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Evaluation of the *H1d* flagellin gene targeting PCR for detection of *Salmonella enterica* serovar Typhi

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ABSTRACT A nested-PCR assay targeting the *H1-d* flagellin gene was evaluated on 46 *Salmonella* strains representing 27 different serovars and 13 non-*Salmonella* strains from eight different bacterial genera. The PCR was highly sensitive as it could detect down to 2 cfu/ml after the nested PCR. The first and second round of PCR amplifications produced the expected amplicons in all the *S.* Typhi as well as *S.* Muenchen and *S.* Stanley strains. The second round of PCR also produced an amplicon of the expected size with the *Citrobacter freundii* strain. These findings indicate that the PCR is not completely specific for *S.* Typhi. The nested-PCR system also posed problems of carryover contaminations. Although highly sensitive, the problem of specificity and carryover contamination questions the reliability of this nested-PCR assay. Therefore there is a need for the development of new PCR assays targeting more specific regions of the *S.* Typhi genome.

ABSTRAK Suatu *nested-PCR* yang mengesan gen *H1-d* flagellin diuji ke atas 46 strain *Salmonella* yang mewakili 27 serovar berlainan dan 13 strain bukan-*Salmonella* daripada 8 genus bakteria yang berlainan. PCR tersebut didapati sangat sensitif kerana berupaya mengesan sehingga 2 cfu/ml selepas pusingan kedua PCR (nested). Pusingan pertama dan kedua PCR menghasilkan amplikon (jujukan DNA hasil PCR) bagi semua strain *S.* Typhi dan juga strain *S.* Muenchen dan *S.* Stanley. Pusingan kedua PCR turut memberikan amplicon yang serupa bagi strain *Citrobacter freundii.* Hasil ujikaji ini munujukkan bahawa PCR tersebut bukan spesifik sepenuhnya bagi *S. Typhi.* Sistem *nested –PCR* in juga memberikan masalah kontaminasi. Walaupun amat sensitif, masalah kekurangan spesifisiti dan kontaminasi menyoal keberkesanan kaedah ini. Oleh yang demikian, usaha untuk menemui kaedah PCR baru yang mengesan bahagian genom *S.* Typhi yang lebih spesifik diperlukan.

(Flagellin gene, S. Typhi, detection)

INTRODUCTION

Typhoid fever is considered to be one of the most important and underreported diseases in developing nations [1]. WHO estimates that 16 million cases of typhoid fever and 600 000 related deaths occur worldwide each year [2]. Clinical diagnosis of typhoid fever is difficult, because the signs and symptoms are not unique and overlap with other common febrile illness [3]. Diagnosis is conventionally based on two methods: a) blood cultures and b) serology; However, these methods either lack sensitivity or specificity or are therefore of little diagnostic value. A dot enzyme immunosorbent assay developed in Malaysia [4, 5] using a 50kD outer membrane protein (OMP) from *S.* Typhi for the evaluation of the presence of IgG and IgM (Typhidot) and the IgM alone (Typhidot-M) also proved to be more specific, sensitive and cost effective compared to other serological methods. However, serological methods such as this has its limitations as they can only be used to detect the presence of *S.* Typhi in human clinical samples, and not in environmental samples such as sewage water and food especially for the detection of the organism during an outbreak. The Polymerase Chain Reaction (PCR) is a promising molecular technique being widely used for the detection of microorganisms in recent years [6].

A number of PCR assays have been developed for the detection of *S.* Typhi [7, 8, 9 and 10]. Of these, the *H1-d* flagellin gene targeting primers developed by Song *et al.* (1993) is the most used PCR method for the identification of *S.* Typhi [11, 12 and 13]. However, Hashimoto *et al.* [8], pointed out that this assay may not be applicable to clinical specimens, as the possibility exists that it may also detect other *Salmonella* serovars which also have the *H1-d* flagellin gene.

As a very limited number of bacterial strains (10 *Salmonella* and 7 non- *Salmonella* strains) were used by Song *et al.* [7] to test the specificity of the PCR assay, we decided to evaluate its specificity on a larger range of *Salmonella* and non- *Salmonella* organisms. We have also tested the sensitivity of the technique using serially diluted whole cells as well as genomic DNA of *S.* Typhi.

