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Detection of Vi-Negative *Salmonella enterica* Serovar Typhi in the Peripheral Blood of Patients with Typhoid Fever in the Faisalabad Region of Pakistan

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The synthesis and transportation proteins of the Vi capsular polysaccharide of *Salmonella enterica* serovar Typhi (serovar Typhi) are encoded by the *viaB* operon, which resides on a 134-kb pathogenicity island known as SPI-7. In recent years, Vi-negative strains of serovar Typhi have been reported in regions where typhoid fever is endemic. However, because Vi negativity can arise during in vitro passage, the clinical significance of Vi-negative serovar Typhi is not clear. To investigate the loss of Vi expression at the genetic level, 60 stored strains of serovar Typhi from the Faisalabad region of Pakistan were analyzed by PCR for the presence of SPI-7 and two genes essential for Vi production: *tviA* and *tviB*. Nine of the sixty strains analyzed (15%) tested negative for both *tviA* and *tviB*; only two of these strains lacked SPI-7. In order to investigate whether this phenomenon occurred in vivo, blood samples from patients with the clinical symptoms of typhoid fever were also investigated. Of 48 blood samples tested, 42 tested positive by *fliC* PCR for serovar Typhi; 4 of these were negative for *tviA* and *tviB*. Three of these samples tested positive for SPI-7. These results demonstrate that *viaB*-negative, SPI-7-positive serovar Typhi is naturally occurring and can be detected by PCR in the peripheral blood of typhoid patients in this region. The method described here can be used to monitor the incidence of Vi-negative serovar Typhi in regions where the Vi vaccine is used.

Salmonella enterica subsp. *enterica* serovar Typhi is primarily but not exclusively the etiologic agent of a systemic infection in humans known as enteric (typhoid) fever. Unlike many other *Salmonella* serovars, serovar Typhi is restricted to human populations. Despite improvements in sanitation and healthcare in many developing countries, typhoid fever remains an important public health problem in areas of undeveloped and developing countries. The World Health Organization estimates that the current annual global burden of typhoid is approximately 22 million new cases, 5% of which are fatal (7, 19).

The pathogenesis of the members of the genus *Salmonella* is attributed, in part, to the acquisition of horizontally transferred DNA, including plasmids, prophage, and gene islands (3, 4, 24, 25, 31). Arguably the most important form of horizontally transferred DNA with respect to the pathogenesis of the salmonellae is the possession of large gene islands that carry genes that are transcribed in a coordinated manor and directly impinge on the pathogenic potential of the bacterium. These gene islands (termed pathogenicity islands) are missing from closely related nonpathogenic strains, are often but not always flanked by small direct repeats, and are frequently associated with tRNA genes (10, 11).

The serovar Typhi CT18 genome sequence identified five previously described *Salmonella* pathogenicity islands (SPIs) and also predicted five more gene islands that had coding sequences implicated in pathogenicity (31). One such island, known as SPI-7, is inserted in between two partially duplicated copies of the $tRNA^{pheU}$ gene located at positions 4409511 and 4543074, respectively, in the serovar Typhi CT18 genome sequence (Fig. 1) (33). SPI-7 encodes genes responsible for several pathogenic traits, including a type IV pilus, implicated in aiding attachment to eukaryotic cells (45), and the *sopE* prophage (27, 28, 41), which harbors a gene encoding an effector protein secreted via a type III secretion system within its tail fiber genes. In addition, SPI-7 carries the *viaB* operon, which encodes the genes responsible for the synthesis and transportation for the virulence (Vi) capsule (15, 42).

The *viaB* operon is only found in organisms that can produce the Vi polysaccharide and has no corresponding homologues in *Escherichia coli*. The organisms that have been documented as producing Vi are *S. enterica* serovars Typhi, Dublin, and Paratyphi C and *Citrobacter freundii* (14). Interestingly, the *Salmonella* serovars that can produce Vi also possess an SPI-7 element. The *viaB* region in serovar Typhi consists of 10 genes. *tviA*, *tviB*, *tviC*, *tviD*, and *tviE* are involved in synthesis of the capsule. Export of the capsule is controlled by the proteins encoded by *vexA*, *vexB*, *vexC*, *vexD*, and *vexE* (42). In addition, *rcsB* and *rcsC* (*viaA*) and the *ompR-envZ* two-component regulatory system regulate the production of the Vi polysaccharide (32).

Traditionally, the production of Vi by serovar Typhi is a distinguishing feature of the bacterium, and agglutination using Vi antisera is a routine procedure for the identification of serovar Typhi in research and diagnostic laboratories (19, 22). The precise biological role of the Vi polysaccharide remains

