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Single-strand conformation polymorphism analysis of variability of the *rpoS* sequence in environmental and clinical isolates of *Salmonella typhi*

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ABSTRACT *rpoS* gene is present in a number of Gram-negative bacteria. It encodes a sigma factor, which is part of an alternative regulatory system that is known to enhance survival of bacteria in adverse conditions. Mutation screening of the downstream region of *rpoS* gene in 26 environmental and clinical strains of *S. typhi* from Santiago, Chile was performed. No polymorphism at the *rpoS* gene was detected among clinical isolates. On the other hand, environmental isolates had four unique single-stranded conformation polymorphism (SSCP) profiles, in which a dominant profile was shared with the human isolates. The close association of the clinical and environmental isolates in the *rpoS* gene may suggest a possible epidemiological link between environmental sources and human infection. In addition, SSCP data also indicates possible mutations within the *rpoS* gene at the downstream region.

ABSTRAK Gen *rpoS* adalah beberapa bacteria Gram-negetif. Ia mengkodkan factor sigma, iaitu sebahagian pengawalturan alternatif, yang meninggikan kemandiran bacteria dalam keadaan yang teruk. Penyaringan mutasi kawasan aliran bawah gen *rpoS* dilakukan terhadap 26 strain klinikal dan persekitaran *Salmonella typhi* yang diperolehi dari Santiago, Chile. Dalam isolat-isolat klinikal, didapati tiada polimorfisma pada gen *rpoS*. Isolat-isolat persekitaran memaparkan empat jenis corak apabila dilakukan teknik 'single-stranded conformation polymorphisma' (SSCP). Antara empat corak polimorfisma ini, terdapat satu corak unggul yang dikongsi sama dengan isolat klinikal. Perkitaran yang rapat dalam gen *rpoS* dikalangan isolat *Salmonella. typhi* dari sumber klinikal dan persekitaran mungkin menyarankan bahawa terdapat sesuatu hubungan epidemiologi di antara sumber persekitaran dan infeksi manusia. Tambahan pula, data SSCP juga menunjukkan mutasi yang mungkin wujud dalam kawasan aliran bawah gen *rpoS*.

(*Salmonella typhi*, *rpoS* gen, SSCP)

INTRODUCTION

In many developing countries, typhoid fever remains an important public health problem, with 16.6 million cases and 600,000 deaths annually (1). In areas where typhoid fever is endemic, water from rivers or lakes, which is used for public consumption and is sometimes contaminated by raw sewage is the main source of infection (2). It has been clearly demonstrated that the incidence of typhoid fever decreases dramatically with the provision of clean water through chlorination and filtration (2). Thus, in many developing countries where the use of raw river water remains widespread, this pathway of transmission remains an important factor in disease epidemiology. However, despite the clear importance of environmental sources of *S. typhi*, little is known about the biochemical and molecular characteristic of such strains of *S. typhi* and the mechanisms of survival employed by this pathogen. The environment has placed these bacteria under stress, which may often result in adaptation by *S. typhi* or other resident microflora in order to increase the probability of survival. One such mechanism that has been postulated involves *rpoS*, which encodes a sigma factor that is known to enhance survival upon exposure to stress (3). The objective of this work was to apply the technique of single-stranded conformation poymorphism (SSCP) to subtype environmental and clinical isolates of *S. typhi*, the causative agent of typhoid fever, and also to screen for mutation of the *rpoS* sequences among

the *S. typhi* strains. Results indicated from SSCP analysis that sequence variation does occur at the downstream region of the *rpoS* gene.

MATERIALS AND METHOD

Bacterial strains. Environmental and human isolates of *S.typhi* were used in this study. A total of 9 environmental strains obtained from sewage or water from the Mapocho River, Santiago, Chile and 17 human isolates from blood from sporadic cases of typhoid fever obtained in 1994 were analysed for polymorphism in the *rpoS* gene. The strains were isolated and identified by standard procedures (4). **DNA preparation.** Genomic DNA extraction from *S. typhi* was carried out by a modified method of Saito and Muira (5). Briefly, 5 ml of an overnight cell culture were harvested and lysed with 1% SDS. The DNA was extracted using phenol-chloroform method, followed by ethanol precipitation. The quality and quantity of genomic DNA was checked by agarose gel electrophoresis (AGE) at 80V for an hour. The genomic DNA was diluted to 100 ng for subsequent PCR amplification.

