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Molecular Analysis of Isolates of *Salmonella typhi* Obtained from Patients with Fatal and Nonfatal Typhoid Fever

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Molecular characterization of a total of 52 human isolates of *Salmonella typhi* from Papua New Guinea was performed by using pulsed-field gel electrophoresis (PFGE) after digestion of chromosomal DNA with three restriction endonucleases, *Xba*I (5′-TCTAGA-3′), *Avr*II (5′-CCTAGG-3′), and *Spe*I (5′-ACTAGT-3′). Of the 52 isolates tested, 11 were obtained from patients with fatal typhoid fever and 41 were obtained from patients with nonfatal disease. The 52 isolates showed limited genetic diversity as evidenced by only three different PFGE patterns detected following digestion with *Xba*I (patterns X1 to X3; *F* [coefficient of similarity] = 0.86 to 1.0), four patterns detected following digestion with *Avr*II (patterns A1 to A4; *F* = 0.78 to 1.0), and two patterns detected following digestion with *Spe*I (patterns S1 and S2; *F* = 0.97 to 1.0). Of the 52 isolates, 37 were phage typed, and all belonged to phage type D2. All 11 isolates obtained from patients with fatal typhoid fever were identical (*F* = 1.0) and possessed the PFGE pattern combination X1S1A1, whereas the 41 isolates from patients with nonfatal typhoid fever had various PFGE pattern combinations, the most common being X2S1A2 (39%), X1S1A1 (24%), and X1S1A2 (15%). Thus, all the isolates from patients with the fatal disease had the X1 and A1 patterns, whereas the majority of the isolates from patients with nonfatal typhoid fever possessed the X2 and A2 patterns. The data suggest that there is an association among strains of *S. typhi* between genotype, as assessed by PFGE patterns, and the capability to cause fatal illness. Analysis of blood and fecal isolates of *S. typhi* from the same patient also indicated that some genetic changes occur in vivo during the course of infection.

In many parts of the developing world, typhoid fever continues to present an important public health challenge, with 16.6 million cases and 600,000 deaths reported annually (13). With regards to its clinical manifestations, it has been reported previously that these can differ markedly in different parts of the world where typhoid fever is endemic. In South America and parts of Southeast Asia (e.g., Malaysia and Thailand), typhoid fever manifests as a relatively mild illness with low fatality rates and minimal neurologic complications. In contrast, in sub-Saharan Africa and Indonesia, severe, and often, fatal disease is frequently seen with higher mortality and is often accompanied by neurologic involvement such as delirium and coma. The reasons for these differences in disease severity are not known but may be related to differences in health care facilities, host immune responses, genetic factors, and also perhaps to differences in strains of *Salmonella typhi* circulating in areas of endemicity. Few studies have been performed to determine if variations in clinical presentation are related to strain differences. Previous studies have, in fact, shown little correlation between strain characteristics and disease severity (6, 8). This is perhaps not surprising, given the small number of strains studied and the well-known observation that *S. typhi* represents a single clone that has shown minimal intraspecies divergence in different parts of the world (14, 16). More recently, studies with newly developed molecular techniques such as pulsed-field gel electrophoresis (PFGE) and ribotyping suggest that the extent of genetic diversity among strains of *S. typhi* is actually greater than previously thought (1, 12, 20, 21) and, more importantly, that the genome of *S. typhi* has undergone major rearrangements (9). It would thus seem appropriate to apply these more discriminative methods in reinvestigating a possible correlation between disease severity and strain differences. In this report, we used PFGE to compare the molecular characteristics of *S. typhi* isolated from Papua New Guinea from patients with fatal and nonfatal typhoid fever and from different body sites in the same patient.

Human isolates of *S. typhi* recovered from either blood or feces (rectal swab) were used in this study. The organisms were isolated, maintained, and identified by standard methods (3). A total of 52 isolates were obtained from 33 patients from among sporadic cases of typhoid fever in the Eastern Highlands Province of Papua New Guinea between February 1992 and April 1994. Of the 33 patients, 19 were males and 14 were females; they ranged in age from 1 to 50 years. Vi phage typing of the isolates was performed according to standard procedures by the Salmonella Reference Centre at the Institute for Medical Research, Kuala Lumpur, Malaysia. Repeated subculturing of isolates was avoided, and stocks of the primary isolates were maintained at −70°C. All *S. typhi* isolates tested were susceptible to ampicillin, amikacin, chloramphenicol, kanamycin, carbenicillin, cephalothin, cefamandole, gentamicin, neomycin, tetracycline, trimethoprim, streptomycin, spectinomycin, sulfonamides, nitrofurans, and nalidixic acid as determined by standard disk diffusion procedures to measure resistance according to National Committee for Clinical Laboratory Standards guidelines. None of the isolates studied contained any plasmids, as determined by a standard alkaline lysis