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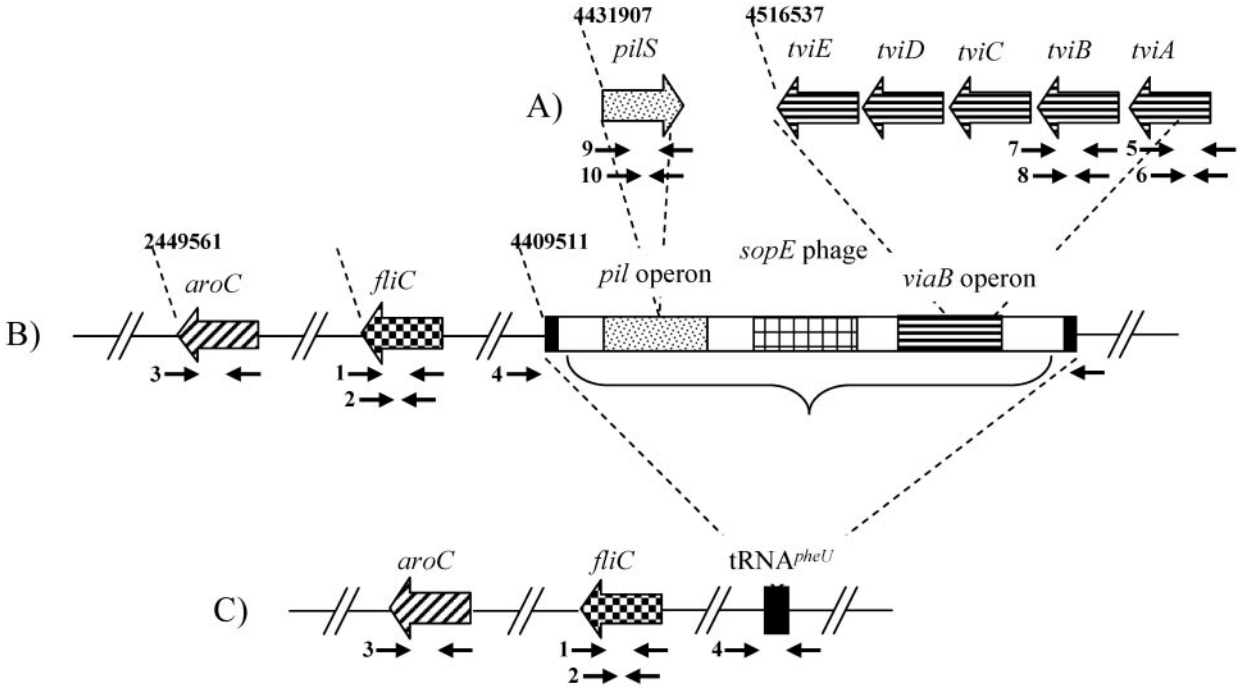

FIG. 1. Map of regions investigated in this study, including SPI-7 and the *viaB* locus. The gene names and regions are highlighted, as are the approximate location of the primer pairs (see Table 1) and the location of the regions on the chromosome (according to the serovar Typhi CT18 genome sequence). (A) Map corresponding to the *pilS* gene and a section of the *viaB* locus (*tviE* to *tviA*) within SPI-7. The schematic locations of primer pairs 9 and 10 (*pilS*), 7 and 8 (*tviB*), and 5 and 6 (*tviA*) are shown. (B) Location of the *aroC* and *fliC* genes and SPI-7 and the primer pairs specific for each region: 3 (*aroC*), 1 and 2 (*fliC*), and 4 (SPI-7 flanking). Regions of interest within SPI-7 are depicted and include the flanking tRNA sequences (black boxes), the *pil* operon (dotted box), the *sopE* phage (checked box), and the *viaB* operon (striped box). (C) Same arrangement as in panel B, but with the absence of SPI-7. Thus, this region would be predicted to generate an amplicon with primer pair 4.

unclear. It is, however, believed to prevent phagocytosis and complement-mediated killing when the bacteria are outside eukaryotic cells but inside the host (36), although there are data that suggest that the rates of internalization of encapsulated and unencapsulated serovar Typhi into macrophages are equivalent (16). These experimental data imply that Vi may be important in the survival of the bacterium inside the macrophage but not in cellular invasion of the macrophage or the intestinal wall. Volunteer studies have indicated that Vi-positive strains of serovar Typhi are more virulent in humans than Vi-negative strains, although Vi production is not essential for the infection process in humans (18).

Despite the role of the Vi antigen as a distinguishing feature of serovar Typhi, serovar Typhi isolates lacking the Vi capsular polysaccharide antigen during slide agglutination with Vi typing antisera is not uncommon. Vi-negative isolates have been reported in several countries, including India and Malaysia (1, 20, 26). In fact, in 2000, serovar Typhi isolates that were Vi negative by molecular probes were responsible for an epidemic of multidrug-resistant typhoid fever in Kolkata, India (37). It is however, possible that Vi agglutination-negative serovar Typhi reported from clinical microbiology laboratories may be Vi positive but demonstrate a downregulation of Vi or loss of *viaB* on culturing. These findings have been substantiated by the description of additional serovar Typhi isolates lacking SPI-7 (29). SPI-7 may therefore be able to excise from the chromosome and act in similar fashion to a conjugative transposon (33). However, all of these studies were performed on serovar Typhi isolates that had been cultured and in many cases stored. The loss of SPI-7 and Vi negativity could therefore arise by selection during isolation or storage. The significance of Vi-negative serovar Typhi in vivo is thus currently ambiguous. Recently, in Karachi, Pakistan, only 1 of more than 2,000 stored clinical isolates of serovar Typhi was found to be Vi negative by immunofluorescence detection of Vi and PCR detection of the SPI-7 locus (43). There was complete correlation between Vi expression and the presence of SPI-7.

We have investigated here the frequency of Vi-negative serovar Typhi among clinical isolates from typhoid patients living within the Faisalabad region of Pakistan. In order to establish the possible presence of Vi-negative serovar Typhi in this region of Pakistan, stored serovar Typhi cultures were screened by PCR for the presence of *viaB* and SPI-7. Also, to investigate the *viaB* region within serovar Typhi strains without the need for culture or storage, PCR was performed directly on total DNA extracted from the blood of patients with suspected typhoid fever (23, 35). Typhoid fever continues to be a major health problem in Pakistan, and these data provide insight into the occurrence of naturally occurring Vi-negative strains in the blood of typhoid patients and in turn may inform future vaccine development (2).

