*Shigella flexneri*	-
23	*Shigella boydii*	-
24	*Shigella dysenteriae*	-
25	*Vibrio cholerae* Ogawa	-
26	*Vibrio cholerae* Non 01/Non 0139	-
27	*Vibrio cholerae* Bengal	-
28	*Vibrio cholerae* Inaba	-
29	*Vibrio parahaemolyticus*	-
30	*Citrobacter freundii 1*	-
31	*Citrobacter freundii 2*	-
32	*Citrobacter freundii 3*	-

[a] A positive PCR result was indicated by crossing point value (C_p) value less or equal to 35.0 with melting curve value 85.0±0.5, [b]American Type Culture Collection.

Table 2: Details of primers designed and used this study.

Primers name	Primers sequence (5'-3')	Target	Amplicon size
SViF	GTTGCCTGCGCTAAA TCTTC	*vexC*	300 bp
SViR	GACCCAGCTTGAACA GTGCT		

In this study, a test named as Vi-qPCR based on real-time PCR platform was developed. Real-time PCR eliminates post-amplification agarose gel analysis thus requires shorter time. This assay produces higher throughput compared to conventional PCR and reduces the possibility of carryover contamination. The Vi-qPCR provides rapid result within approximately 3 h including the DNA extraction step per sample. This is an added advantage especially during an outbreak when non-invasive sample collection and rapid results are required to contain the disease. In addition, the test is specific for only *S.* Typhi detection and is confirmed by the *in-vitro* and *in-silico* analysis. This assay was designed based on detection of the specific DNA sequence of the *vexC* gene encoding the Vi antigen which is located on the ViaB region. The amplification with the primer set, SViF and SViR led to highly specific results for *S*. Typhi detection. In fact, this method demonstrated negative results for all tested Vi-negative strains. A whole-genome sequencing study of *S*. Typhi has revealed the presence of 601 genes on 82 genetic islands which were absent in the *S*. Typhimurium genome (Parkhill *et al*., 2001). *Salmonella* pathogenicity island 7 (SPI-7) occupy most of these islands and consists of *S*. Typhi-specific DNA and ViaB region that is responsible for the production of Vi antigen (Hashimoto *et al*., 1993). In order to assess the ability of the test in *S.* Typhi detection of stool samples, the Vi-qPCR assay was challenged with serially diluted spiked stool. The test was able to detect up to 13±1.4 (1×10^1) *S.* Typhi CFU/mL in stool sample. A study by Kumar *et al.* (2006) reported that their multiplex PCR test could detect down to 10^{-1} bacteria (70 CFU/100 mL) in water samples, 4.8×10^1 CFU/mL bacteria in milk and 2.0×10^1 CFU/mL bacteria in meat rinse samples. Later, Hatta *et al.* (2007) developed a nested PCR for the detection of *S.* Typhi and suggested that most of typhoid patients excreted high number of *S*. Typhi in their urine or stools. The nested PCR was able to detect the *S*. Typhi DNA more in the urine (65.6%) than in stool samples (39.5%) of the patients diagnosed with typhoid fever. The sensitivity is lower compared to Vi-qPCR that was developed in this study. The detection limit of Vi-qPCR is acceptable and advantageous. It has been reported that depending on composition of normal flora, nutrition lifestyle and environment of the patient, stool samples contain highly variable components. The components such as bile salts, polysaccharides from vegetables, haemoglobin, heparin urea and glycolipids are potential PCR inhibitors (Oikarinen *et al.*, 2009; Schrader *et al.*, 2012). Presence of inhibitors may reduce PCR sensitivity (Hatta *et al.*, 2007; Oikarinen *et al.*, 2009).

Table 3: The growth and Vi-qPCR detection of *S*. Typhi in artificially seeded stool of selenite F culture.

S. Typhi CFU/g	~$1´10^9$	$1´10^8$	$1´10^7$	$1´10^6$	$1´10^5$	$1´10^4$	$1´10^3$	$1´10^2$	$1´10^1$	$1´10^0$
Colony-forming unit (Mean±SD)	TNTC[1]	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	147±12.7	13±1.4	2±1.4
Vi amplicons[2]	2	+	+	+	+	+	+	+	+	-3

[1]Too Numerous To Count (colony count more than 300), [2]*S*. Typhi Vi amplicons results from Vi-qPCR using three independent cultures DNA templates.