PCR amplification. Amplification of the downstream region of *rpoS* gene was carried out using an automated DNA thermocycler (Perkin Elmer 480). The oligonucleotide primer sequences used were as reported by Jordan et al. (6). Reaction mixtures of 50 µl, containing 20pmol (each) primers, 200 µM (each) deoxynucleoside triphosphates, 1.25 mM $MgCl_2$, and 5.0 µl of 10x buffer supplied with DyNAzyme™ II DNA Polymerase, and 100 ng DNA template were prepared. Following a pre-denaturing step at 95°C for 5 min., PCR was performed with 32 cycles of 95°C for 1 min., 55°C for 1 min., and 72°C for 1 min. and a final extension of 72°C for 5 min. After PCR, 10 µl of the amplified product was subjected to electrophoresis at 100 V on a 1.0% agarose gel (type II medium electrophoresis grade, Sigma, USA) in a 0.5 X TBE (45 mM Tris, 45 mM boric acid, 10 mM EDTA, pH 8.0) buffer system. Following electrophoresis, the gel was stained in ethidium bromide (1 µg/ml) and photographed under UV light.

SSCP analysis of PCR amplicon. Single-stranded nucleic acids were separated on vertical gels (16 cm x 16 cm x 1 mm) under constant temperature and voltage. Ten µl of PCR products were mixed with 5 µl of formamide loading dye. The samples were heated at 95°C for 3 minutes to generate single strands and then snapped chilled immediately on ice before loading. The samples were then loaded into the wells of pre-chilled 1 X Mutation Detection Enhancement (MDE) gel (7). Electrophoresis was performed at constant voltage of 300 V for 2-4 hours at 15°C. The gel was stained with GelStar nucleic acid stain (FMC) and was viewed under ultra-violet illumination.

RESULTS

PCR amplification of *rpoS* gene in *S.typhi*. Optimization of PCR conditions was carried out to amplify the *rpoS* gene. Different concentrations of $MgCl_2$ ranging from 0.5 to 3.0 mM were used for optimization. It was found that internal priming occurred at high Mg^{2+} concentration. No polymorphism was detected among the clinical and environmental strains when PCR products were electrophoresed using agarose gel electrophoresis. All the strains showed an identical profile consisting of four bands (1 specific and 3 non-specific) (Figure 1). Following optimization at 1.25 mM $MgCl_2$ and 20.0 pmol of each primer, a single PCR product of 364 bp was obtained on a 1.5 % agarose gel for all the isolates tested. This was the expected amplification product generated by the specific primers that annealed to the downstream region of the *rpo*S gene. The size of the amplicon (364 bp) was verified by relative comparison with a 100 bp DNA size marker (Figure 2A&B). The amplicon was then subjected to SSCP analysis. In the event of repeated failure to obtain a specific 364 bp amplicon (as occurred for some isolates), the desired DNA band was eluted and purified for further analysis.

SSCP analysis. The PCR product was then analyzed by SSCP to detect any nucleotide differences in the *rpoS*. PCR products for the downstream region (1334-1698 bp) of the 26 strains of *S. typhi* were analyzed. Possible sequence variations were determined by comparison of their relative mobility with each other. Four unique SSCP profiles were obtained (Figure 3). All clinical isolates and five out of nine environmental isolates shared one profile (profile 1), two environmental isolates (1678 and 1684) had the second profile (profile 2) while another two environmental isolates had two unique profiles (profile 3 and 4), respectively. This showed that the environmental isolates were

either identical to the human isolates or had acquired certain alterations in their nucleotide sequence within the *rpoS* gene, which consequently produced polymorphisms. The sequence variation will be confirmed by direct DNA sequencing of the unique bands.