MATERIALS AND METHODS

Bacterial strains and clinical samples. This study was carried out with 60 isolates of serovar Typhi collected from hospitals in the Faisalabad region (population of approximately 10 million) of Pakistan between March 2002 and Sep-

TABLE 1. Oligonucleotides used in this study[a]

Target gene	Primer pair[b]	Annealing temp (°C)	Elongation time (min)	Predicted amplification size (bp)	Primer	Sequence (5′-3′)	Source or reference
fliC	1	53	1	495	ST1 ST2	TATGCCGCTACATATGATGAG TTAACGCAGTAAAGAGAG	Song et al. (40)
fliC nested	2	50	1	363	ST3 ST4	ACTGCTAAAACCACTACT TGGAGACTTCGGTCGCGTAG	Song et al. (40)
aroC	3	57	2	639	aroCsfor aroCsrev	GGCACCAGTATTGGCCTGCT CATATGCGCCACAATGTGTTG	Kidgell et al. (21)
tRNA^{pheU}	4	57	2	1,275	DE0032-F DE0083-R	GCTCAGTCGGTAGAGCAGGGGATT TCATCTTCAGGACGGCAGGTAGAATG	Pickard et al. (33)
tviA	5	55	2	599	V1 V2	GTTATTTCAGCATAAGGAG ACTTGTCCGTGTTTTACTC	Hashimoto et al. (13)
tviA nested	6	55	1	307	V3 V4	GTGAACCTAAATCGCTACAG CTTCCATACCACTTTCCG	Hashimoto et al. (13)
tviB	7	57	2	846	tviB-F tviB-R	CGAGTGAAACCGTTGGTACA CAATGATCGCATCGTAGTGG	Wain et al. (43)
tviB nested	8	60	2	774	tviB-in-F tviB-in-R	GAATCGGGGAGATATTGTGG TGCCATACTCTCGTCTTACC	This study
pilS	9	58	1	502	pilS-F pilS-R	GTATCAACATTAAATCCATGC CGTTACTTTCGCATCGGTGTG	This study
pilS nested	10	57	1	335	pilS-F-inner pilS-R-inner	ATCATTGGGGTGATAGCC GCAGATTGCGGAACTTTG	This study

[a] The name, predicted amplification size (according to the serovar Typhi CT18 genome sequence), annealing temperature, elongation time, sequence, and primer pair number (as demonstrated in Fig. 1) of the oligonucleotides used for PCR amplification in this study are given.
[b] See Fig. 1.

tember 2002. Strains were isolated from unvaccinated patients clinically diagnosed with typhoid fever, i.e., fever for ≤3 days with enlarged spleen, headache, malaise, abdominal discomfort, and/or agitation. Initially, the strains were cultured from blood samples and identified by conventional biochemical and serologic methods (8). After primary isolation, serovar Typhi strains were plated on MacConkey agar, subcultured in Trypticase soy broth overnight, and tested for Vi antigen (antisera; Bio-Stat); aliquots were preserved in 20% glycerol and stored at −20°C for further use. When required, an aliquot of the stored serovar Typhi isolates was revived in Trypticase soy broth for 24 h at 37°C, and total genomic DNA was extracted from the Trypticase soy broth by the conventional phenol-chloroform method (38).

This study also included the assessment of blood samples taken in February 2005 from 48 unvaccinated patients suspected of having typhoid fever. Patients were of both sexes and a broad age range; these patients had 2 ml of blood collected in tubes containing anticoagulant (20 mM potassium EDTA) The blood was stored at 4°C and prepared for PCR within 48 h of collection.

DNA extraction from blood samples. DNA from blood samples was extracted by procedure described by Haque et al. (12). Briefly, 1 ml of blood containing 20 mM potassium EDTA as anticoagulant was centrifuged at 10,000 rpm (Sorvall Legend RT) for 5 min. Plasma was separated for serology. The pellet was resuspended in 1 ml of lysis buffer (0.2% Triton X-100 in Tris-HCl [pH 8.0]). The mixture was gently aspirated several times to encourage efficient hemolysis. The tube was centrifuged at 12,000 rpm (Sorvall Legend RT) for 6 min, the supernatant was discarded, and the procedure was repeated. The pellet was washed with distilled water. The supernatant was removed, and the pellet was subsequently resuspended in 20 to 30 μl of distilled water. The tubes were sealed and then sterilized in boiling water for 20 min.

PCR conditions and primers. The oligonucleotides utilized in this study were supplied by Sigma (Dorset, United Kingdom) and are presented in Table 1. The PCR and thermal cycling conditions for DNA from stored bacterial cultures were as follows. A total of 100 μl of a PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 150 pmol of each primer, 95 nmol of each deoxynucleoside triphosphate, 1 μl of *Taq* polymerase (Fermentas), 20 μl of template, and distilled water up to 100 μl. The reaction mixture was subjected to 30 cycles of 94°C for 30 s, 50 to 60°C for 30 s (see Table 1 for primer annealing temperatures), and 72°C for 1 to 2 min (see Table 1 for primer extension times), followed by 5 min at 72°C (MasterCycler; Eppendorf, Hamburg, Germany). Samples were separated immediately by gel electrophoresis on 2% agarose gels at 100 V for 60 min and then photographed using Eagle Eye (Stratagene).

