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ORIGINAL ARTICLE

The discrimination of d-tartrate positive and d-tartrate negative S. *enterica* subsp. *enterica* serovar Paratyphi B isolated in Malaysia by phenotypic and genotypic methods

Norazah AHMAD *MD, PhD*, Shirley TANG Gee Hoon *BSc**, Mohamed Kamel ABD GHANI *MD, MSc** and KOH Yin Tee *DipMLT*

*Bacteriology Unit, Institute for Medical Research, Kuala Lumpur and *Department of Biomedical Science, Faculty of Allied Health Sciences, University Kebangsaan Malaysia, Kuala Lumpur.*

Abstract

Serotyping is not sufficient to differentiate between Salmonella species that cause paratyphoid fever from the strains that cause milder gastroenteritis as these organisms share the same serotype *Salmonella* Paratyphi B (*S*. Paratyphi B). Strains causing paratyphoid fever do not ferment d-tartrate and this key feature was used in this study to determine the prevalence of these strains among the collection of *S*. Paratyphi B strains isolated from patients in Malaysia. A total of 105 isolates of *S*. Paratyphi B were discriminated into *d*-tartrate positive (dT^+) and *d*-tartrate negative (dT^-) variants by two lead acetate test protocols and multiplex PCR. The lead acetate test protocol 1 differed from protocol 2 by a lower inoculum size and different incubation conditions while the multiplex PCR utilized 2 sets of primers targeting the ATG start codon of the gene STM3356. Lead acetate protocol 1 discriminated 97.1% of the isolates as *S*. Paratyphi B dT^+ and 2.9% as dT^- while test protocol 2 discriminated all the isolates as S. Paratyphi B dT^+. The multiplex PCR test identified all 105 isolates as *S*. Paratyphi B dT^+ strains. The concordance of the lead acetate test relative to that of multiplex PCR was 97.7% and 100% for protocol 1 and 2 respectively. This study showed that *S*. Paratyphi B dT^+ is a common causative agent of gastroenteritis in Malaysia while paratyphoid fever appears to be relatively uncommon. Multiplex PCR was shown to be a simpler, more rapid and reliable method to discriminate *S*. Paratyphi B than the phenotypic lead acetate test.

Keywords: S. Paratyphi B, *d*-tartrate, lead acetate test, multiplex PCR

INTRODUCTION

Serotyping of *Salmonella* species could not differentiate the virulent causative agent of paratyphoid fever from the causative agent of *Salmonella* gastroenteritis which was both serotyped as *S. enterica subsp. enterica serovar* Paratyphi B (*S*. Paratyphi B). S. Paratyphi B can be differentiated into *d*-tartrate fermenting and non-fermenting via the fermentation of dextrorotatory [(L(+)]-tartrate (d-tartrate).[1] The *d*-tartrate non-fermenting (dT^-) variant *S*. Paratyphi B is the causative agent of paratyphoid fever while the *d*-tartrate fermenting (dT^+) variant *S*. Paratyphi B (formerly called *Salmonella* Java) only triggers gastroenteritis with the symptoms of vomiting, abdominal pain and diarrhea in humans.[2]

Recently, *S*. Paratyphi B dT^+ variant has become increasingly important throughout the world especially in the developing countries including Malaysia. Moreover, the total number of isolates of *S*. Paratyphi B received by Salmonella Serotyping Laboratory, Institute for Medical Research, Kuala Lumpur has increased dramatically from 37 isolates in 2006 to 105 isolates in 2009 (unpublished data). A dramatic increase in the incidence of *S*. Paratyphi B was also reported in other countries such as France, Italy and Canada.[3,4,5,6]

The lead acetate test which differentiates *d*-tartrate fermenting (dT^+) from *d*-tartrate non-fermenting (dT^-) bacteria has been incorporated in most identification schemes.[7] Currently, the lead acetate test described by Alfredsson *et al*. is used as the standard method by the WHO for

Address for correspondence and reprint requests: Dr Norazah Ahmad, Bacteriology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur. Tel: 03-26162650. Fax: 03-26919716. Email: norazah@imr.gov.my

discrimination of dT+ and dT– *Salmonella* strains.[8] A modified lead acetate test which differed in inoculum size and shorter incubation conditions had also been shown to discriminate dT+ strains from dT– *S*. Paratyphi B strains.[9] Marlony *et al* also developed multiplex PCR method to rapidly identify dT+ and dT– *Salmonella* strains.[9]

The aim of this study was to determine whether the causative agent of paratyphoid fever is common among the *S*. Paratyphi B isolated from clinical cases in Malaysia using lead acetate test protocols and multiplex PCR and to determine the concordance of the phenotypic method compared to that of multiplex PCR.

