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CLONAL RECONQUEST OF ANTIBIOTIC-SUSCEPTIBLE *SALMONELLA ENTERICA* SEROTYPE TYPHI IN SON LA PROVINCE, VIETNAM

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Abstract. In the last three decades, high rates of resistance to common first-line antimicrobial agents have been reported in *Salmonella enterica* serotype Typhi (Typhi), the causative organism of typhoid fever (TF), in many regions of the world, especially in South East Asia. Analysis of Typhi strains isolated from outbreaks and sporadic cases of TF in Son La province, northwest Vietnam, in 2002 revealed that 94.5% (85/90) of the isolates were fully susceptible to amoxicillin, chloramphenicol, cotrimoxazole, tetracycline, and nalidixic acid. There was a clear decline in the occurrence of multi-drug resistant (MDR) Typhi isolates collected in this province in 2002 (4.4%) compared with the period 1995–1999 in the same province (30.8–100%). By using molecular (IS*200* profiling, *Pst*I-ribotyping, *Xba*I-pulsed-field gel electrophoresis, and haplotyping) and phage-typing methods, we showed that the Typhi isolates from Son La province in 2002 were genetically related; however, they were unrelated to the previous MDR clones established in Vietnam.

INTRODUCTION

Typhoid fever (TF) remains a major health problem in the world, with an estimate of > 20 million cases resulting in > 200,000 deaths during 2000, mostly in developing countries.[1] Treatment with appropriate antibiotics is essential for recovery. However, treatment has become progressively more problematic with the gradual emergence of antimicrobial resistance.[2] In the last three decades, high rates of resistance to common first-line antimicrobial agents have been reported in *Salmonella enterica* serotype Typhi (hereafter referred to as Typhi) in many regions of the world.[2] Multiple resistance to ampicillin, chloramphenicol, cotrimoxazole, and tetracycline (ACSxtTe resistance type) is encoded by large conjugative plasmids mostly belonging to the incompatibility complex group *Inc*HI.[3–6] The Indian subcontinent and Southeast Asian countries are particularly affected by multidrug resistant (MDR) Typhi strains.[2,7–9] In Vietnam, the spread of MDR Typhi (ACSxtTe R-type) was first reported in 1992–1993 in the southern part of the country.[10] In 1994, > 80% of Typhi isolates were MDR in southern Vietnam, but only 5% and 10% of MDRST strains were isolated in the center and the north, respectively.[8] During the period 1995–2002, a study found that > 90% of sporadic and epidemic Typhi strains from the center and the north were MDR.[8] The economic reforms in Vietnam in the early 1990s had resulted in a boom in private pharmacies and all first line antibiotics for TF could be bought as over-the-counter medicines without prescriptions, leading to misuse and abuse of these drugs.[11] Because of the widespread occurrence of MDR Typhi strains, quinolones and fluoroquinolones, in particular, were used in the first-line treatment of adults in several countries, including Vietnam. However, the emergence of MDR Typhi isolates with an additional chromosomally encoded resistance to nalidixic acid (MDR-Nal^R) and with reduced susceptibility to ciprofloxacin has been increasingly reported since the beginning of the 1990s on the Indian subcontinent and afterwards in different Asian countries.[12–17] Several reports indicated that MDR-Nal^R Typhi strains were associated with slower clinical responses to fluoroquinolones or treatment failures.[12,18,19] In Vietnam, MDR-Nal^R Typhi isolates were first reported in 1993 and increased dramatically in 1997 in the south of the country.[12,20] As outbreaks of TF are reported every year in various parts of Vietnam, the emergence of MDR-Nal^R Typhi isolates is of great concern because TF caused by such isolates would require treatment with expensive, third-generation cephalosporins that are unaffordable for most people in Vietnam. Furthermore, the average cost of admission to Vietnamese hospitals (including bed fees, health care professionals, and cost of treatment) for a patient infected with MDR-Nal^R Typhi has been estimated to be USD 50 compared with USD 22 for a patient infected with a susceptible strain.[21]

In northern Vietnam, MDR Typhi strains were reported in most provinces: Lao Cai, Lai Chau, Thanh Hoa, and Son La, since the mid-1990s (H. Tran, unpublished results). From July to December 2002, a hospital-based study aiming at identifying risk factors associated with TF in Son La province was conducted by some of us (H.H.T., B.M.N., G.B., and P.J.G.).[22] During the study period, three probable outbreaks were detected in three geographically distinct districts: Quynh Nhai district (23 cases, attack rate: 1.6%), in Phu Yen district (32 cases, attack rate: 2.9%), and in Thuan Chau district (28 cases, attack rate: 2.9%). Three risk factors were statistically associated with TF: no education (odds ratio [OR] = 2.0, 95% confidence interval [CI] 1.0–3.7), contact with typhoid case (OR = 3.3, 95% CI 1.7–6.2), and drinking untreated water from streams or wells (OR = 3.9, 95% CI 2.0–7.5).[22] As only five cases had a history of travel to Son La, the provincial town, the sources of infection were attributed to carriers from each community. We present here a microbiological com-

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parative study (antibiotyping, molecular, and phage typing) of *S. enterica* serotype Typhi isolates collected during the previous study to explore how this could help to understand the mode of acquisition of TF in Son La province in 2002.