For amplification where total DNA from whole blood acted as a template, the PCR and cycling conditions were as follows. A total of 100 μl of PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 pmol of each primer, 125 nmol of each deoxynucleoside triphosphate, 1.5 μl of *Taq* polymerase (Fermentas), 20 μl of template (purified total DNA from whole blood), and distilled water (up to 100 μl). The reaction mixture was subjected to 30 cycles of 94°C for 1 min, 50 to 60°C for 30 s (see Table 1 for primer annealing temperatures), and 72°C for 1 to 2 min (see Table 1 for primer extension times), followed by 7 min at 72°C (Mastercycler). PCR products were handled as described above.

RESULTS

Detection of serovar Typhi from stored isolates by PCR. Blood samples from patients suspected of having typhoid fever are routinely cultured in order to confirm the clinical diagnosis. The identification of serovar Typhi is confirmed by standard laboratory procedures including biochemical testing and agglutination using relevant antisera (09 and Vi). In Faisalabad a significant proportion (ca. 15%) are agglutination negative for Vi, suggesting that these serovar Typhi isolates cannot produce the Vi antigen. In order to explore this phenomenon, 60 stored serovar Typhi samples, isolated between March 2002 and September 2002 in the Faisalabad region of Pakistan, were recultured and investigated.

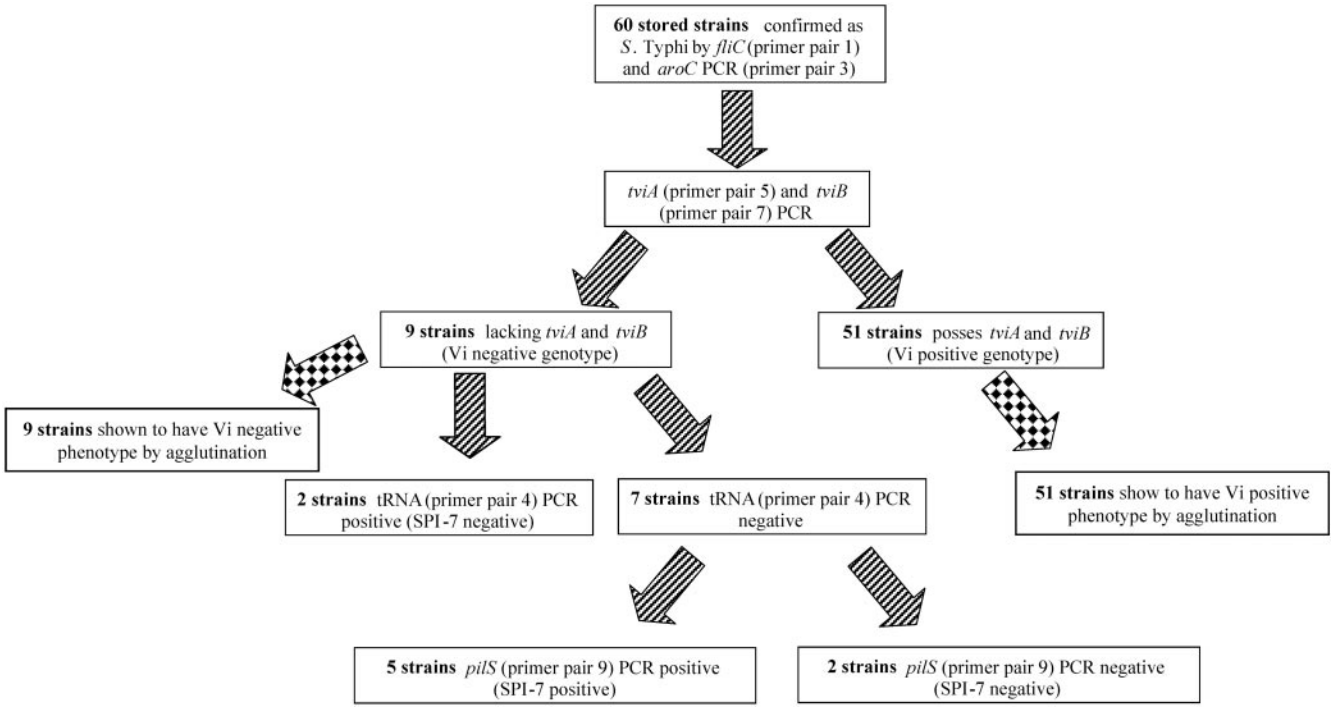

FIG. 2. Sequential investigation of the Vi status of stored serovar Typhi strains from Faisalabad, Pakistan. A flow diagram shows the results of investigating the ability of 60 stored isolates from Faisalabad, Pakistan, to express Vi. The 60 strains were investigated at all regions displayed in Fig. 1, which included *fliC* (primer pair 1), *aroC* (primer pair 3), *tviA* (primer pair 5), *tviB* (primer pair 7), tRNA ^{pheU} (primer pair 4), and *pilS* (primer pair 9).