MATERIALS AND METHODS

Bacterial strains and serotyping

The test strains were from various clinical specimens isolated by microbiology laboratories of government and private hospitals in Malaysia and sent for serotyping at the Salmonella Serotyping Reference Centre in Bacteriology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. These strains were serotyped as *S*. Paratyphi B when they exhibited the antigenic formula O1, 4,(5), 12: Hb:1,2 using the Kauffmann-White scheme. A total of 105 clinical strains of *S*. Paratyphi B were collected in the year 2009. The strains were kept at -80°C until further use.

Reference strains

The positive and negative control strains used in both the lead acetate test and multiplex polymerase chain reaction (m-PCR) were *S*. Paratyphi B dT+ NCTC 5706 and dT– NCTC 3176 respectively.

Lead acetate test protocols

Two different phenotypic lead acetate test protocols were performed for all the strains. Protocol 1 was performed following the protocol described by Alfredsson *et al*.[8] Ten grams of peptone (Difco) powder was weighed and dissolved in 1 L of distilled water. After autoclaving at 121°C for 15 minutes, potassium sodium tartrate tetrahydrate (10 g) was added to the base medium to give a final concentration of 1%. The pH of the base medium was adjusted to pH7.4 with 1N of sodium hydroxide. Bromothymol blue sodium salt was added as an indicator to a final concentration of 0.0023%. The broth was dispensed in 8ml aliquots into cotton-wool-stoppered round-bottomed test tubes and sterilized at 110°C for 10 minutes. Bacterial suspension equivalent to 0.5 McFarland Standard was prepared in 0.85 % sodium chloride. Fifty microliters of the bacterial suspension was inoculated into test tubes containing 8ml of base medium. A set of the culture was incubated for 3 days and another set for 6 days, aerobically without shaking at 37°C. After incubation, the cultures were tested for *d*-tartrate utilization by adding saturated aqueous lead acetate solution in the proportion of 0.1ml per 1ml of culture. The resulting precipitate was homogenized by brief mixing. *d*-tartrate fermenting *S*. Paratyphi B strains were indicated by the formation of a small precipitate after 1-2 hours of lead acetate addition. The presence of a bulky precipitate after 1-2 hours of lead acetate addition indicated that the strain was a *d*-tartrate non-fermenting *S*. Paratyphi B strain. The *S*. Paratyphi B dT+ and dT– reference strains were included in the lead acetate test for comparison.

Protocol 2 was performed as described by Malorny *et al*.[9]. Protocol 2 differed from protocol 1 in the inoculation size and incubation conditions. A loopful of bacteria grown aerobically for 18-24 hours on blood agar was inoculated into the base medium. The cultures were incubated in 5% CO_2 at 37°C instead of air.

DNA extraction

The genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions.

Multiplex polymerase chain reaction (m-PCR)

Multiplex PCR was carried out using 2 sets of primers. Primer ST11 (5'-AGC CAA CCA TTG CTA AAT TGG CGCA- 3') and ST15 (5' –GGT AGA AAT TCC CAG CGG GTA CTG- 3') were genus specific for *Salmonella*.[10] The resulting PCR product size for these primers was 429 bp.

The second primer pair 167 (5' –CAC ATT ATT CGC TCA ATG GAG- 3') and 166 (5' –GTA AGG GTA ATG GGT TCC- 3') were used to detect the presence or absence of ATG start codon for the gene encoding the putative cation transporter in the *S*. Paratyphi B, resulting in a 290-bp PCR fragment if the strain was *d*-tartrate-positive.[9] Twenty-five μl PCR cocktails were prepared consisting of sterile nuclease-free water, 1X Colorless GoTaq® Flexi Buffer (Promega), 3mM $MgCl_2$ solution, 200 μM of each dNTP, 0.4 μM of primers 166 and 167, 0.1 μM of primers ST11 and ST15, 2.5U GoTaq® DNA polymerase

TABLE 1: Number of strains tested positive or negative using lead acetate test protocol 1 and 2 after 3 and 6 days of incubation

Incubation time	*d*-tartrate fermentation			
	Protocol 1		Protocol 2	
	dT^+	dT^-	dT^+	dT^-
Day 3	73	32	99	6
Day 6	102	3	105	0

(Promega), and 1 μl of the DNA template. The m-PCR assay was conducted under the following conditions: 95 °C for 1 minute followed by three cycles of 95 °C (30s), 61 °C (30s) and 72 °C (30s) and 27 cycles of 95 °C (30s), 50 °C (30s) and 72 °C (30s). A final extension was carried out at 72 °C for 4 minutes. The 290-bp PCR product was purified using QIAquick PCR purification kit (Qiagen) and sent for DNA sequencing.