MATERIALS AND METHODS

Study place. The research was conducted in Son La province, in northwest Vietnam, 320 km from the capital Hanoi. This province, 80% covered by mountains, is difficult to access. Son La town is the main town of this province, subdivided into nine districts. The standard of living of the population is very low, and income per capita is among the poorest compared with other provinces in Vietnam (USD 130/person/year).[23] TF is of great concern in Son La province with outbreaks reported in several districts from 1998 to 2001.

Patients and sampling. The patients in this study were recruited for a confirmed TF in provincial and district hospitals of Son La province between 1 July and 30 December 2002, as reported previously.[22] One blood and one stool sample were obtained for each patient. Diagnosis of TF was made by isolation of Typhi from blood and/or from stool associated with clinical symptoms compatible with recent TF infection, i.e. fever over 38°C for more than 3 days with no other evident diagnosis to explain this fever.

Origin and identification of Typhi isolates. Blood samples (5 mL for patients older than 5 years old, and at least 2 mL for children under 5 years old) were inoculated into Brain Heart Infusion broth (Difco, Detroit, MI) and incubated at 35–37°C for 10 days. Vials were checked for growth twice daily on the first 2 days, once on day 3 and day 4, and once on day 10. Positive vials were subcultured on blood, MacConkey, and *Salmonella–Shigella* agar plates (SS agar, Difco). A stool sample was collected at the same time as the blood sample. Stool (3 g) was inoculated into selenite broth enrichment medium (Difco), incubated at 35–37°C for 18–24 hours and then subcultured on MacConkey and SS agar. Identification of Typhi was performed in Son La Health Center Microbiology Department using biochemical tests and agglutination with O, H, and Vi antisera (Difco). Suspected Typhi strains were sent for confirmation to the National Reference Laboratory of Enteric Pathogens, National Institute of Hygiene and Epidemiology (NIHE), Hanoi, Vietnam. Molecular and phage-typing studies were carried out at the French National Reference Center for *Salmonella* (NRC-Salm), Institut Pasteur, Paris, France. Typhi strains 162/95, 230/95, 14/96, 119/96, 339/98, 358/98, and CM2664 collected in Vietnam from 1995 to 2002 and used as comparison strains (CS) were from the NRC-Salm collection. Typhi reference strain Ty-2 was from the WHO Collaborating Center for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed on Typhi isolates from Son-La, 2002 using the Paper Disc Method (PDM-Biodisk, Stockholm, Sweden) at the National Reference Laboratory for Enteropathogenic Bacteria, Norwegian Institute of Public Health, Oslo, Norway, using Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards) guidelines.[24,25] The following disks were used: amoxicillin (10 μg), amoxicillin + clavulanic acid (20 μg + 10 μg), streptomycin (30 μg), tetracycline (30 μg), ceftriaxone (30 μg), azithromycin (15 μg), chloramphenicol (30 μg), sulfamethoxazole (23,8 μg), trimethoprim (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg), and ciprofloxacin (10 μg). *Escherichia coli* ATCC 25922 was used as a control.

To assess temporal changes that may have occurred in the resistance profile of Typhi in Son La province, we also present the results of antimicrobial susceptibility testing of 116 Typhi strains collected during outbreaks or routine surveillance in this province between 1995 and 1999. Isolates were sent to NIHE for confirmation and antibiotic susceptibility testing using the Paper Disc Method (Bio-Rad) on Mueller-Hinton agar according to CLSI guidelines.[24,25]

Phage typing. Vi-phage typing of the 90 Typhi isolates from Son La province, 2002 and of the eight CS followed a standardized methodology as described previously.[8] Phage suspensions were kindly provided by the Health Protection Agency (Colindale, United Kingdom).

IS*200* profiling. IS*200* profiling using *Pst*I (Roche, Mannheim, Germany) for the cleavage of the genomic DNA was performed on 33 selected Typhi isolates from Son La province, 2002, and on eight CS, as described previously.[26]

Ribotyping. The membranes used for IS*200* profiling were reprobed with digoxigenin (DIG)-labeled OligoMix5 probe as described previously.[26] Fifteen Typhi isolates, which exhibited different representative ribotypes, were subjected to the RiboPrinter microbial characterization system (Qualicon, Wilmington, DE), a fully automated and standardized ribotyping method for creating a database. Ribotype numbering was generated by this system. Image normalization and construction of similarity matrices were carried out using BioNumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Ribotype profiles were compared with the RiboPrinter database of the NRC-Salm (1997–2004, 339 *Pst*I-ribotypes of Typhi).