Initially, it was essential to confirm that the cultured bacteria were serovar Typhi; this was accomplished via PCR targeted to the *aroC* and *fliC* genes. The *aroC* gene and the *fliC* gene were amplified with the primer pairs aroCsfor-aroCsrev (primer pair 3) and ST1-ST2 (primer pair 1), respectively (Table 1 and Fig. 1). The *aroC* primers are specific for *Salmonella*, have been utilized in previous studies (21, 43), and are predicted to generate an amplicon of 639 bp with *Salmonella* DNA. The *fliC* primers amplify a serovar Typhi-specific region in the flagellin gene and have been previously described as being highly sensitive for the detection of serovar Typhi within clinical specimens (9, 17, 39, 40). The *fliC* primers (primer pair 1) are predicted to produce an amplicon of 495 bp with serovar Typhi DNA (Table 1). All 60 cultured bacteria gave amplifications of the predicted size with both the *aroC* and the *fliC* primers, thus confirming that all 60 strains were serovar Typhi (Fig. 2).

PCR detection of *viaB* operon. A recent study of Vi-negative serovar Typhi in Karachi, Pakistan, demonstrated that Vi-negative serovar Typhi could be detected by a multiplex PCR method. In order to utilize the methodology described by Wain et al. (43) on clinical samples with small amounts of DNA, the PCRs were not multiplexed, and each set of primers was used individually. Detection of the *viaB* operon was performed on the 60 stored serovar Typhi isolates by PCR amplification of two genes essential for Vi production; *tviA* and *tviB* (Fig. 1). Primer pair 5 is specific to the DNA sequence within the *tviA* gene and is predicted to generate an amplicon of 599 bp if *tviA* is present. The primers specific for the *tviB* gene were tviB-F and tviB-R (primer pair 7) and were predicted to generate an amplicon of 846 bp if *tviB* is present (Table 1). The 60 strains confirmed as serovar Typhi by *aroC* and *fliC* PCR amplification were subjected to PCR with the *tviA* and *tviB* specific primers. Of the 60 strains confirmed as serovar Typhi, 9 (ca. 15%) failed to give any amplification with the *tviA* primers. Also, the same nine strains failed to give any visible amplification for *tviB* (Fig. 2). Since *tviA* and *tviB* are essential for Vi production, these nine strains were thought to be incapable of Vi expression. Agglutination with Vi antisera was in accordance with these results. In conclusion, nine strains were found to lack the *tviA* and *tviB* genes and were therefore genotypically Vi negative. These same nine strains were found to be Vi negative by agglutination and were therefore both genotypically and phenotypically Vi negative. The remaining 51 strains were found to possess both *tviA* and *tviB* genes and were found to be phenotypically Vi positive by Vi agglutination.

Assessing the presence or absence of SPI-7. The *viaB* locus is located on SPI-7, which is inserted between two partially duplicated tRNA^{pheU} genes (as discussed above); however, SPI-7 may be unstable and can potentially excise from the chromosome (5, 29). This mechanism was hypothesized to be responsible for the inability of the nine samples above to generate a visible amplification for *tviA* and *tviB*. These nine samples were subjected to PCR to confirm the presence or the absence of SPI-7. Primers DE0032-F and DE0083-R (primer pair 4) have been previously utilized to demonstrate the lack of an insertion at the tRNA^{pheU} locus (33, 43). These primers are predicted to generate a PCR amplicon of 1,275 bp (Table 1) if the island is absent. SPI-7 is 134 kb in length; therefore, the presence of the island is outside the constraints of the PCR. In addition, the presence of the island was confirmed by PCR to

a separate loci within SPI-7, a gene within the type IV pilus cluster that encodes the major pilin subunit, *pilS* (44, 45). The *pilS* primers (pilS-F and pilS-R [primer pair 9]) were predicted to generate an amplicon of 502 bp if the gene (and therefore the island) was present.

Two of the nine confirmed serovar Typhi strains gave amplification across the tRNA gene that indicated the absence of SPI-7 (Fig. 1 and 2). In addition, these two isolates failed to give any amplification with the *pilS* primers, thus confirming the absence of SPI-7. The remaining seven strains failed to give any amplification with primers DE0032-F and DE0083-R, suggesting the presence of SPI-7. This was subsequently confirmed by the amplification of the *pilS* gene in five strains; this result suggested that SPI-7 was present but that there had been a deletion within SPI-7 that included the *viaB* locus. Two strains, however, failed to give any amplification with the tRNA primers or the *pilS* primers, suggesting the absence of SPI-7 with rearrangement around the $tRNA^{pheU}$, which resulted in the failure of the DE0032-F and DE0083-R to anneal, or the loss of two regions (*pil* and *tviB*) within SPI-7. Of these nine samples, two were confirmed to be lacking SPI-7, five appeared to have part of SPI-7 but not *tviB*, and two did not produce amplification across the tRNA gene or at two sites within SPI-7.

These results indicate that serovar Typhi which is unable to produce Vi can be detected in stored cultures from this region in Pakistan. It is also apparent that there appear to be three distinct mechanisms. One such mechanism has been extensively described and involves the excision and consequential loss of SPI-7. The second mechanism suggests that SPI-7 has again been lost, but a rearrangement at the tRNA locus prevents the production of a visible PCR amplicon across the tRNA junction. The final mechanism appears to be novel, whereby the presence of SPI-7 is suggested, but the *viaB* operon cannot be detected, which implies a deletion that removes part or all of the *viaB* operon. Further characterization of cultured isolates was not considered useful, and so DNA was extracted directly from patient's blood. The detection of serovar Typhi isolates that are lacking the genes responsible for Vi production can be detected on cultured strains via a simple PCR assay. However, it is not clear whether the loss of the genes responsible for Vi production is due to the lack of a positive selective pressure by culturing and/or storage or whether these strains are circulating in populations where typhoid is endemic. In order to investigate this, a PCR assay was performed directly on DNA extractions from blood samples of patients suspected of contracting typhoid. This procedure was selected in order to remove the influence of culturing and storage.

