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Rapid confirmation of five non-typhoidal *Salmonella* (NTS) serotypes using a low-cost portable-DIY OpenPCR© thermocycler

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ABSTRACT

Aims: OpenPCR is a low cost yet accurate thermocycler which can be self-built. The aim of the study is to highlight a low-cost alternative method for rapid confirmation of five predominant non-typhoidal *Salmonella* (NTS) serotypes using a multiplex PCR on a portable-DIY OpenPCR© thermocycler.
Methodology and results: Eight multiplex polymerase chain reaction (mPCR) samples containing genomic DNA of *S.* Agona selectively placed on the wells of the conventional PCR and OpenPCR© thermocyclers showed uniform heating in both thermocyclers. The limit of detection was similar for both thermocyclers for all five serotypes. The limit of detection for *S*. Typhimurium, *S*. Agona and *S*. Weltevreden was 10 pg/µL whereas the limit of detection for *S*. Enteriditis and *S*. Heidelberg was 1 pg/µL and 100 pg/µL, respectively. This assay incorporated a panel of unique genes; *STM4495*, *SEN1392*, *SeHa-C4893*, *SeAg-B1096* and *SENTW-3241* which were previously identified to be specific for *S*. Typhimurium, *S*. Enteritidis, *S*. Heidelberg, *S*. Agona, and *S*. Weltevreden, respectively, as well as the pan-*Salmonella* gene *invA* as internal control (IC) and pan-bacteria gene 16S rRNA to serve as amplification control (AC). The analytical specificity of the mPCR assay was found to be 100% for all five NTS using OpenPCR© thermocyclers.
Conclusion, significance and impact of study: The feasibility and low cost of the OpenPCR© thermocycler makes this device an ideal alternative for mPCR assay for rapid confirmation of NTS serotypes.

Keywords: Non-typhoidal *Salmonella*, NTS, OpenPCR, Multiplex PCR, thermocycler, thermal cycler

INTRODUCTION

Globally, non-typhoidal *Salmonella* (NTS) causes an estimated 93.8 million illnesses, of which an estimated 80.3 million are foodborne, and 155,000 death cases each year (Haeusler and Curtis, 2013). In the United States, NTS causes approximately 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths annually (CDC, 2013). The predominant serotypes causing Non-Typhoidal Salmonellosis include *S*. Enteritidis, *S*. Typhimurium, *S*. Welteverden, *S*. Agona, and *S*. Heidelberg (Learn-Han *et al.*, 2008). In most cases, outbreaks of NTS infections are usually caused by *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serotype Enteritidis (*S.* Enteritidis) (Jaya and Harita, 2013). Cryptic outbreaks of *S*. Agona, *S*. Heidelberg and *S*. Weltevreden have also been previously reported (Taylor *et al*., 1998; D'ortenzio *et al*., 2008; CDC, 2014). In third-world regions, such as in sub-Saharan Africa, NTS frequently presents itself in a more virulent form, i.e. septicemia in the bloodstream of both adults and children (Graham and English, 2009). Generally, NTS is detected conventionally using a series of microbiological and biochemical tests (Dunbar *et al*., 2003), followed by antisera serotyping (Acheson and Hohmann, 2001; Nori and Thong, 2010; Gordon, 2011). The whole process is time-consuming, tedious and requires skilled manpower. However, the advent of the polymerase chain reaction (PCR) technology has simplified this process.

PCR is a commonly used tool for diagnosis of many diseases through detection by amplification of specific regions of genes of the pathogen (Park *et al.*, 2012). PCR consists of three steps, denaturation of the double-stranded DNA, annealing of the primers to the DNA template, and finally the extension to resynthesize the DNA. By repeating the thermal cycles of reaction, exponential copies of the original DNA target can be produced to facilitate detection (Park *et al.*, 2015). Multiplex PCR (mPCR) is an efficient technique to detect several DNA sequences simultaneously, due to its specificity and sensitivity in detecting small amounts of DNA through amplification of a specific DNA segment. In addition, mPCR can distinguish closely related organisms