MATERIALS AND METHODS

A total of 46 *Salmonella* strains representing 27 different serovars including *S.* Typhi and 13 non-*Salmonella* strains belonging to 8 different genera were included in this study (Table 1). The first round of PCR primer sequences amplifying a 458 bp region were ST 1: 5'-ACT GCT AAA ACC ACT ACT-3', and ST 2: 5'-TTA ACG CAG TAA AGA GAG-3'. Nested primers amplifying a 343-bp region were ST 3: 5'-AGA TGG TAC TGG CGT TGC TC-3' and ST 4: 5'-TGG AGA CTT CGG TCG CGT AG-3'.

Amplification was carried out in a total volume of 25 µl containing 25 pmol of each primer, 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.7 U *Taq* DNA polymerase and 1 x PCR buffer. The PCR cycling parameters used was 30 cycles of denaturation at 94 °C, for 1 min, annealing at 57 °C (first round PCR) and 68 °C (nested PCR) for 1 min 15 sec and extension at 72 °C for 3 min were carried out. Five µl of the amplified product was used as template for the nested PCR. A negative control containing the same reaction mixture except the DNA template was included in every experiment to detect possible cross contamination.

For the specificity test, a single bacterial colony was picked from LB agar plate, boiled in 50 µl distilled water for 10 min and immediately cooled on ice for 5 min. After a short spin, 4 µl of this solution was used in PCR. To determine the detection limit of the technique, genomic DNA, extracted as previously described [12] as well as a broth culture of *S.* Typhi grown up to its log phase, were serially diluted to a final concentration from 100 ng to 1 fg and 2 x 10^9 to 2 cfu/ml respectively, and subjected to PCR analysis. Both specificity and sensitivity experiments were repeated at least twice under identical conditions in independent runs. Amplicons of the desired size from respective strains were also sequenced and the DNA sequences submitted to the National Center for Biotechnology Information (NCBI) for a nucleotide comparison against available database sequences using the Basic Local Alignment Search Tool (BLAST).

RESULTS AND DISCUSSION

The first PCR produced amplification products of 458 bp from all the *S.* Typhi as well as the *S.* Muenchen and *S.* Stanley strains, but not from the other organisms. With the nested PCR, amplification products of 343 bp were detected from all *S.* Typhi, *S.* Muenchen, *S.* Stanley and *Citrobacter freundii* strains, but not from the other strains (Table 1, Figure 1).

The limit of detection of the PCR assay was about 2 x 10^5 CFU/ml with the first round of PCR and increased to about 2 CFU/ml after the nested PCR. When serial dilutions of genomic DNA was used, a sensitivity of 1 ng was observed with the first round PCR, while the nested PCR detected up to 1 fg of DNA.

According to Song *et al.* [7], the PCR assay only detected *S.* Muenchen with the second pair of primers. In this study however, both first and second PCRs detected *S.* Muenchen and *S.* Stanley (*S.* Stanley was not included in Song's study). When sequenced, the first round PCR-amplicon from *S.* Typhi was 462 bp long and 100 % homologous to the *Salmonella* Typhi flagellin gene (STYFLAGLN locus, accession no. L21912.1). Nucleotide sequences of the amplicons from *S.* Muenchen and *S.* Stanley, both 462 bp long, shared a 98 % identity with the *S.* Typhi flagellin gene (STYFLAGLN locus, accession no. L21912.1). The *S.* Stanley amplicon sequence had a similarity of 99 % i.e. 441 nucleotides over a length of 445 nucleotides while the *S.* Muenchen amplicon sequence had a similarity of 98 % i.e. 440 nucleotides over a

length of 445 nucleotides with the *S.* Muenchen *H1-d* gene phase 1-d flagellin (SMH1D locus, accession no. X03395.1).

Table 1. Specificity of the PCR for the detection of the *H1d flagellin* gene of *S.* Typhi.

Strain	No. of strains tested	PCR results	
		1st PCR	2nd PCR
S. Typhi	6	+	+
S. Typhimurium	2	-	-
S. Paratyphi A	2	-	-
S. Paratyphi B	2	-	-
S. Paratyphi C	2	-	-
S. Enteritidis	1	-	-
S. Weltevreden	1	-	-
S. Newport	6	-	-
S. Virchow	1	-	-
S. Hadar	1	-	-
S. Infantis	1	-	-
S. Matopeni	1	-	-
S. Bareilly	1	-	-
S. Raus	1	-	-
S. Kentucky	1	-	-
S. Waycross	1	-	-
S. Muenchen	6	+	+
S. Chingola	1	-	-
S. Lomita	1	-	-
S. Hvittingfos	1	-	-
S. Blockley	1	-	-
S. Stanley	1	+	+
S. Thompson	1	-	-
S. Corvallis	1	-	-
S. Haifa	1	-	-
S. Bovismorbifcans	1	-	-
S. Dublin	1	-	-
Citrobacter freundii	1	-	+
Escherichia coli	2	-	-
Listeria monocytogenes	1	-	-
Pseudomonas aeruginosa	1	-	-
Klebsiella pneumoniae	1	-	-
Shigella flexneri	1	-	-
Shigella dysenteriae	1	-	-
Shigella sonnei	1	-	-
Shigella boydii	1	-	-
Enterobacter cloacae	1	-	-
Enterobacter gergoviae	1	-	-
Vibrio parahaemolyticus	1	-	-
Vibrio cholerae	1	-	-