RESULTS

Most of the strains were isolated from stool (67.6%) while the rest were from rectal swabs (19%), blood (7.6%), tissues (3.8%) and urine (1.9%). There were limited clinical data but most of the strains were isolated from patients with diarrhea, abdominal pain, nausea and vomiting.

Delay in d-tartrate fermentation was more pronounced in some strains using protocol 1 compared to protocol 2. Only 73 out of 105 strains were dT^+ after 3 days of incubation using protocol 1. The number increased to 102 strains at the end of 6 days of incubation. In contrast, with protocol 2, more strains (99) were dT^+ at 3 days of incubation and all 105 strains were dT^+ by 6 days of incubation (Table 1). Therefore more strains were shown to be dT^+ if protocol 2 was used compared to protocol 1.

Multiplex PCR results showed that all 105 *S.* Paratyphi B isolates tested in this study were S. Paratyphi B dT^+ strains.

The sequence data of dT^+strain (TA111) was compared to the sequence database of the *S. enterica* serotype Paratyphi B dT^+ (NCTC 5706) to check for sequence homology in the intergenic region of STM 3357 and STM 3356. The percentage of identity between isolate TA111 with NCTC 5706 was 100 %. This result supported the finding of *d*-tartrate test and confirmed that clinical sample TA111 was *S.* Paratyphi B dT^+. This result is in agreement with the findings reported by Malorny et al (2003)[9].

The concordance of the two *d*-tartrate test protocols relative to that of multiplex PCR is presented in Table 2. The concordance of the d-tartrate test compared to multiplex PCR increased to 94.3% and 100% after 3 and 6 days of incubation when protocol 2 was followed compared to a lower concordance with protocol 1.

DISCUSSION

The lead acetate test is a conventional biochemical test which is used to discriminate dT^+ and dT^- *Salmonella* strains.[7,8,9] The differences in the inoculation and incubation conditions of the two lead acetate test protocols

TABLE 2: The discrimination of *S. enterica* serotype Paratyphi B dT^+ by two *d*-tartrate test protocols and multiplex PCR method

No. of strains determined to be dT^+ by:				
Multiplex PCR	Lead acetate test			
	Protocol 1		Protocol 2	
	3 days	6 days	3 days	6 days
105	73	102	99	105
Concordance of lead acetate test relative to that of m-PCR	69.5%	97.1%	94.3%	100%

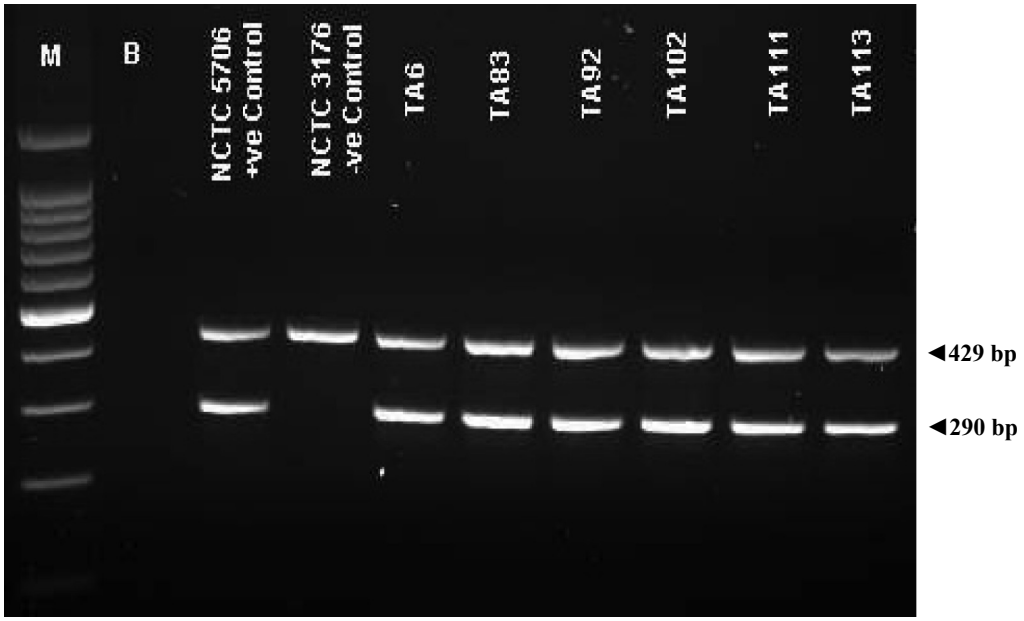

FIG. 1: Multiplex PCR for discrimination of *S*. Paratyphi B dT$^+$ and dT$^-$ strains. Lane 1: 100 bp marker; Lane 2: PCR mix without DNA; Lane 3: positive control strain (*S*. Paratyphi B dT$^{+)}$; Lane 4: negative control strain (*S*. Paratyphi B dT$^-$); Lane 5-10: clinical isolates TA6, TA83, TA92, TA102, TA111 and TA113 as *S*. Paratyphi B dT$^+$ strains.