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in a single reaction (Tennant *et al*., 2010). Despite being a cheaper diagnostic tool to other available methods, the cost of running a PCR for disease diagnosis may not be completely cost efficient. This is because the cost of a PCR machine and reagents are higher compared to the microscopy method (Morgan *et al*., 1998). In addition, the PCR thermocycler has an in-built software for thermal cycling condition control. The OpenPCR© thermocycler initiative was established to address this problem in which an external software can be used in a mobile phone or laptop to manipulate the thermal cycling conditions. Also, the software needed to automate the OpenPCR© thermocycler is open source which is freely available online software, there is no overhead cost associated with the manufacturing of this equipment, making the machine about 10 times cheaper compared to the conventional PCR machine. Moreover, it is available as a DIY kit that can be easily assembled manually (http://openpcr.org/). Therefore, the OpenPCR© thermocycler can be a cheaper alternative if it is able to meet the stringent requirements of the laboratory assay (Park *et al*., 2012; 2015).

Therefore, in this study, the sensitivity and the accuracy of the diagnostics of five non typhoidal *Salmonella* using OpenPCR© thermocycler were compared with the conventional PCR thermocycler employing the mPCR technique. This study is first of its kind in comparing open and conventional PCR thermocycler utilizing the multiplex PCR method in terms of their feasibility and cost. The main objective of this study is to provide a portable, cheaper and cost-effective option which facilitates point-of-care testing of NTS in third world countries and rural areas.

MATERIALS AND METHODS

Bacterial strains

In this study, the bacterial isolates of the five NTS reference strains obtained from American Type Culture Collection (ATCC) with a panel of 36 (10 non-*Salmonella*, 26 other-*Salmonella*) archived clinical isolates were used for diagnostic sensitivity and specificity tests. After reviving the bacteria from the glycerol stock and plating on nutrient agar, a single colony was inoculated into 10 mL of nutrient broth and incubated for 18 h (overnight) at 37 °C.

DNA extraction kit

A volume of 1.5 mL of overnight bacterial culture (OD between 1.0 to 2.0) was transferred into a sterile 1.5 mL micro-centrifuge tube. The bacterial culture was pelleted down by centrifugation (18,000 ×*g*, 1 min) and the supernatant was discarded. Bacterial DNA was then extracted using G-spin™ Genomic DNA Extraction Kit [for Bacteria] (iNtRON Biotecnology, South Korea). The concentration and purity of the extracted DNA was determined using Nanodrop (Thermofisher, USA).

Multiplex PCR reaction

The conditions and parameters of mPCR for the detection of NTS utilized in this experiment were previously optimized by Tan (2015). The primer sequences used in this study are listed down in Table 1. While 5 sets of specific primers (SEN, STM, SAGO, SWEL and SHEL-1) were used to detect the respective bacterial target, extra sets of *inv*A and 16S rRNA primers were used as positive control (Figure 1). The optimized final concentration of primers are listed in Table 1. PCR assay mastermix was setup in a 20 µL reaction. Firstly, 5× Green GoTaq® Flexi Buffer was added for a final concentration of 1×. Then 25 mM $MgCl_2$ was added for a final concentration of 3.0 mM. Then, 10 mM dNTP mix was added to a final concentration of 0.16 mM and 80% glycerol was added to a final concentration of 5%. Followed by this, respective primer sets were added according to final concentrations in Table 1 from a working solution of 10 µM then 2 µL DNA template was added for each target gene, respectively. Each mPCR was carried out simultaneously in a conventional PCR machine and OpenPCR© machine using parameters listed in Table 2.

Open PCR and conventional thermocyclers

In this study, the BioRad (MyCycler™ , USA) conventional thermocycler has 96 wells and DIY OpenPCR© (http://openpcr.org/) machine on the other hand comes with 16 wells. These two thermocyclers were used to run the mPCR assay. The BioRad thermocycler contains an in-built programming of temperatures, times, and protocol options. Weighing around 10 kg, the BioRad thermocycler is 20 cm in height and 24 cm in width. Unlike the conventional PCR machine, OpenPCR© thermocycler was operated manually using OpenPCR© application along with Adobe Air installed in a disconnected host. The OpenPCR© application is compatible with both Microsoft and Mac. The OpenPCR© application is able to manage the temperature, time and protocol options. The OpenPCR© thermocycler weighs around 3.5 kg, with the height of 25 cm, width of 13 cm and a depth of 20 cm.