Sensitivity of PCR (*fliC* and *tviA* genes) for application on blood samples. The detection of serovar Typhi circulating in peripheral blood has been demonstrated by several studies to be a highly sensitive and reproducible method of diagnosis (9, 17, 40). Indeed, a nested PCR detecting the *fliC* (H-d1) has been implied to be the "gold standard" in typhoid detection, giving a higher sensitivity than that of blood culture and the Widal test (35). Due to the nature of the PCR, this method is specific for serovar Typhi and will not generate an amplification with other invasive *Salmonella* strains, such as serovar Paratyphi A or Sendai (17).

In order to develop a methodology that could be utilized for the detection of Vi-negative serovar Typhi in blood samples, nested PCR for the *fliC* gene was compared with nested PCR of the *tviA* gene as described in Hashimoto et al. (13). DNA was extracted from a culture of a reference serovar Typhi strain by phenol-chloroform method, and serial dilutions were made in distilled water. Each dilution was used as a template for PCR. Both conventional PCR and nested PCR were performed on the serial dilutions for the *fliC* gene by using primer pair 1 (ST1-ST2 [primary]) and primer pair 2 (ST3-ST4 [nested]) (Table 1) and also for the *tviA* gene using primer pair 5 (V1-V2 [primary]) and primer pair 6 (V3-V4 [nested]) (Table 1 and Fig. 1). The predicted sizes of the amplicons for serovar Typhi were 495 and 363 bp (*fliC* [conventional/nested]) and 599 and 307 bp (*tviA* [conventional/nested]), respectively. The calculations were made according to the recommendations of Song et al. (40).

The results of the experiments assessing the sensitivity of the *fliC* primers and the *tviA* nested primer pairs are shown in Fig. 3. The amplicons from the conventional PCR and the nested PCR from both the *fliC* primers the *tviA* primers were of the expected size. The results presented in Fig. 3 demonstrate that conventional PCR for *fliC* can detect serovar Typhi DNA in 10^5 CFU/ml, whereas the nested *fliC* PCR is significantly more sensitive and can detect serovar Typhi DNA in a concentration as low as 5 CFU/ml. The nested *tviA* PCR was equal in sensitivity to the nested *fliC* PCR. These results are in agreement with the results of previous studies on the sensitivity of nested PCR for the detection of serovar Typhi in clinical samples (13, 35, 40).

Detection of Vi-negative serovar Typhi in blood samples from typhoid patients. The nested PCR for the *tviA* and *tviB* genes was used to test whether serovar Typhi lacking genes essential for Vi could be detected in the peripheral blood of typhoid patients. Blood samples were collected from 48 patients with clinically diagnosed typhoid fever admitted to various hospitals in the region during February 2005. Blood samples were taken from these patients, and total DNA was extracted.

Of the 48 blood samples, 42 tested positive for serovar Typhi DNA by means of the nested *fliC* (primer pairs 1 and 2, Table 1) PCR. The 42 samples that were confirmed of containing serovar Typhi were then investigated further in order to detect the presence or absence of the *viaB* locus. Nested PCR for the *tviA* and *tviB* genes was performed. Four samples (9%) tested negative by nested PCR for both *tviA* and *tviB* (Fig. 4.). In addition, three of these samples tested positive for the *pilS* gene by nested PCR (Fig. 4), thus indicating the presence of SPI-7 but the absence of the *viaB* locus. The remaining sample did not produce any amplification for *tviA*, *tviB*, or *pilS*. None of these four samples produced any visible amplification across the tRNA junction with primer pair 4. These PCR assays were repeated and performed simultaneously with the control *fliC* primers to confirm the result; the repeated assay was in complete accordance with the initial experiment.

DISCUSSION

The characterization of serovar Typhi for expression of Vi capsular polysaccharide is necessary to define the role of Vi in