DISCUSSION

Several methods, both conventional and molecular based, were widely applied to characterize *Salmonella* spp., within and among serovars. Most of the techniques are restricted to the chromosomal level. Polymorphisms are interpreted in a broader spectrum and relies upon properties conferred by the chromosome as a whole. In this study, molecular subtyping of *S. typhi* was based on polymorphisms at the nucleotide level. Any nucleotide changes will result in sequence variations and therefore, lead to alterations in acid amino sequences and possible differential protein expression. Here, SSCP analysis was chosen as means of detecting single-point mutation due to its simplicity, sensitivity and versatility. The power of this PCR-based method lies in its capability to locate silent and nonsense mutation rapidly prior to sequencing which is more time-consuming (6,7).

Target sequence studied in this present work was the *rpoS* gene (σ^s, σ^{38} or KatF) *of S. typhi. rpoS* plays an important role in determining the survival of bacteria and its morphological changes in extreme environments, including nutrition deprivation, long-term starvation, oxidative stress, thermal stress, irradiative acidic surroundings, osmotic shock and others (8,9). Genetic variation of *rpoS* between clinical and environmental isolates detected by SSCP would suggest an explanation towards the significance of this gene in the tolerance and adaptation of *S. typhi* in the presence of natural selection. Agarose gel electrophoresis of PCR amplicons could not discriminate between clinical isolates and environmental isolates because the amplification patterns were identical. SSCP analysis was used to further evaluate the possible variation of *rpoS* sequence. There were altogether four genetic profiles from the 17 clinical and 9 environmental strains. A dominant SSCP profile (profile 1) (85%) was observed in all the 17 clinical isolates and 5 (5/9) environmental strains. Apparently, only the single stranded DNA of the environmental isolates carried nucleotide changes in their sequences compared to the clinical strains.

The indistinguishable SSCP profiles shown by some of the environmental strains strongly suggested that genetic diversity at *rpoS* existed among these strains as compared to the clinical isolates obtained during the same time period in the same locality. The close association of the clinical and environmental isolates in the *rpoS* gene may suggest a possible epidemiological link, at the nucleotide level, between water supplies contaminated by sewage and human infection with *S. typhi*. On the other hand, three unique SSCP profiles had been detected from four environmental isolates. Each pattern reflected changes in the nucleotide sequence. The change could be inherent in the *S. typhi* strains in human hosts, or could have only been induced during the period of transmission from fecal to sewage source. The three SSCP profiles among the environmental isolates suggested that different types or levels or pressure had induced the bacteria strains to change accordingly. Whether these sewage-isolated strains could survive or function in human hosts would require more detailed study.

In conclusion, PCR-SSCP is a useful method in the evaluation of genetic polymorphism of *rpoS* gene among human and environmental isolates of Chilean *S. typhi*. The presence of a common profile among the environmental and clinical strains of *S. typhi* may suggests a possible transmission route employed by the bacteria from fecal to sewage source and vice versa. Mutations in the *rpoS* nucleotide sequences further suggests a possible link to selective advantage of individual *S. typhi* and its resistance against multiple stresses needs to be further investigated. The study reiterates the usefulness of PCR-SSCP technique for rapid mutation detection.

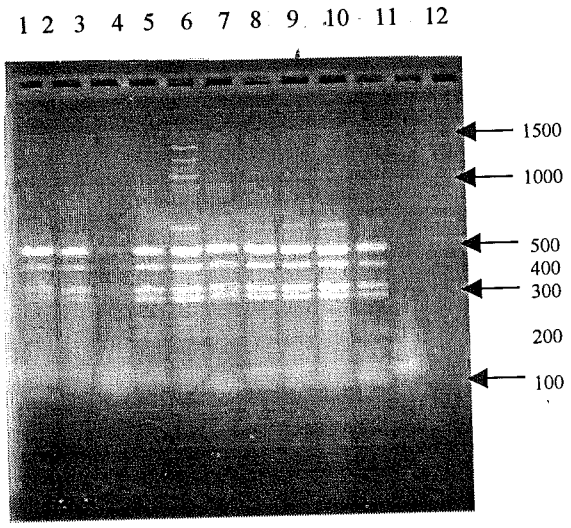

Figure 1. Amplification products of representative environmental and clinical *S.typhi* isolates (before optimisation) (1.5% agarose gel, 0.5x TBE). Lane1: 3101, lane2: 3105, lane3:3106, lane 4:3107, lane 5:3119, lane 6:3120, lane7:1680, lane8:1686, lane9:1688, lane10:1690, lane 11: negative control, lane 12: 100 base pair DNA ladder marker. Numbers at the side indicate size of marker bands in base pairs (bp).