Heating block uniformity between open PCR and conventional PCR thermocycler

The ability of OpenPCR© to accurately hold a specific annealing temperature were tested by comparing the results of 8 mPCR reactions at designated regions in the heating blocks in OpenPCR© with 8 mPCR reactions at designated regions in the heating blocks of the conventional PCR thermocycler (BioRad MyCycler™, USA) as shown in Figure 2. These mPCR reactions were run using the genomic DNA from *S*. Agona as the test sample. The thermal cycling was performed in both the machines and the PCR reactions were visualized in an agarose gel after electrophoresis.

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Table 1: List of primers with the optimized primer concentrations used for the multiplex PCR assay.

Target	Primers	Sequence (5'→ 3')	Product size (bp)	Final concentration (µM)
Salmonella Enteritidis	SEN	F: GCTGCAGATGTACTGTGCTTTC R: CCAGGCGACTCTACTTATCCAG	692	0.125
Salmonella Typhimurium	STM	F: GCAGCCGATGATGTAGCTTA R: TTACGCTGCGGGATTAATGT	495	1.000
Salmonella Weltevreden	SWEL	F: CACCACCTGACACAACCAGT R: GTGGTGCCTGTGACGAATAA	349	0.125
Salmonella Agona	SAGO	F: TCGCTCTTTCTGCCATACCT R: CTCCTGAATCTGCTGGTGGT	438	0.125
Salmonella Heidelberg	SHEI-1	F: GGCGTGGTATTTGTCGAAGT R: ATAAACGCCCATCGTTCTCA	626	0.125
Pan *Salmonella* (IC)	invA	F: GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	284	0.060
All bacteria species (amplification control)	16S rRNA	F: CAGGCCTAACACATGCAAGTC R: GGGCGGTGTGTACAAGGC	1362	0.050

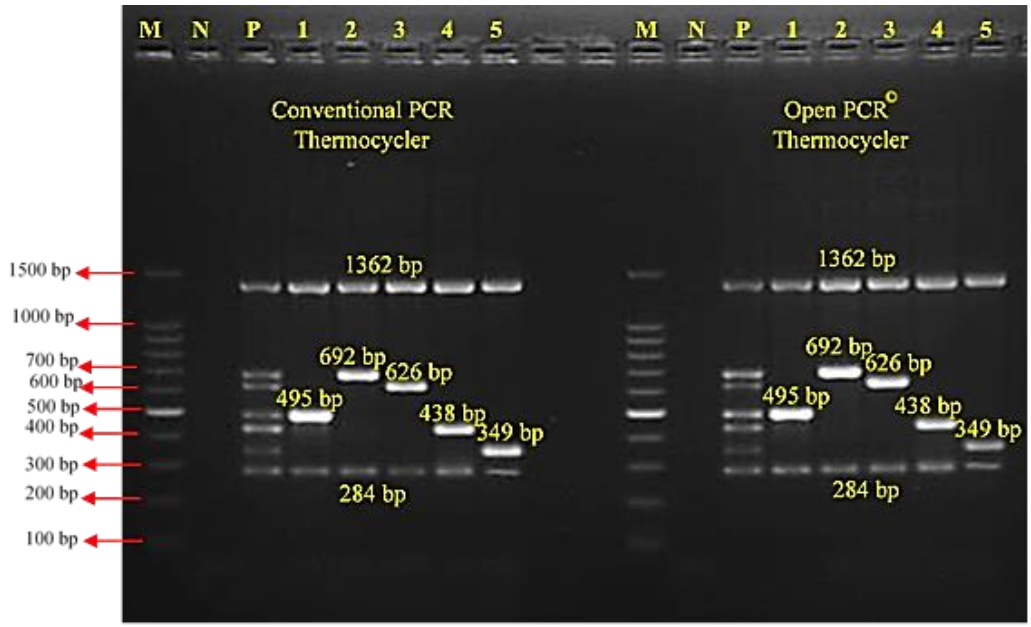

Figure 1: Simulatenous detection of the five predominant NTS by mPCR using conventional and OpenPCR© thermocyclers. Lane M: 100 bp DNA ladder (Promega); Lane N: No template control (ddH_2O); Lane P: Pooled DNA Templates; Lane 1*: S.* Typhimurium ATCC 14028 (495 bp); Lane 2: *S*. Enteritidis ATCC 13076 (692 bp); Lane 3: *S*. Heidelberg ATCC 8326 (626 bp); Lane 4: *S.* Agona ATCC 51957 (438 bp); and Lane 5: *S*. Weltevreden ATCC 6534 (349 bp).