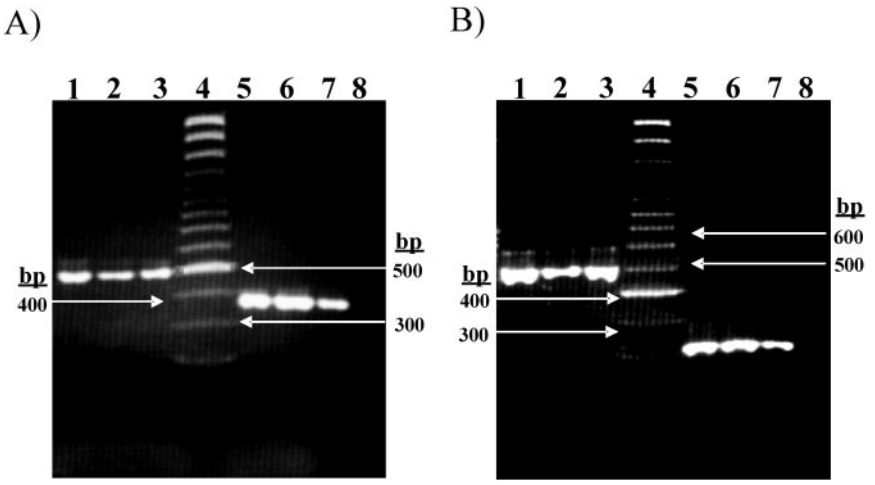

FIG. 3. Investigating the sensitivity of the *fliC* and *tviA* nested PCR. (A) PCR amplification of the *fliC* gene with primer pairs 1 and 2 (Table 1 and Fig. 1). Lanes 1, 2, and 3 show PCR amplification of the *fliC* gene with primer pair 1 with 10^7, 10^6, and 10^5 CFU/ml, respectively. The size of the amplicon was 495 bp. Lanes 5, 6, and 7 show PCR amplification with primer pair 2 with product from primer pair 1 as the template (nested) with 10^2, 10, and 5 CFU/ml, respectively. The size of the amplicon was 363 bp. Lane 4 is Generuler SM 0323 (Fermentas), and lane 8 is a negative control (no template). (B) PCR amplification of the *tviA* gene with primer pairs 5 and 6 (Table 1 and Fig. 1). Lanes 1, 2, and 3 show PCR amplification of the *tviA* gene with primer pair 5 with 10^7, 10^6, and 10^5 CFU/ml, respectively. The size of amplicon was 599 bp. Lanes 5, 6, and 7 show PCR amplification of *tviA* with primer pair 6 with products from primer pair 5 as a template (nested) with 10^2, 10, and 5 CFU/ml, respectively. The size of amplicon was 307 bp. Lane 4 is Generuler SM 0323 (Fermentas), and lane 8 is a negative control (no template).

the pathogenesis and epidemiology of typhoid fever. Serovar Typhi lacking Vi capsular polysaccharide antigen has been known and reported worldwide for several decades. However, most of the reports of the Vi-negative isolates are based on the serological tests with Vi typing antisera and, since Vi expression is particularly sensitive to the osmolarity of the selected growth media, this would be a phenotypic rather than a genotypic event (32). Recently, molecular evidence of the loss of Vi antigen has suggested that Vi-negative strains can be derived by the excision of SPI-7 (30) or by a spontaneous base change in the *viaB* operon (5, 29, 43). It has been postulated that after long-term storage or repeated culturing on laboratory media Vi-negative strains would predominate. This spontaneous loss of Vi expression upon culture and/or storage implies a strong positive selective pressure that serves to maintain the Vi capsule in the natural niche of serovar Typhi. This theory raises doubt as to the existence of Vi-negative strains of serovar Typhi in the field. Typhoid is a common disease in Pakistan and, using stored cultures and blood samples from typhoid patients, a two-pronged strategy was adopted to investigate

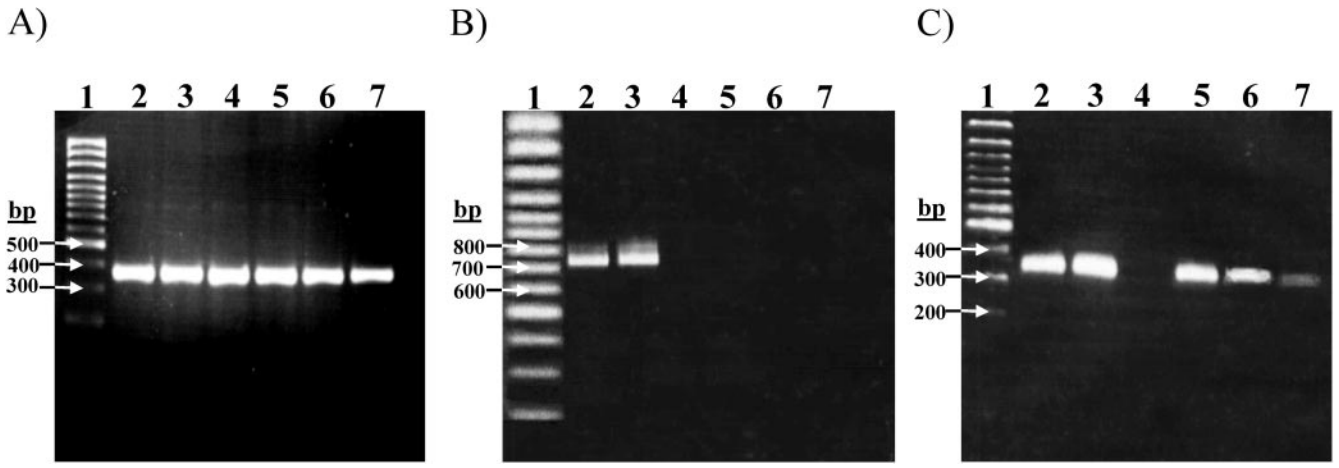

FIG. 4. Detection of Vi-negative serovar Typhi in the peripheral blood of typhoid patients by nested PCR. (A) The detection of serovar Typhi in the blood of patients suspected of having typhoid fever by nested *fliC* PCR on total DNA extracted from the blood of patients. Primer pair 1 (Table 1 and Fig. 1) was used for the primary reaction, and primer pair 2 was used for the secondary reaction; the product from primary reaction was used as a template in the secondary reaction. Lanes 2 to 7 show nested *fliC* PCR from six typhoid patients blood samples with amplification size 363 bp. Lane 1 is Generuler SM 0323 (Fermentas). (B) Nested *tviB* PCR with primer pairs 7 (primary) and 8 (nested); the lanes and samples correspond to those in panel A. (C) Nested *pilS* PCR with primer pairs 9 (primary) and 10 (nested). Lanes and samples correspond to those in panel A.

whether serovar Typhi that is unable to express Vi could be detected in this region without culture and storage.