showed that some strains may exhibit delay in *d*-tartrate fermentation and this could lead to a false negative result. The delay was observed more remarkably in protocol 1 compared to protocol 2 where more strains showed negative results after 3 days but improved detection was observed after 6 days of incubation. Barker reported that 8 *Salmonella* strains were *d*-tartrate negative after 24 hours of incubation but became *d*-tartrate positive after 48 hours of incubation.[1] Additionally, Challinor and Rhodes stated that if the test is negative after 24 hours incubation, a further 48 hours growth should be examined, so as not to miss a slowly developing positive reaction.[11]

The higher inoculum size as in protocol 2, led to the higher production of *d*-tartrate dehydrase enzyme and thus increased the *d*-tartrate fermentation in the test medium. It was also easier to inoculate a loopful of bacteria directly into the test medium for protocol 2 as compared to preparation of the bacterial suspension following protocol 1. *d*-tartrate dehydrase of *S*. Paratyphi B dT$^+$ strain is an oxygen-labile enzyme and it is obvious that the poorly aerated conditions and the low inoculum size of bacteria in protocol 1 are not sufficient to activate *d*-tartrate dehydrase for the fermentation process. In this study, the use of protocol 2 has enabled the identification of 100% of the strains as d-tartrate fermenters which showed that these strains were not the causative agent for paratyphoid fever. The lead acetate test however is labour intensive, time consuming and require prolonged incubation period. Delays in determining the *d*-tartrate fermentation in the lead acetate test may also hinder rapid response to an outbreak of disease and/or its epidemiologic surveillance.

The multiplex PCR technique was shown to rapidly identify *Salmonella* species and simultaneously discriminate *S. enterica* serotype Paratyphi B dT$^+$ and dT$^-$ strains. In this study, the multiplex PCR identified all 105 strains as *S*. Paratyphi B dT$^+$ strains. The multiplex PCR also prevents delays and the generation of false negative results. In this study, 3 strains would have been mistakenly identified as *S*. Paratyphi B dT$^-$ strains if protocol 1 was followed.

This study has highlighted the presence of high numbers of *d*-tartrate fermenting (dT$^+$) *S*. Paratyphi B causing gastroenteritis in Malaysia. This variant differs from the causative agent of paratyphoid fever (*S*. Paratyphi B dT$^-$ variant) which causes a more severe infection. The causative agent of paratyphoid fever causes a more severe infection and since humans are the only reservoir for S. Paratyphi B dT$^-$ bacteria, this has major public health implications because

identifying the human source is pertinent in preventing transmission and spread of the disease, especially if it involves food handlers and contamination of water supply by carriers or patients. The detection by PCR is helpful in investigations because it can provide more rapid results compared to phenotypic methods. The identification of dT^- strains indicates that the infection is caused by Salmonella Paratyphi B from a human source and early identification of human source can help in the rapid implementation of control measures. Humans are the only reservoir for *S*. Paratyphi B dT^- bacteria thus the main source of infection is the stool of infected persons which can contaminate water and food. Paratyphoid fever is uncommon in Malaysia, most probably because of good sanitation and access of clean running water. In a 7-year-study on travel-associated typhoid and paratyphoid fevers in various regions, typhoid and paratyphoid fever were not observed among travellers returning from Malaysia.[12] S. Paratyphi B dT^+ variant (formerly *S. java*) has its reservoir in animals thus food of animal origin could be the source of infection. The increasing number of dT^+ isolates received at our institution showed that it should not be underestimated as a significant cause of gastroenteritis. This serovar should be considered a threat to public health and control measures should be undertaken to prevent the spread of this emerging pathogen in the food chain.

The results of this study also verified the usefulness of the multiplex PCR in determining whether the isolates received were the more virulent causative agent of paratyphoid fever or the less virulent causative agent of gastroenteritis. This multiplex PCR method has become the method of choice for discrimination of *S. enterica* serotype Paratyphi B dT^+ and dT^- strains in our institution.

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