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Table 2: PCR thermocycling conditions.

Segment	Temperature (°C)	Duration (sec)	Cycles
Initial denaturation	95	30	1
Denaturation	95	30	
Annealing	60	30	30
Extension	72	60	
Final extension	72	300	1
Final hold	4	∞	1

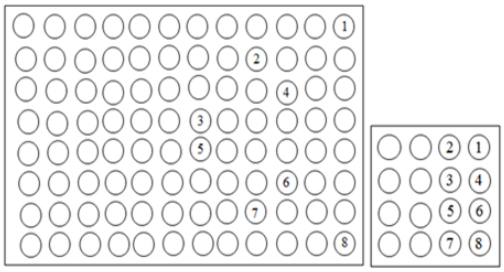

Figure 2: The numbers in (a) conventional PCR and (b) OpenPCR© thermocycler indicates the selected wells used for mPCR assay using *S.* Agona genomic DNA to test the heating uniformity of the blocks.

Gel electrophoresis

PCR amplicons of all mPCR reactions were assessed using 1.5% (w/v) agarose gel with 1 µL of 25% (v/v) ethidium bromide. Gel electrophoresis was done at 70V for 100 min.

Analytical sensitivity of the mPCR assay

The analytical sensitivity in this study is defined as the lowest concentration of *Salmonella* DNA sample that is able to yield a positive result by the mPCR assay. Genomic DNA from the ATCC reference strains of *S*. Enteritidis, *S*. Typhimurium, *S*. Weltevreden, *S*. Agona and *S*. Heidelberg were extracted and pooled to obtain a standard concentration of 100 ng/µL. Then, genomic DNA from all five serotypes was subjected to dilutions to obtain a range of concentration from 100 ng/µL to10 fg/µL.

Analytical specificity test of the mPCR assay

Analytical specificity tests of the mPCR assay were performed on various clinical isolates (Table 3) to assess the performance of the OpenPCR© thermocycler in detecting and differentiating the serotypes in a panel containing a total of 36 samples, 26 from known *Salmonella* species and 10 non-*Salmonella* enteric pathogens.

RESULTS

Figure 2 shows the performance comparison between the conventional and OpenPCR© thermocyclers for detection of five NTS by an mPCR assay. There are no significant differences between both machines as the visual inspection of the gel image showed similar amplification performance. The number and size of amplicons obtained for each reaction were similar between the conventional and OpenPCR© machines.

Figure 3 shows the comparison of the block heating uniformity between the OpenPCR© and the conventional PCR thermocyclers (BioRad MyCycler™, USA). All bands of the target gene were observed in all the reactions (with respective internal and amplification controls) for both thermocyclers. As observed in the figure, similar results were observed in both thermocyclers indicating uniform heating for both thermocyclers. This would prove that the OpenPCR© thermocycler was successful in amplifying the target gene by holding a specific annealing temperature in different segments of the heating block. Moreover, the efficiency of the thermocyclers was tested through multiplex PCR as this method allows the simultaneous amplification of multiple sequences of target DNA in a single reaction. This technique has also been commonly utilized to detect multiple agents that cause similar or identical clinical syndromes. Both thermocyclers produced similar results although the conventional PCR thermocycler had a larger capacity to run samples (96 versus 16 wells).

Figures 4A to 4E show the DNA detection sensitivity for five NTS (*S.* Enteritidis, *S.* Typhimurium, *S.* Weltevreden, *S.* Agona and *S.* Heidelberg). Figure 4F shows the sensitivity of DNA detection level test for five NTS (*S.* Enteritidis, *S.* Typhimurium, *S.* Weltevreden, *S.* Agona and *S.* Heidelberg). As shown in all six gel images, the detection for five NTS similar in both OpenPCR© and conventional thermocyclers as no significant performance differences were observed. Figures 5A and 5B show the DNA specificity for 10 non-*Salmonella* pathogens and Figures 6A and 6B show the DNA specificity for 26 known *Salmonella* serotypes. Comparison of specific for these strains were done between the conventional and OpenPCR© thermocyclers and similar results were observed.