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procedure and by PFGE of undigested DNA (see below). DNA for PFGE analysis was prepared by a modification of the method of Smith et al. (17) as described previously (20). Selection of restriction endonucleases for digestion of chromosomal DNA was based on the percent G+C content of 50 to 54% previously reported for *Salmonella* spp. The following restriction endonucleases were used: *Xba*I (5′-TCTAGA-3′), *Avr*II (5′-CCTAGG-3′), and *Spe*I (5′-ACTAGT-3′) (New England Biolabs, Beverly, Mass., and Stratagene Co., La Jolla, Calif.). PFGE of inserts was performed by using the contour-clamped homogeneous electric field method on a CHEF DR-II or DR-III system (Bio-Rad Laboratories, Richmond, Calif.) in gels of 1% agarose in 0.5× Tris-borate-EDTA buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) for 28 to 30 h at 200 V at a temperature of 14°C, with ramped pulse times varying according to the enzymes used (ranging from 2 to 50 s). The gels were stained with ethidium bromide and photographed with a UV transilluminator (wavelength, 302 nm; Spectroline). The DNA size standards used were a lambda ladder consisting of concatemers starting at 48.5 kbp and increasing to approximately 1,000 kbp (Bio-Rad Laboratories) and a midrange II PFG Marker (size range, 24 to 291 kbp) (New England Biolabs). Size determination of DNA fragments was carried out by measuring migration distances of individual bands and referring these to a standard curve which plotted migration distances against log molecular weight of the DNA size standards. For each strain, with various restriction endonucleases, between 11 and 24 bands were normally seen, and all visible bands were included in determining PFGE patterns. The existence of doublets (comigrating bands) was also taken into account by referring to band intensity. Several electrophoretic runs with ramped pulse times varying between 2 and 50 s were performed to obtain optimal times in which individual bands were clearly separated. DNA fragment patterns were visually assessed, and distinct patterns were assigned an arbitrary restriction endonuclease analysis (REA) pattern. Isolates were considered to be genetically similar or identical if there was complete concordance of DNA fragment profiles, and they were considered to be genetically different if there was a difference of one or more DNA bands. REA patterns generated by PFGE for various isolates were compared, and the similarity of fragment length patterns between two strains is scored by the Dice coefficient, also known as a coefficient of similarity (5). This coefficient, F, expresses the proportion of shared DNA fragments in two isolates and was calculated with the following formula: $F = 2n_{xy}/(n_x + n_y)$ where n_x is the total number of DNA fragments from isolate X, n_y is the total number of DNA fragments from isolate Y, and n_{xy} is the number of fragments identical in the two isolates. An F value of 1.0 indicates that the two isolates have identical REA patterns, and an F value of 0 indicates complete dissimilarity.

PFGE of the 52 human isolates of *S. typhi* from Papua New Guinea was performed after digestion of chromosomal DNA with three restriction endonucleases, *Xba*I (5′-TCTAGA-3′), *Avr*II (5′-CCTAGG-3′), and *Spe*I (5′-ACTAGT-3′). Stable and reproducible PFGE patterns consisting of between 15 and 22 bands were observed (Fig. 1), and the various patterns were assigned an arbitrary pattern type (Fig. 2). The 52 isolates analyzed showed limited genetic diversity, as evidenced by only three different PFGE patterns detected following digestion with *Xba*I (patterns X1 to X3; $F = 0.86$ to 1.0), four patterns detected following digestion with *Avr*II (patterns A1 to A4; $F = 0.78$ to 1.0), and two patterns detected following digestion with *Spe*I (patterns S1 and S2; $F = 0.97$ to 1.0) (Table 1) (Fig. 2). Among these 52 isolates, eight different PFGE pattern combinations were detected; X1S1A1 was the most common