Initially, we sought to determine whether Vi-negative serovar Typhi could be detected in stored serovar Typhi strains from the Faisalabad region of Pakistan. The stored strains (more than 1 year old) were recultured, and the identity was confirmed by PCR targeting of the *aroC* and *fliC* genes (Fig. 1). Of 60 strains examined, 9 were negative for both the *tviA* and *tviB* genes (Fig. 2) and were therefore designated as genotypically Vi negative. *tviA* and *tviB* were both selected to ensure that Vi negativity was not due to spontaneous mutation; if both genes were missing, we assumed that the genes had been lost. Our results showed the presence of Vi-negative serovar Typhi isolates that had lost a significant amount, if not all, of the *viaB* operon.

Further investigations were carried out to assess the nature of the deletion and to detect the presence or absence of SPI-7. Using the methodology of Wain et al. (43), primer pair 4 (Fig. 1) confirmed the presence or absence of SPI-7. If missing, an amplicon would be produced across the tRNA junction (1,275 bp) and, if present, the distance between the primers is such that an amplicon would not be generated. The presence of SPI-7 was also confirmed by using an additional second primer pair from another region within SPI-7. Two serovar Typhi strains were found to have lost SPI-7. In addition, two stored strains were PCR negative for *tviA*, *tviB*, and *pilS* but did not produce any PCR product across the tRNA^{pheU} junction. These strains may have also undergone rearrangement that has deleted SPI-7 and a portion of the adjacent region. One of the SPI-7-negative serovar Typhi strains investigated by Nair et al. (29) appeared to have also lost an adjacent section at 5′ end of the island which encompassed the *phoN* gene. Moreover, detailed microarray investigations have predicted that some *Salmonella* isolates that are without SPI-7 are also missing genes adjacent to the 5′ side of tRNA^{pheU} (6, 34). The results shown here can be explained by these data; SPI-7 is absent but, due to a further deletion event including *phoN*, a PCR amplification across the tRNA junction cannot be generated.

The remaining five strains appeared to retain the island but have somehow deleted all or part of the *viaB* locus. These results are in contrast to a recent study whereby only 1 in 2,000 stored strains from the Aga Kahn University in Karachi, Pakistan, tested negative for *tviB*, having lost the whole of SPI-7 (43). A rearrangement or deletion within or adjacent to the *viaB* locus may be a novel mechanism of Vi negativity, although the exact method remains unclear and requires further investigation. It is known that insertion sequence elements within the *viaB* locus can diminish and also completely prevent Vi production; this has been shown to be the case in *Citrobacter freundii* (30) and recently in the serovar Typhi sequence strain CT18 (Stephen Baker, unpublished data). However, this mechanism could not be confirmed in isolates in the present study.

PCR amplification on the total DNA from blood samples revealed that 4 samples of the 42 that were confirmed to contain serovar Typhi did not contain the *tviA* or *tviB* genes. Three of these samples tested positive for the *pilS* gene, demonstrating the presence of SPI-7. The data presented here suggest that serovar Typhi that is incapable of Vi production (genotypically Vi negative) can be detected in the peripheral blood of typhoid patients by nested PCR in this region of Pakistan. In addition, it would appear that the mechanism responsible for this genotype in these cases is based upon the deletion of the *viaB* operon and not excision of the whole of SPI-7. Furthermore, this implies that the strains found to be lacking the *viaA* and *viaB* genes after culture and storage may have been missing these genes prior to isolation, and the loss of these genes may be independent of isolation and culture. It is logical to think that under the correct conditions, environmental loss of one operon (*viaB*) of 15 kb is much more likely than the loss of the whole island (SPI-7) of 134 kb. Our results appear to support this assumption; moreover, serovar Typhi strains circulating within this geographic region of Pakistan may be more prevalent due to the loss of Vi expression by this mechanism. However, the exact nature and size of the deletion could not be established and is currently under investigation.

The data presented here may pose further questions on the role of Vi in serovar Typhi infection. It has been established previously that Vi is not essential for the development of typhoid fever (18). This appears to be the case for other human-restricted invasive *Salmonella* serovars, such as serovars Paratyphi A and Sendai, which both induce a disease clinically indistinguishable from typhoid independent of Vi production. Indeed, is now known that in this region of Pakistan serovar Typhi with the inability to produce Vi can be detected without culturing; therefore, these serovar Typhi strains may be circulating in this typhoid-endemic region. It is currently unknown whether these circulating Vi-negative serovar Typhi strains are more or less virulent than their Vi-positive counterparts. The trigger behind the development and the spread of these serovar Typhi Vi-negative organisms also remains unclear. These findings suggest the molecular surveillance of Vi production by serovar Typhi in regions where Vi-based vaccines are used would be sensible.

Our findings show that Vi-negative serovar Typhi strains are not only artifacts of storage but can exist naturally. However, although deletion of SPI-7 was shown to play a role in some cases, the majority of the samples tested positive for SPI-7, and a localized deletion within or around the *viaB* locus appears to be more common. It is also noteworthy that typhoid vaccination in this region of Pakistan is not routine, and none of the patients involved in the present study was vaccinated against typhoid. We do not know the transmission potential of these Vi-negative strains, but the implications of these data require further investigation, in particular, the accurate estimation of circulating Vi-negative serovar Typhi strains in Vi-vaccinated, recently vaccinated, and unvaccinated populations. The methodology presented here will be useful in this effort.

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