DISCUSSION

Salmonella Enteritidis, *S*. Typhimurium, *S*. Welteverden, *S*. Agona, and *S*. Heidelberg are the five predominant NTS causing foodborne disease with high morbidity rate in most developing countries. mPCR is one of the most powerful tools for diagnosis of many diseases including salmonellosis through detection of several pathogens at a time by amplification of specific genes. However, the conventional PCR thermocycler is costly and heavy making it unsuitable for low-resource laboratory settings and not feasible for the field test.

Both the OpenPCR© and conventional PCR

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Table 3: Summary of the analytical specificity of the mPCR using OpenPCR© thermocycler.

Bacterial species		No. of strains tested positive for each primer in mPCR						
		STM	SEN	SHEI-1	SAGO	SWEL	invA	16S rRNA
1	*S.* Typhimurium	1/1	0/1	0/1	0/1	0/1	1/1	1/1
2	*S.* Enteritidis	0/1	1/1	0/1	0/1	0/1	1/1	1/1
3	*S.* Heidelberg	0/1	0/1	1/1	0/1	0/1	1/1	1/1
4	*S.* Agona	0/1	0/1	0/1	1/1	0/1	1/1	1/1
5	*S.* Weltevreden	0/1	0/1	0/1	0/1	1/1	1/1	1/1
6	*S.* Typhi	0/1	0/1	0/1	0/1	0/1	1/1	1/1
7	*S.* Paratyphi A	0/1	0/1	0/1	0/1	0/1	1/1	1/1
8	*S.* Paratyphi B	0/1	0/1	0/1	0/1	0/1	1/1	1/1
9	*S.* Paratyphi C	0/1	0/1	0/1	0/1	0/1	1/1	1/1
10	*S.* Poona	0/1	0/1	0/1	0/1	0/1	1/1	1/1
11	*S.* Hadar	0/1	0/1	0/1	0/1	0/1	1/1	1/1
12	*S.* Tshiongwe	0/1	0/1	0/1	0/1	0/1	1/1	1/1
13	*S.* Kissi	0/1	0/1	0/1	0/1	0/1	1/1	1/1
14	*S.* Assinie	0/1	0/1	0/1	0/1	0/1	1/1	1/1
15	*S.* Richmond	0/1	0/1	0/1	0/1	0/1	1/1	1/1
16	*S.* Uppsala	0/1	0/1	0/1	0/1	0/1	1/1	1/1
17	*S.* Kibi	0/1	0/1	0/1	0/1	0/1	1/1	1/1
18	*S.* Bardo	0/1	0/1	0/1	0/1	0/1	1/1	1/1
19	*S.* Virchow	0/1	0/1	0/1	0/1	0/1	1/1	1/1
20	*S.* Bordeaux	0/1	0/1	0/1	0/1	0/1	1/1	1/1
21	*S.* Newport	0/1	0/1	0/1	0/1	0/1	1/1	1/1
22	*S.* Emek	0/1	0/1	0/1	0/1	0/1	1/1	1/1
23	*S.* Braenderup	0/1	0/1	0/1	0/1	0/1	1/1	1/1
24	*S.* Regent	0/1	0/1	0/1	0/1	0/1	1/1	1/1
25	*S.* Oslo	0/1	0/1	0/1	0/1	0/1	1/1	1/1
26	*S.* Standley	0/1	0/1	0/1	0/1	0/1	1/1	1/1
27	*Escherichia coli*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
28	Enterohemorrhagic *E. coli*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
29	Enteropathogenic *E. coli*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
30	*Shigella* d*ysentriae*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
31	*S. flexneri*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
32	*S. sonnei*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
33	*S. Boydii*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
34	*Aeromonas Hydrophila*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
35	*Yersinia enterocolitica*	0/1	0/1	0/1	0/1	0/1	0/1	1/1
36	*Klebsiella pneumonia*	0/1	0/1	0/1	0/1	0/1	0/1	1/1

1 = Detected; 0 = Not detected.

thermocyclers successfully showed similar limit of detection of DNA. The limit of detection for *S*. Typhimurium, *S*. Agona and *S*. Weltevreden was found to be 10 pg/µL. On the other hand, the limit of detection for *S*. Enteriditis and *S*. Heidelberg was 1 pg/µL and 100 pg/µL, respectively Similarly, Park *et al.* (2012) and Park *et al.* (2015) demonstrated that the OpenPCR© had similar accuracy as the conventional PCR when HPV DNA was used as DNA template. The Open PCR thermocycler also exhibits a 100% specificity tested against a panel of 26 other *Salmonella* serotypes and 10 non-*Salmonella* strains as shown in Table 3.