(+) indicates presence and (-) indicates absence of desired amplicon

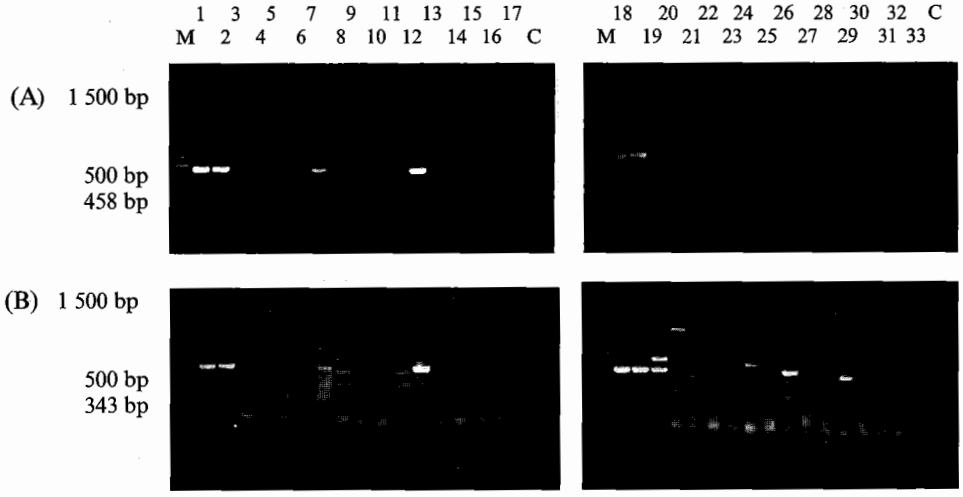
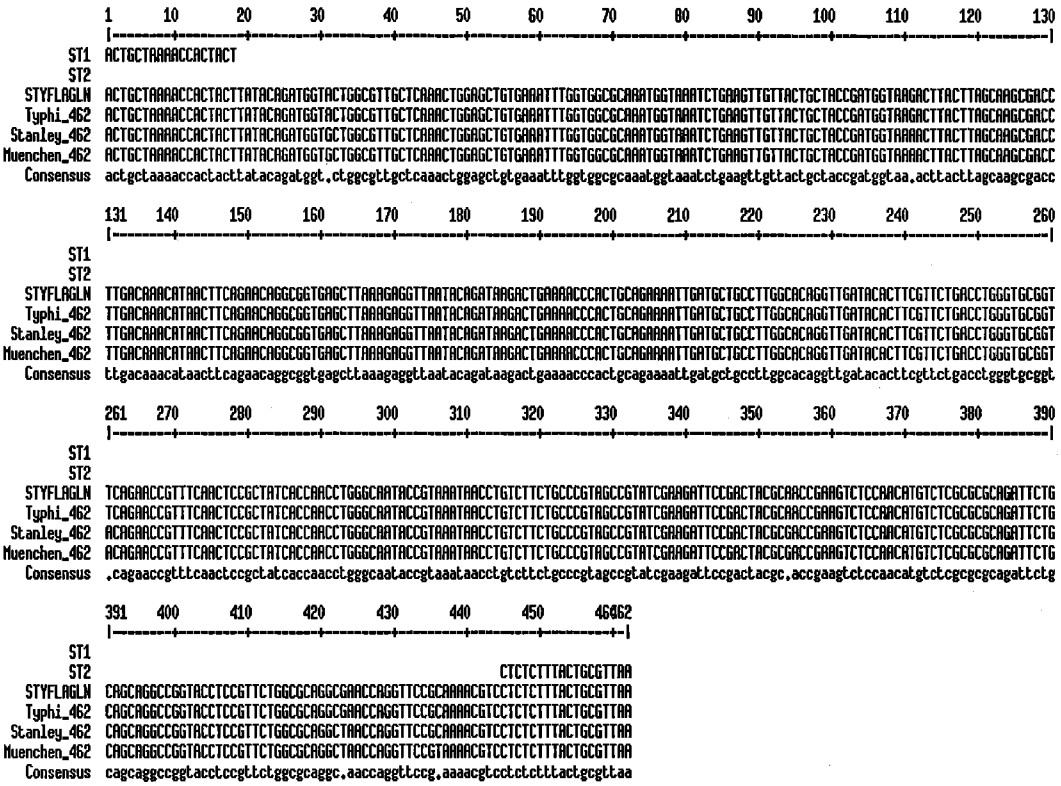


