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Short Communication

Further Evaluation of a Multiplex PCR for Differentiation of *Salmonella* Paratyphi A from Other *Salmonellae*

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SUMMARY: *Salmonella enterica* serovar Paratyphi A is a causative agent of paratyphoid fever. The clinical syndrome caused by paratyphoid fever overlaps with other febrile illnesses and cannot be distinguished from typhoid fever. Conventional methods used for diagnosis are time consuming, costly, and labor-intensive. We evaluated the specificity, sensitivity, and application of a multiplex polymerase chain reaction (PCR) previously developed by the method (Ou, H.Y., Teh, C.S.J., Thong, K.L., et al., J. Mol. Diagn., 9, 624-630, 2007) using 6 *S*. Paratyphi A, 22 *S*. Typhi, and 85 other *Salmonella* serovars as well as 36 non-*Salmonella* strains. The detection limit of the multiplex PCR was 4×10^4 cfu ml^{-1}. In a blind test of the other 50 strains, this multiplex PCR correctly identified the only *S*. Paratyphi A in the panel of strains. The sensitivity of this PCR using spiked blood and stool samples was 1×10^5 cfu ml^{-1} and 2×10^5 cfu ml^{-1}, respectively, but increased to 1×10^4 cfu ml^{-1} and 2×10^3 cfu ml^{-1} after 5-h enrichment. We believe that this multiplex PCR is a promising technique for the specific and sensitive detection of *S*. Paratyphi A in clinical, environmental, and food samples.

Salmonella enterica serovar Typhi and *Salmonella enterica* serovar Paratyphi A are two infectious agents responsible for enteric fever, which remains endemic in many parts of the world in which sanitary conditions remain poor and the water supply is not treated (1). Typhoid fever was generally considered as a more critical illness than paratyphoid fever, but is gradually being superseded by paratyphoid fever in the past decade (2). *S*. Paratyphi A is becoming predominant in some provinces in China, and an increasing number of cases has been reported in other parts of Asia (3,4).

Clinical diagnosis can be difficult, as the symptoms associated with paratyphoid fever are not unique and overlap with those of other febrile illness, especially those of typhoid fever. Patients with *S*. Paratyphi A infection are said to develop more numerous rose-colored spots than do patients with *S*. Typhi infection, but difficulties with quantification arise in dark-skinned patients (5). The isolation and identification of *S*. Paratyphi A from clinical specimens will confirm the diagnosis of enteric fever, but *S*. Paratyphi A, unlike other strains of *Salmonellae*, typically does not produce hydrogen sulphide and therefore is often missed in the laboratory (6). The diagnostic significance, as determined by serological assays such as the Widal test, can be confirmed only when a 4-fold increase in antibodies is demonstrated over a 2- to 3-week period (7).

Hirose et al. (8) has proposed a multiplex PCR assay to identify *S*. Typhi and *S*. Paratyphi A by the use of the following 5 genes: *tyv*, *fliC-d*, *viaB*, *fliC-a*, and *prt*. Detection of *tyv*, *fliC-d*, *viaB*, and *prt* will correctly identify *S*. Typhi, while *fliC-a* and *prt* correctly identify *S*. Paratyphi A. We had previously developed a multiplex polymerase chain reaction (PCR) targeting 4 different genes (9) as an alternative approach to differentiate *S*. Paratyphi A from other *Salmonellae*. In the present study, we report the application of this multiplex PCR on more bacterial cultures and using spiked blood and stool samples in order to further evaluate its specificity and sensitivity.

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The multiplex PCR used in this study, previously described by Ou et al. (9), consists of four sets of primers targeting *stkF* (159 bp), *spa2473* (324 bp), *spa2539* (484 bp), and *hsdM* (627 bp) genomic locations; results were interpreted as Paratyphi A-positive when specific fragments corresponding to these 4 targets were produced. The specificity of this multiplex PCR was determined by testing a collection of 149 bacterial strains. The multiplex PCR assay correctly identified all 6 strains of *S*. Paratyphi A, and demonstrated a specificity of 100% when all 4 targeted amplicons were produced. One amplicon of 159 bp was produced for *S*. Paratyphi B, *S*. Paratyphi C, *S*. Albany, and *S*. Virchow. *S*. Bareilly yielded 2 amplicons (159 bp and 627 bp). *S*. Typhi, another *Salmonella* spp., and all non-*Salmonella* strains showed no amplification (Fig. 1). The procedure was repeated twice and all results were confirmed as reproducible.

The multiplex PCR assay was tested in a blinded manner on 50 bacterial cultures. In the panel of strains, only one yielded 4 bands of interest, another yielded 2 amplicons (159 bp and 627 bp), and 6 strains yielded one amplicon each (159

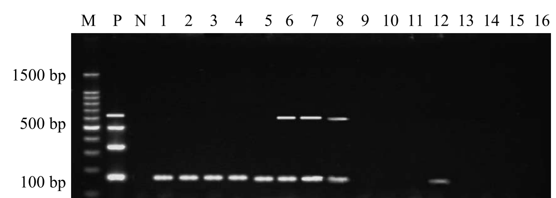

Fig. 1. Multiplex PCR results of *Salmonella* and non-*Salmonella* spp. Lanes: M, molecular size marker (100-bp DNA ladder); P, positive control (*S*. Paratyphi A ATCC 9150); N, negative control (non-*S*. Paratyphi A strain), lanes 1-3, *S*. Paratyphi B; lane 4, *S*. Paratyphi C; lane 5, *S*. Albany; lanes 6-8, *S*. Bareilly; lanes 9-10, *S*. Typhi; lane 11, *S*. Typhimurium; lane 12, *S*. Virchow; lane 13, *S*. Enteritidis; lanes 14-16, non-*Salmonella* strains.

bp). Negative results were obtained for all remaining strains. Thus, the strain which yielded 4 PCR amplicons was considered to be *S*. Paratyphi A. The PCR data was then compared with the identity of the strains, and the results revealed that this multiplex PCR assay enabled the differentiation of *S*. Paratyphi A from all other strains tested, and the test was 100% specific. Moreover, the positive predictive value was 100%.