An OpenPCR© is ten times cheaper compared to the conventional PCR machine and is portable. This enables transportation of this machine to remote field locations as it weighs less than 3.5 kg (OpenPCR©). The portability, simple mode of operation and low cost of the OpenPCR© thermocycler makes it a practical solution for field test. In this study, an mPCR assay was successfully performed using the OpenPCR© thermocycler. This shows that this machine is able to provide a low-cost solution for rapid confirmation of 5 NTS serotypes causing foodborne diseases.

CONCLUSION

In conclusion, an optimized NTS-mPCR assay performed on a DIY OpenPCR© thermocycler shows that this machine can be used as a low- cost alternative option for rapid detection of the five predominant NTS serotypes. This study shows the mPCR of the five NTS serotypes using OpenPCR© thermocycler has a similar performance in terms of detection, sensitivity and specificity in comparison with the established conventional PCR machine. Finally, the limit of detection for *S*. Typhimurium,

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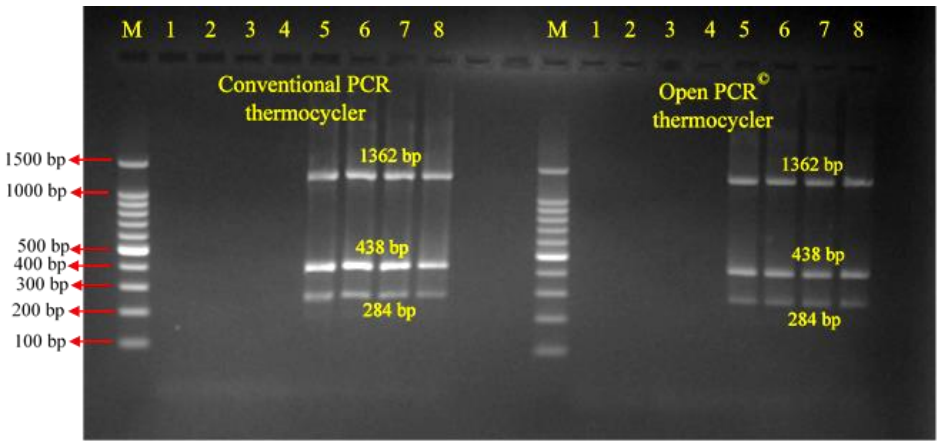

Figure 3: mPCR result using *S.* Agona to assess the block heating uniformity between OpenPCR© and conventional PCR thermocyclers. M: 100 bp DNA ladder, 1-4: negative control (ddH_20); 5-8: *S.* Agona ATCC 51957.