Figure 1. Representative gels showing specificity of the PCR for the detection of the *H1d flagellin* gene of *S.* Typhi, non-typhoidal *Salmonella* and other *Enterobacteriacea* (A): Amplification products 458 bp with the external primers ST1 &ST2. (B) Amplification products of 343 bp with the internal primers ST3 & ST4.

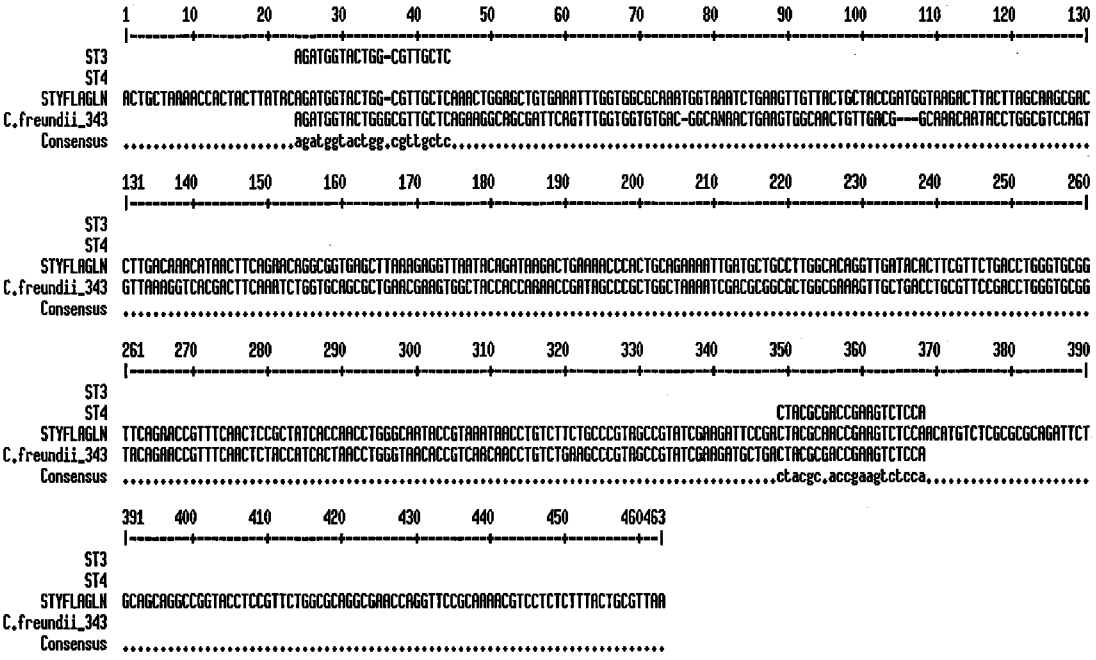
M: 100 bp Marker, lanes 1 -33: *S.* Typhi, *S.* Typhi, *S.* Bareilly, *S.* Rau, *S.* Kentucky, *S.* Waycross, *S.* Muenchen, *S.* Chingola, *S.* Lomita, *S.* Hvittingfos, *S.* Blockley, *S.* Stanley, *S.* Thompson, *S.* Corvallis, *S.* Haifa, *S.* Bovismorbificans, *S.* Dublin, *S.* Typhi, *S.* Typhi, *Citrobacter freundii, Escherichia coli, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella flexneri, Shigella dysenteriae, Shigella sonnei, Shigella boydii, Enterobacter cloacae, Enterobacter gergoviae, Vibrio parahaemolyticus, Vibrio cholerae,* and C: Negative control