The procedure described by Pathmanathan et al. (10) was used for sensitivity tests using bacterial cell dilutions and spiked stool samples. Briefly, an overnight culture of *S*. Paratyphi A ATCC 9150 was serially diluted 10-fold, and 100 μl of each dilution was boiled, snap-cooled, and centrifuged to yield the bacterial cell template, while another 250 μl of each bacterial dilution was spiked into a 250-μl stool sample (provided by a healthy volunteer) which had been diluted 10-fold with BHI broth to minimize inhibition during PCR. To determine the sensitivity of the assay with the spiked blood sample, an aliquot of 100 μl of *S*. Paratyphi A ATCC 9150 cultured overnight was spiked with 900 μl of blood from a healthy volunteer and the sample was serially diluted 10-fold. The spiked blood and stool samples were incubated at 37°C for 5 h. PCR was performed on samples obtained at 0- and 5-h time points. An aliquot of 100 μl from each dilution was plated onto an LB plate, and the plates were incubated overnight at 37°C in order to determine the cell density.

PCR for bacterial cell dilutions and spiked stool samples was carried out under the optimized conditions as described in Ou et al. (9), while BloodDirect™ PCR Buffer Kit (Novagen, Madison, Wis., USA) was used for direct PCR with blood samples. Five microliters of 5 $\times$ blood buffer 1 (Novagen), 5 μl of 5 $\times$ blood buffer A (Novagen), 200 μM of each dNTP (Promega, Madison, Wis., USA), 2.5 U ml^{-1} *Taq* DNA polymerase (Promega), 0.4 μM of each primer, 1 μl of spiked blood sample (from each dilution), and an appropriate amount of deionised water in a total volume of 25 μl was used for the PCR.

The sensitivity of the multiplex PCR was tested using the *S*. Paratyphi A ATCC 9150 isolate on 10-fold dilutions of the bacterial culture. The sensitivity was found to be 4×10^4 cfu ml^{-1}, i.e., equivalent to approximately 200 cfu per PCR (4×10^4 cfu $ml^{-1} \times 5$ μl). The sensitivity of multiplex PCR using the spiked blood sample was 1×10^5 cfu ml^{-1} (approximately 100 cfu per PCR as 1×10^5 cfu $ml^{-1} \times 1$ μl) which increased to 1×10^4 cfu ml^{-1} after 5 h of enrichment (10 cfu per PCR as 1×10^4 cfu $ml^{-1} \times 1$ μl). The sensitivity of the multiplex PCR using the spiked stool sample was 2×10^5 cfu ml^{-1} (approximately 1,000 cfu per PCR as 2×10^5 cfu $ml^{-1} \times 5$ μl). As expected, the sensitivity also increased to 2×10^3 cfu ml^{-1} after 5 h of enrichment (approximately 10 cfu per PCR as 2×10^3 cfu $ml^{-1} \times 5$ μl).

PCR is a better alternative than conventional methods, as it targets the DNA of an organism. The multiplex PCR used in the present study enabled the detection of *S*. Paratyphi A in both blood and stool samples, thus increasing the efficiency of detection without sacrificing specificity or sensitivity. As no amplification of any *S*. Typhi strains tested was observed, the present results demonstrate that this approach successfully distinguished *S*. Paratyphi A from *S*. Typhi. As the targeting of multiple independent protein coding sequences (CDS) using a multiplex approach for the detection and identification of a single type of bacterium will enhance the specificity of a test, multiplex PCR was used here to detect *S*. Paratyphi A (9). Therefore, the development of a nested PCR for screening was not considered necessary.

Due to the poor isolation of *S*. Paratyphi A in Malaysia, only 6 strains were available. Although the multiplex PCR showed 100% specificity for *S*. Paratyphi A, a larger sample size would have been better for evaluating the efficiency of this multiplex PCR. Although the incidence of paratyphoid fever in Malaysia is low, human beings are still the only known reservoir, and transmission occurs through food and water contaminated by ill patients or by chronic carriers. Therefore, two groups of people are generally implicated in the transmission of this disease. The first group is travellers returning from endemic countries, but the risk to this group appears to vary by geographic region visited (e.g., India and Nepal, where the disease is highly prevalent). The second group consists of migrant workers arriving from endemic regions; of special importance are people employed in the food and beverage sector (1). Typhoid vaccine, which is recommended to all travellers to endemic countries, offers no protection against *S*. Paratyphi infections. *S*. Paratyphi A has become the predominant cause of enteric fever among vaccinated travellers, and hence consideration should be given to the development of an effective vaccine against this pathogen.

In conclusion, any successful disease surveillance program starts with adequate diagnosis. Physicians must become aware of the rising incidence of Paratyphoid A fever. Correct diagnosis is not only important for treatment, but also essential for monitoring and transmission. The multiplex PCR developed in this study offers rapid identification and verification of *S*. Paratyphi A infections and therefore offers an option for improved control and surveillance of this important pathogen (9).

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