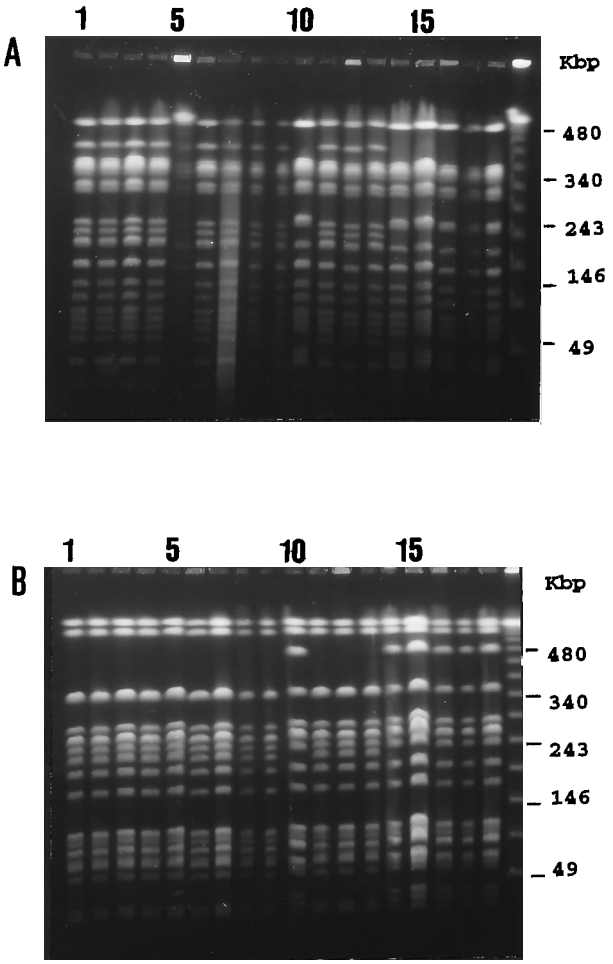

FIG. 1. Agarose gel showing the *Xba*I (A) and *Avr*II (B) digestion patterns of *S. typhi* isolates from patients with fatal and nonfatal typhoid fever. Lanes 1 to 9 and 12, isolates from patients with fatal disease; lanes 10 and 11 and 13 to 18, isolates from patients with nonfatal disease; and lane 19, PFGE marker (lambda concatemers).

(40%) followed by X2S1A2 (31%) and X1S1A2 (11%) (Table 1). For the 41 isolates obtained from patients with nonfatal typhoid fever, eight different PFGE pattern combinations were detected; X2S1A2 was the most common pattern combination, accounting for 39% of the isolates, followed by X1S1A1 (24%) and X1S1A2 (15%) (Table 1). All of the 11 isolates obtained from patients with fatal typhoid fever were identical ($F = 1.0$) and had the X1S1A1 pattern combination (Table 1). The most notable difference between the isolates from patients with fatal disease and those with nonfatal disease is in the *Xba*I and *Avr*II PFGE patterns, with all the isolates from patients with fatal disease having the X1 and A1 patterns and the majority of the isolates from patients with nonfatal disease having the X2 and A2 patterns (Table 1). The X1 (e.g., Fig. 1A, lanes 1 to 9) and X2 (e.g., Fig. 1A, lanes 10 and 14 to 18) patterns differ by 5 bands ranging in size from approximately 49 to 400 kbp (Fig. 2). For *Avr*II digestion, the A1 PFGE pattern (e.g., Fig. 1B, lanes 1 to 9) differs from the A2 pattern (Fig. 1B, lanes 10 and 14 to 18) by 3 bands ranging in size from approximately 40 to 480 kbp (Fig. 2). Isolates obtained from blood and feces from the same patient were also compared. It was found that in 15 patients, the PFGE pattern combinations of the blood and fecal isolates were identical (X2S1A3, X1S1A1, X2S1A2,