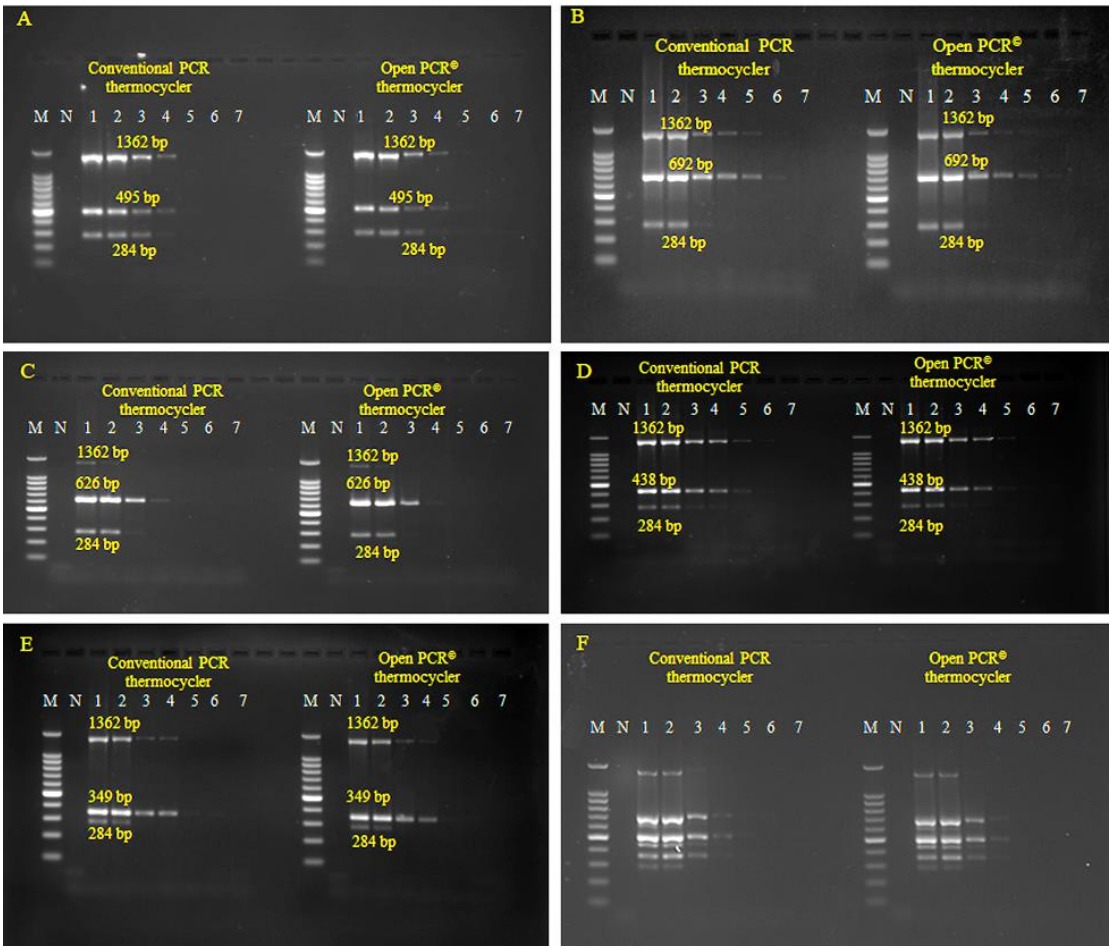

Figure 4 (A-F): Analytical sensitivity gel electrophoresis images for DNA detection level for five NTS). (A) *S.* Typhimurium, (B) *S.* Enteritidis, (C) *S.* Heidelberg, (D) *S.* Agona, and (E) *S.* Weltevreden (F) Pooled DNA. Lane M: 100 bp DNA Ladder (Promega); Lane N: No template control (ddH_2O); Lane 1: 100 ng/µL; Lane 2: 10 ng/µL; Lane 3: 100 pg/µL; Lane 4: 10 pg/µL; Lane 5: 1 pg/µL; Lane 6: 100 fg/µL; Lane 7: 10 fg/µL.

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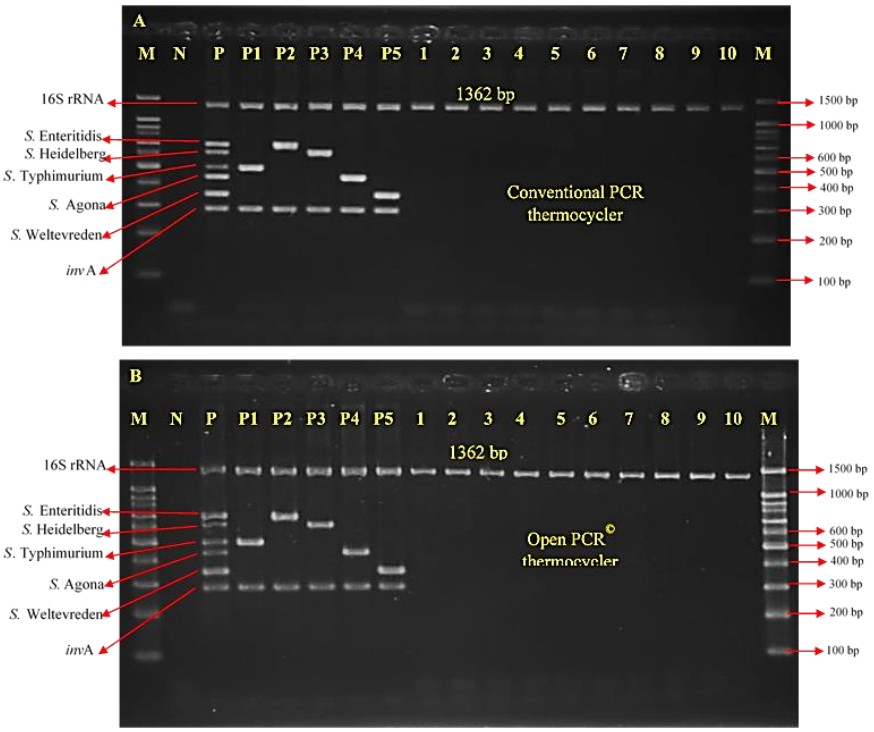