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CONCLUSION

The protocol developed in this study is rapid, sensitive and specific for the early diagnosis and chronic carrier detection of typhoid fever. Further application of this approach will be targeted to clinical blood, urine and stool samples in order to obtain data on clinical validation performance.

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REFERENCES

Abdullah, J., Saffie, N., Sjasri, F. A., Husin, A., Abdul-Rahman, Z., Ismail, A., Aziah, I. and Mohamed, M. (2014). Rapid detection of *Salmonella* Typhi by loop-mediated isothermal amplification (LAMP) method. *Brazilian Journal of Microbiology* **45, 1385-1391.**

Ali, A., Haque, A., Haque, A., Sarwar, Y., Mohsin, M., Bashir, S. and Tariq, A. (2009). Multiplex PCR for differential diagnosis of emerging typhoidal pathogens directly from blood samples. *Epidemiology & Infection* **137, 102-107.**

Ambati, S. R., Nath, G. and Das, B. K. (2007). Diagnosis of typhoid fever by polymerase chain reaction. *The Indian Journal of Pediatrics* **74, 909-913.**

Bhutta, Z. A. (2006). Current concepts in the diagnosis and treatment of typhoid fever. *British Medical Journal* **333, 78-81.**

Brooks, W. A., Hossain, A., Goswami, D., Sharmeen, A. T., Nahar, K., Alam, K., Ahmed, N., Naheed, A., Nair, G. B., Luby, S. and Breiman, R. F. (2005). Bacteremic typhoid fever in children in an urban slum, Bangladesh. *Emerging Infectious Diseases* **11, 326-329.**

Chaudhry, R., Chandel, D. S., Verma, N., Singh, N., Singh, P. and Dey, A. B. (2010). Rapid diagnosis of typhoid fever by an in-house flagellin PCR. *Journal of Medical Microbiology* **59, 1391-1393.**

Crump, J. A. and Mintz, E. D. (2010). Global trends in typhoid and paratyphoid fever. *Clinical Infectious Diseases* **50, 241-246.**

Crump, J. A., Luby, S. P. and Mintz, E. D. (2004). The global burden of typhoid fever. *Bulletin of the World Health Organization* **82, 346-353.**

Elizaquivel, P. and Aznar, R. (2008). A multiplex RTi-PCR reaction for simultaneous detection of Escherichia coli O157: H7, *Salmonella* spp. and *Staphylococcus aureus* on fresh, minimally processed vegetables. *Food Microbiology* **25, 705-713.**

Fan, F., Du, P., Kan, B. and Yan, M. (2015). The development and evaluation of a loop-mediated isothermal amplification method for the rapid detection of *Salmonella enterica* serovar Typhi. *PLoS ONE* **10(4): e0124507.**

Farrell, J. J., Doyle, L. J., Addison, R. M., Reller, L. B., Hall, G. S. and Procop, G. W. (2005). Broad-range (Pan) *Salmonella* and *Salmonella* serotype Typhi–specific Real-Time PCR assays: Potential tools for the clinical microbiologist. *American Journal of Clinical Pathology* **123, 339-345.**

Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S. and Gordon, M. A. (2012). Invasive non-typhoidal *Salmonella* disease: An emerging and neglected tropical disease in Africa. *The Lancet* **379, 2489-2499.**

Germini, A., Masola, A., Carnevali, P. and Marchelli, R. (2009). Simultaneous detection of *Escherichia coli* O175: H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. *Food Control* **20, 733-738.**

Hashimoto, Y., Itho, Y., Fujinaga, Y., Khan, A. Q., Sultana, F., Miyake, M., Hirose, K., Yamamoto, H. and Ezaki, T. (1995). Development of nested PCR based on the *ViaB* sequence to detect *Salmonella* Typhi. *Journal of Clinical Microbiology* **33, 775-777.**

Hashimoto, Y., Li, N., Yokoyama, H. and Ezaki, T. (1993). Complete nucleotide sequence and molecular characterization of *ViaB* region encoding Vi antigen in *Salmonella* Typhi. *Journal of Bacteriology* **175**, **4456-4465.**