Song and co-workers did not obtain a positive result with any of the non-*Salmonella* bacteria. In the present study, interestingly, *Citrobacter freundii* produced a 341 bp amplicon with the second pair of primers. It had a similarity of 88 % i.e. 113 nucleotides over a length of 127 nucleotides with the *S.* Muenchen *H1-d* gene for phase-1-d flagellin (SMH1D locus, accession no. X03395.1) and a similarity of 87 % i.e. 111 nucleotides over a length of 127 nucleotides with the *S.* Typhi flagellin gene (STYFLAGLN locus, accession no. L21912.1). The amplicon sequences obtained from the first amplification of *S.* Typhi, *S.* Muenchen and *S.* Stanley strains as well as the amplicon sequence obtained from the second round amplification of *Citobacter freundii* were aligned with the *S.* Typhi flagellin gene sequence (STYFLAGLN locus, accession no. L21912.1) (Figure 2) using the multiple sequence alignment service at Multalin version 5.4.1 [15].

As stated by Song *et al.* [7], flagellar antigen is not a structure specific to *Salmonella* species and d antigen is also present in many *Salmonella* species other than *S.* Typhi. It is to be noted that both *S.* Muenchen and *S.* Stanley are *Salmonella* serovars that contain the d antigen. Therefore, as suggested by Hashimoto *et al.* [8], it is possible that the *H1d* flagellin gene targeting PCR amplifies other *Salmonella* serovars where the *H1d* flagellin gene exists. Likewise, it has been established that *Citrobacter freundii*, also a member of the Enterobacteriaceae, is closely allied biochemically with *Salmonella* and cultures of the *Citrobacter* group may have both O and H antigens related to those of *Salmonella* cultures [16]. Hence, it can be concluded that the *H1d* flagellin gene targeting PCR evaluated in the present study is not 100 % specific to *S.* Typhi.



(**A**) Sequences aligned: **ST1**: external primer (forward) , **ST2**: external primer (reverse comlimentary), **STYFLAGLN**: *S.* Typhi flagellin gene sequence from GenBank, **Typhi_462**: 462 bp PCR product from *S.* Typhi, **Stanley_462**: 462 bp PCR product from *S.* Stanley, **Muenchen_462**: 462 bp PCR product from *S.* Muenchen



(**B**) Sequences aligned: **ST3**: nested primer (forward), **ST4**: nested primer (reverse complimentary), **STYFLAGLN**: *S.* Typhi flagellin gene sequence from Gen Bank, ***C. freundii*_343**: 343 bp PCR product from *Citrobacter freundii*

Figure 2. The amplicon sequences of *S.* Typhi, *S.* Muenchen and *S.* Stanley (A) and of *Citrobacter freundii* (B) were aligned with the S. Typhi flagellin gene sequence available at Genbank, using Multalin version 5.4.1 (Corpet, 1988).

A major problem faced with this nested PCR assay was carryover contamination which often resulted in false positivity (results not shown). This is not something unexpected, as nested PCR systems are known to be error-prone due to random contamination caused by multiple pipetting steps between first round and second round amplifications [17]. In this study, a negative control was included in every PCR reaction and therefore contaminations could be identified immediately, although it could not be entirely eliminated.

Several other research groups have developed PCR assays using different sets of primers to detect *S.* Typhi. Hashimoto and co-workers [8] developed a nested PCR assay based on the *viaB* sequence of *S.* Typhi. However, this method was not 100% specific as it also detected *S.* Paratyphi C. Chaudry *et al.* [18] developed a *dH* flagellin gene targeting PCR for the detection of *S.* Typhi. Yet again, the primers were not completely specific for *S.* Typhi as they also detected other *Salmonella* strains containing *dH* flagellin gene such as *S.* Stanley, *S.* Livingstone and *S.* Schwarzengrund. A nested PCR targeting the spacer region between *23S rRNA* and *5S rRNA* was described by Lim *et al.*. Although the identity of the amplicons was confirmed by sequencing, the specificity of the method was not evaluated on other bacterial strains. More recently, a multiplex PCR targeting the *tyv*, *prt*, *viaB* and *fliC* genes was developed for the simultaneous detection of *S.* Typhi and *S.* Paratyphi A [10]. This method utilizes 5 pairs of primers in one PCR and therefore is complex and tedious.

In the present study it can be concluded that, although highly sensitive, the problem of specificity and carryover contamination questions the reliability of this nested-PCR assay. Therefore, there is a great need for the development of new PCR assays targeting more specific regions of the S. Typhi genome in order to facilitate rapid, specific and sensitive diagnosis of typhoid fever.

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