Figure 5 (A-B): Analytical specificity test of the mPCR assay using 10 non-*Salmonella* enteric pathogens with positive controls. M: 100 bp DNA ladder, N: -ve control; P: pooled DNA; P1: *S*. Typhimurium ATCC 14028; P2: *S*. Enteritidis ATCC 13076; P3: *S*. Heidelberg ATCC 8326; P4: *S*. Agona ATCC 51957; P5: *S*. Weltevreden ATCC 6534; (1-10): non *Salmonella* spp.

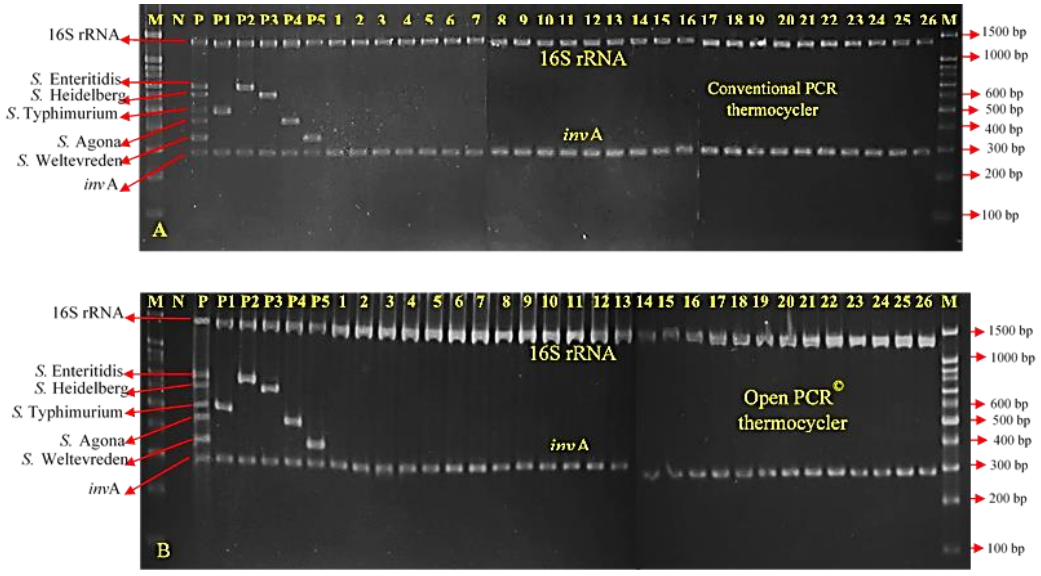

Figure 6 (A-B): Analytical specificity test of the mPCR assay using 26 known *Salmonella* species. M: 100 bp DNA ladder, N: negative control; P: pooled DNA; P1: *S*. Typhimurium ATCC 14028; P2: *S*. Enteritidis ATCC 13076; P3: *S*. Heidelberg ATCC 8326; P4: *S*. Agona ATCC 51957; P5: *S*. Weltevreden ATCC 6534; (1-26): other *Salmonella* spp.

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S. Agona and *S*. Weltevreden was determined to be 10pg/µL whereas the limit of detection for *S*. Enteriditis and *S*. Heidelberg was determined to be 1 pg/µL and 100 pg/µL, respectively

This cost reduced and rapid diagnostic equipment available in a portable design (OpenPCR© thermocycler) would act as revolutionary step forward in global health diagnostics concerning NTS especially in the developing countries. Thus, the OpenPCR© thermocycler was found to be preferable due to low-cost and has good sensitivity and specificity for amplification of nucleic acid targets. The machine is also suitable for diagnostics of other infectious diseases in developing countries.

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