Hatta, M. and Smits, H. L. (2007). Detection of *Salmonella* Typhi by nested polymerase chain reaction in blood, urine, and stool samples. *The American Journal of Tropical Medicine and Hygiene* **76, 139-43.**

Hirose, K., Hashimoto, A., Tamura, K., Kawamura, Y., Ezaki, T., Sagara, H. and Watanabe, H. (2002). DNA sequence analysis of DNA gyrase and DNA topoisomerase IV quinolone resistance-determining regions of *Salmonella enterica* serovar Typhi and serovar Paratyphi A. *Antimicrobial Agents and Chemotherapy* **46, 3249-3252.**

Kolyva, S., Waxin, H. and Popoff, M. Y. (1992). The Vi antigen of *Salmonella* Typhi: Molecular analysis of the viaB locus. *Journal of General Microbiology* **138(2), 297-304.**

Kumar, A., Balachandran, Y., Gupta, S. and Khare, S. (2010). Quick PCR based diagnosis of typhoid using specific genetic markers. *Biotechnology Letters* **32, 707-712.**

Kumar, S., Balakrishna, K. and Batra, H. V. (2006). Detection of *Salmonella enterica* serovar Typhi (*S.* Typhi) by selective amplification of *invA*, *viaB*, *fliC-d* and *prt* genes by polymerase chain reaction in mutiplex format. *Letters in Applied Microbiology* **42, 149-154.**

Kumar, S., Rizvi, M. and Berry, N. (2008). Rising prevalence of enteric fever due to multidrug-resistant *Salmonella*: An epidemiological study. *Journal of Medical Microbiology* **57, 1247-1250.**

Levy, H., Diallo, S., Tennant, S. M., Livio, S., Sow, S. O., Tapia, M., Fields, P. I., Mikoleit, M., Tamboura, B., Kotloff, K. L. and Lagos, R. (2008). A PCR method to identify *Salmonella enterica* serovars Typhi, Paratyphi A and Paratyphi B among *Salmonella* isolates from the blood of patients with clinical enteric fever. *Journal of Clinical Microbiology* **46, 1861-1866.**

Massi, M. N., Gotoh, A., Bishnu, A., Kawabata, M., Shirakawa, T. and Hatta, M. (2003). Rapid diagnosis of typhoid fever by PCR assay using one pair of primers from flagellin gene of *Salmonella* typhi. *Journal of Infection and Chemotherapy* **9, 233-237.**

Moganedi, K. L., Goyvaerts, E. M., Venter, S. N. and Sibara, M. M. (2007). Optimisation of the PCR-invA primers for the detection of *Salmonella* in drinking and surface waters following a pre-cultivation step. *Water SA* **33, 195-202.**

Naheed, A., Ram, P. K., Brooks, W. A., Mintz, E. D., Hossain, M. A., Parsons, M. M, Luby, S. P. and Breiman, R. F. (2008). Clinical value of Tubex™ and Typhidot® rapid diagnostic tests for typhoid fever in an urban community clinic in Bangladesh. *Diagnostic Microbiology and Infectious Disease* **61, 381-386.**

Nakhla, I., El Mohammady, H., Mansour, A., Klena, J. D., Hassan, K., Sultan, Y., Pastoor, R., Abdoel, T. H. and Smits, H. (2011). Validation of the Dri-Dot Latex agglutination and IgM lateral flow assays for the diagnosis of typhoid fever in an Egyptian population. *Diagnostic Microbiology and Infectious Disease* **70, 435-441.**

Nizami, S. Q., Bhutta, Z. A., Siddiqui, A. and Lubbad, L. (2006). Enhanced detection rate of typhoid fever in children in a periurban slum in Karachi, Pakistan using polymerase chain reaction technology. *Scandinavian Journal of Clinical and Laboratory Investigation* **66, 429-436.**

Oikarinen, S., Tauriainen, S., Viskari, H., Simell, O., Knip, M., Virtanen, S. and Hyöty, H. (2009). PCR inhibition in stool samples in relation to age of infants. *Journal of Clinical Virology* **44, 211-214.**