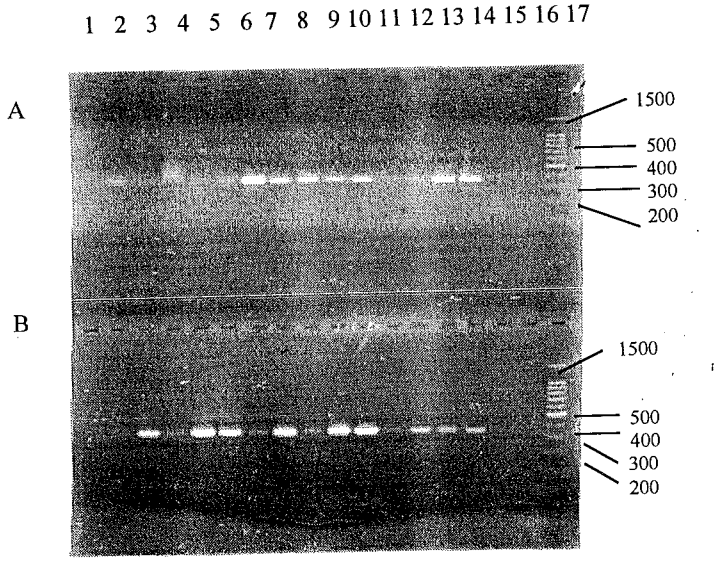

Figure 2. Agarose gel of the 364 bp of the *rpo*S gene in representative environmental and clinical isolates *S. typhi* (1.5% agarose gel, 0.5x TBE). 2A (upper panel): lane1: 1677, lane 2: 1678, lane3:1680, lane 4: 1682, lane 5:1684, lane 6: 1686, lane 7: 1688, lane 8:1690, lane9:3101, lane10: 3102, lane11:3103, lane12:3104, lane13: 3105, lane 14:3107, lane 15-16:negative control, lane 17:100 base pair DNA ladder marker. 2B (lower panel): lane 1:1681, lane2: 3106, lane3: 3109, lane4: 3110, lane 5; 3111, lane 6: 3112, lane7:3113, lane 8: 3114, lane 9:3115, lane10:3116, lane 11:3117, lane12:3118, lane13: 3119, lane 14: 3120,lanes 15-16: negative, lane 17: 100 base pairs DNA standard marker.

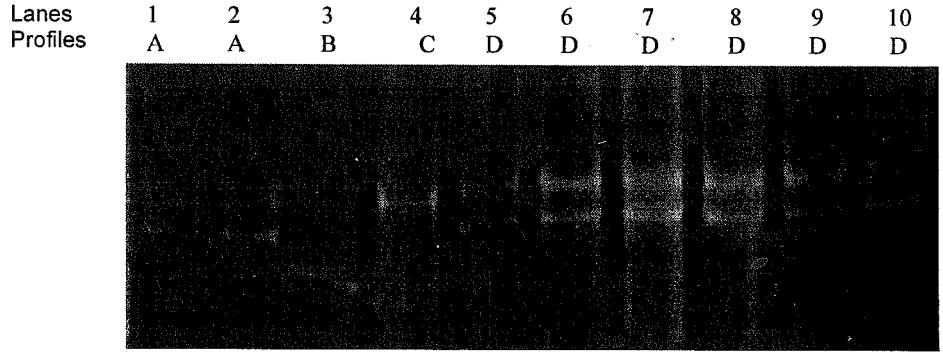

Figure 3. SSCP profile of the amplified downstream region of *rpo*S gene in *S. typhi* on 1x MDE gel. Lanes 1-10: strains 1678, 1684, 1685, 1688, 3103, 3104, 3107, 3109, 3124, 3132 A = profile 1, B = profile 2, C = profile 3 and D = profile 4

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