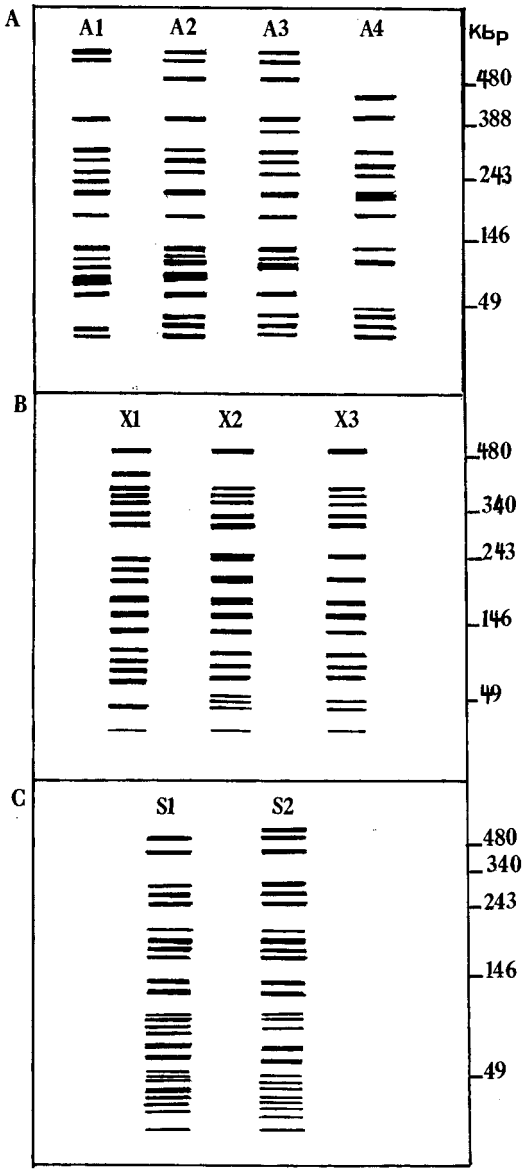

FIG. 2. Schematic representation of the various PFGE patterns produced following digestion of *S. typhi* chromosomal DNA with three restriction endonucleases, *Avr*II (A), *Xba*I (B), and *Spe*I (C).

X1S1A2, and X2S1A1), but a different pattern combination, involving the three restriction endonucleases, was noted in 4 patients (blood-feces patterns, X3S1A2-X3S1A4, X1S1A2-X2S1A2, X2S1A2-X2S2A2, and X1S1A1-X2S1A1). Thirty-seven of the 52 isolates were phage typed, and all belonged to phage type D2.

Previous studies designed to differentiate *S. typhi* strains by the technique of multilocus enzyme electrophoresis concluded that *S. typhi* represents a single clone that has shown minimal intraspecies divergence in its spread to different parts of the world (14, 16). More recently, the development of PFGE, in which large DNA fragments are separated after digestion of chromosomal DNA with infrequently cutting restriction endonucleases, has provided a new approach to the molecular typing and differentiation of individual strains of a variety of bacterial pathogens (2, 10, 19). Using this approach, studies of *S. typhi* isolates from different parts of Southeast Asia suggested that the extent of genetic diversity among strains of *S. typhi* is actually greater than previously thought (20, 21). The considerable genetic diversity among *S. typhi* strains has also been shown by ribotyping (1, 12). An important finding of the present study is the fairly limited amount of genetic diversity observed among the *S. typhi* isolates from Papua New Guinea compared with the diversity noted previously among Southeast Asian (20, 21) and South American *S. typhi* isolates (20a). The *F* values observed with the Papua New Guinea isolates (F = 0.78 to 1.0) are considerably higher than those observed with the Southeast Asian (F = 0.53 to 1.0) (21) or South American (F = 0.61 to 1.0) isolates. The limited diversity observed is most probably related to the fact that, although typhoid fever is now highly endemic in the highlands region, the disease was introduced into Papua New Guinea only relatively recently. Typhoid has become a problem only in the last 10 years and was rarely seen prior to 1985. The area of the study has a population of approximately 20,000, is urban-periurban-rural in nature, and has a highway running through it. The population is generally very mobile, and it is thus unlikely that isolation and limited mobility are the causes of the limited diversity seen among the isolates. Presumably, the *S. typhi* strains currently circulating in Papua New Guinea were derived from a single introduced strain. These strains have now spread, with the development of some limited genetic variation, but there has been little selective pressure to develop genetic variation.