Parikh, R., Mathai, A., Parikh, S., Sekhar, G. C. and Thomas, R. (2008). Understanding and using sensitivity, specificity and predictive values. *Indian Journal of Ophthalmology* **56, 45-50.**

Park, S. H., Kim, H. J., Cho, W. H., Kim, J. H., Oh, M. H., Kim, S. H., Lee, B. K., Ricke, S. C. and Kim, H. Y. (2009). Identification of *Salmonella enterica* subspecies I, *Salmonella enterica* serovars Typhimurium, Enteritidis and Typhi using multiplex PCR. *FEMS Microbiology Letters* **301, 137-146.**

Parkhill, J., Dougan, G., James, K. D., Thomson, N. R., Pickard, D., Wain, J., … and Barrell, B. G. (2001). Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* **413, 848-852.**

Parry, C. M., Wijedoru, L., Arjyal, A. and Baker, S. (2011). The utility of diagnostic tests for enteric fever in endemic locations. *Expert Review of Anti-infective Therapy* **9, 711-725.**

Pui, C. F., Wong, W. C., Chai, L. C., Lee, H. Y., Noorlis, A., Zainazor, T. C., Tang, J. Y., Ghazali, F. M., Cheah, Y. K., Nakaguchi, Y. and Nishibuchi, M. (2011). Multiplex PCR for the concurrent detection and differentiation of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium. *Tropical Medicine and Health* **39, 9-15.**

Schadich, E., Dzubak, P. and Hajduch, M. (2016). Role of *Salmonella* Typhi Vi antigen and secretory systems on immune response. *Current Pharmaceutical Design* **22, 6251-6260.**

Schrader, C., Schielke, A., Ellerbroek, L. and Johne, R. (2012). PCR inhibitors–occurrence, properties and removal. *Journal of Applied Microbiology* **113, 1014-1026.**

Sharma, K. B. and Arya, S. C. (1995). Detection of *Salmonella* Typhi by nested PCR based on the ViaB sequence. *Journal of Clinical Microbiology* **33, 775-777.**

Song, J. H., Cho, H., Park, M. Y., Na, D. S., Moon, H. B. and Pai, C. H. (1993). Detection of *Salmonella* Typhi in the blood of patients with typhoid fever by polymerase chain reaction. *Journal of Clinical Microbiology* **31, 1439-1443.**

Tennant, S. M., Toema, D., Qamar, F., Iqbal, N., Boyd, M. A., Marshall, J. M., Blackwelder, W. C., Wu, Y., Quadri, F., Khan, A. and Aziz, F. (2015). Detection of typhoidal and paratyphoidal *Salmonella* in blood by real-time polymerase chain reaction. *Clinical Infectious Diseases* **61, 241-250.**

Wain, J., Bay, P. V. B., Vinh, H., Duong, N. M., Diep, T. S., Walsh, A. L., Parry, C. M., Hasserjian, R. P., Ho, V. A., Hien, T. T. and Farrar, J. (2001). Quantitation of bacteria in bone marrow from patients with typhoid fever: Relationship between counts and clinical features. *Journal of Clinical Microbiology* **39, 1571-1576.**

Wain, J., Hendriksen, R. S., Mikoleit, M. L., Keddy, K. H. and Ochiai, R. L. (2015). Typhoid fever. *Lancet* **385, 1136-1145.**

Wang, R. F., Cao, W. W. and Cerniglia, C. E. (1997). A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *Journal of Applied Microbiology* **83, 727-736.**

WHO (2003). Background document: The diagnosis, treatment and prevention of typhoid fever.

Zaki, S. A. and Karande, S. (2011). Multidrug-resistant typhoid fever: A review. *The Journal of Infection in Developing Countries* **5, 324-337.**

Zhang, J. Y., Dong, L. W., Ren, Q., Wang, X. Z., Yang, Y., Zhou, W., Zhu, C. H., Meng, X. and Zhu, G. Q. (2014). Simple and rapid detection of *Salmonella* by direct PCR amplification of gene *fimW*. *Current Microbiology* **69, 429-435.**

Zhu, Q., Lim, C. K. and Chan, Y. N. (1996). Detection of *Salmonella* Typhi by polymerase chain reaction. *Journal of Applied Microbiology* **80, 244-251.**

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