In light of the observation that there appear to be various degrees of disease severity in various regions of endemicity, it seems appropriate and logical to extend the use of these discriminatory methods to the analysis of strains of *S. typhi* obtained from patients with different degrees of disease severity. Previous studies in which a limited number of strains were characterized with a variety of methods failed to find an association between strain characteristics and disease severity. One study found that strains causing mild and severe disease belonged to the same phage type (4). A study performed by Heneine et al. (8) characterized four *S. typhi* isolates from patients with severe and mild typhoid fever by using several methods, including multilocus enzyme electrophoresis, protein profiles, ribotyping, plasmid analysis, and phage typing. No association between the characteristics of these clones and disease severity was found (8). Franco et al., utilizing envelope protein profiles, restriction fragment length polymorphism of chromosomal DNA, and immune response to envelope proteins, also found no clear correlation between strain characteristics and disease severity (6). A more recent study, however, reported an association between flagellar serotypes and decreased severity of illness and invasiveness (7). In the present study, there appeared to be a correlation between molecular characteristics of *S. typhi*, as assessed by PFGE, and the ability to cause fatal or nonfatal disease. While the X2S1A2 PFGE pattern combination was found among the majority (39%) of isolates from patients with nonfatal disease, all the isolates from patients with fatal typhoid fever had the X1S1A1 pattern combination. When the total number of isolates was taken into consideration, 40% of all 52 isolates studied possessed the X1S1A1 pattern combination. In overall terms, this would mean that no more than 40% of the 11 isolates (i.e., 4 isolates) from patients with fatal disease would be expected to have this pattern combination. Instead, all 11 isolates had the X1S1A1 pattern combination. The true significance of the association between PFGE pattern and fatal disease is not known at this stage. Interpretation of the results is also hampered by insufficient information concerning the fatal cases together with the fact that many patients died at home and the

TABLE 1. *F* values and PFGE patterns of *S. typhi* isolates from Papua New Guinea

Disease severity	No. of isolates tested	*F* value with[a]:			PFGE pattern combination[b]
		*Xba*I	*Spe*I	*Avr*II	
Fatal	11	1.0 (1)	1.0 (1)	1.0 (1)	X1S1A1 (11)
Nonfatal	41	0.86–1.0 (3)	0.97–1.0 (2)	0.78–1.0 (4)	X2S1A2 (16) X1S1A1 (10) X1S1A2 (6) X2S1A1 (3) X2S1A3 (2) X2S2A2 (2) X3S1A2 (1) X3S1A4 (1)
Total	52	0.86–1.0 (3)	0.97–1.0 (2)	0.78–1.0 (4)	All of the above

[a] Numbers in parentheses refer to the number of different PFGE patterns detected.
[b] For schematic diagram of PFGE patterns, see Fig. 2. Numbers in parentheses indicate the number of isolates possessing that particular pattern combination.

cause of death is not known. Obviously, an important question related to the results of the present study is whether the *S. typhi* strains isolated from these patients with fatal disease really represent a highly virulent strain with the capacity to produce fatal disease or whether the deaths were due to other contributing factors. With an overall case fatality rate for the region estimated at 3%, it is likely that the deaths observed were simply due to an inevitable result of having typhoid, rather than any unusual virulence trait associated with the infecting strains. However, the association between molecular profile by PFGE and fatal disease has been noted and observed and should be investigated further, e.g., to determine any phenotypic correlates which may alter virulence of individual strains of *S. typhi*.

The results of the present study also show, following analysis of blood and fecal isolates of *S. typhi* from the same patient (from sporadic cases), that some genetic changes occur in vivo during the course of infection. Such variation may arise as a result of selective pressure due to antibiotic therapy or host immune responses. The in vivo emergence of polymorphisms detected by PFGE has also been described for *Pseudomonas aeruginosa*, for which up to 20% genomic divergence occurred over several months (18). In a similar vein, PFGE was used to demonstrate that subclonal variation of *Yersinia enterocolitica* may also occur in vivo (11). When Southern blot hybridization with a species-specific probe was used, pathogenic strains of *Candida albicans* were also shown to be genetically unstable in vivo during recurrent vaginitis and it was shown that drug treatment can result in the selection of variants of the original infecting strain (15).

The differences observed in the present study in the molecular structure of the *S. typhi* genome between the strains that cause fatal disease and those that cause nonfatal disease is striking but, perhaps, not surprising in light of recent developments. Recent work by Liu and Sanderson (9) showed clearly that the *S. typhi* genome had undergone major rearrangements not seen in other *Salmonella* spp. This observation, taken together with previous studies indicating considerable genetic diversity in regions of endemicity (20, 21) and some in vivo genetic variation, would suggest a considerable degree of genomic plasticity in the *S. typhi* genome which may be significant in the virulence of this important human pathogen.

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