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis (PFGE) of *Xba*I (Roche)-digested genomic DNA was carried out on a subset of 29 Typhi from Son La, 2002, and on eight CS, as described previously.[27] The running conditions and the molecular size marker (*Xba*I-digested DNA from *S. enterica* serotype Braenderup H9812) were the same as described in the standardized PulseNet protocol.[28] Image normalization and construction of similarity matrices were carried out using BioNumerics 4.0. Bands were assigned manually, and clustering was performed using the unweighted pair-group method with arithmetic averages (UPGMA) based on the Dice similarity index, utilizing an optimization parameter of 0.5% and a 1% band-position tolerance. Each profile that differed by one or more bands was assigned a type.

Haplotyping by denaturing high-performance liquid chromatography (DHPLC) analysis. Fifty-two polymorphic coding gene fragments (Table 1) were amplified from 37 strains, including a subset of 31 Typhi from Son La and 6 CS, as described previously.[29] PCR products were amplified over 25 cycles in 25 μL volumes, containing 15 ng of DNA from each of 4 test strains plus a reference strain (CT18), polymerase (1.25 units, Optimase, Transgenomic, Omaha, NE), as well as specific primers (320 nM, Table 1) and dNTPs (0.2 mM). Duplex obtained after the reannealing of denatured PCR fragments were analyzed by DHPLC with a DNA-SepR Cartridge (WaveR Nucleic Acid Fragment Analysis System, Transgenomic) at the temperatures indicated in Table 1. Ac-

TABLE 1
Polymorphic gene fragments tested by denaturing HPLC analysis on Typhi strains isolated from Son La province, Vietnam, 1995–2002

Gene	Fragment length (bp)	DHPLC temp (°C)	5′–3′ Forward/reverse primers
STY0006	483	60.7	CAGTTTCCGTTGTCGCAG/TCGTATCCCCCTCACCCT
STY0135	497	60.8	TACGTGACGTCCGATGTAGG/GGATAGACATGCTCGCTGC
STY0175	428	61.4	CACATCTCTCCGGGCATCTA/CCTGCAAAGATGCCTTCC
STY0214	482	63.2	TGGGACGTCATGAATGGC/CGCCTGAGTCACATTCAGAG
STY0321	440	64.0	GTTACTGCACCGCACACAGA/CAGGGAAGTGGTCTTCGTTG
STY0336	462	60.0	CCAGGGACTTATCGAGTGGA/GACACGCCACGCACCTATAT
STY0573	429	62.9	GATAACCCGTTGGTGGGG/ACCGTGACGATGGACCAC
STY0831	436	62.9	CCCGGCAACTATGAGCTG/GCCCTATGGTCTGAATCAGG
STY0961	434	61.4	CTGTATGGCACCGGTCAAC/GGAACCCAGACTTCGAGGT
STY0962	500	60.5	GACGCAAGAAGAGTGGATGC/CGATATGAACTTCGCCGC
STY1097	467	62.1	TACCCGTGAACACGTTCG/GGAACGTACGGGCAGTAATC
STY1121	435	59.6	CGCATTTGATCCTCGACC/ACTCACGCATAACGGGCA
STY1286	415	62.9	GAACTCCTGGCAGCTTGC/CACCGACTCTCACACCCAA
STY1327	411	62.4	CTCCAGAATTGGCTGTAGGG/GAGCTGCTGACCCGACTA
STY1556	432	61.9	GGTTTTCATCCCCGGAAC/GGGCGTAACTTCGTCACCTT
STY1693	430	60.7	CAGCTCAGGGTCCCCATA/AACTGCACCGTGTGCCTT
STY1720	411	59.3	CCTGCGGCCATATAAGCTAC/TCAGGAGGCAAGCAATGC
STY1808	435	61.2	GCATGGTCCTGACATTCCC/CGAGTATGGTTCGGCAGG
STY1919	436	63.4	ACAGTTCGGCCTACGAGGT/GCCACCTGAGGAATAGCG
STY1947	454	62.7	GACCGCCCACAGGTTTTC/GCCGGAACCTCTGATTGA
STY1948	418	61.0	CGACAACCTCGGGTTTGAC/CAACAAGGGCTGGGACTC
STY2093	416	61.3	GTTAACTCACCTGTCTGCCC/CCTCACGGCGGTACTAAGGT
STY2211	462	61.7	CTCGTAACCCTCCACAGTGC/AGACGGCAGACGGTCTGTT
STY2281	651	65	GTCGGTCTGTATATTCCCGG/GGTAATCGCATCCACCAAATC
STY2389	500	60.7	CGAGCCGAGTTTACACTGC/CGGGAGCAATTACGTGTG
STY2441	476	62.9	CGTATGTTCGCTGGACTCG/CAGACTGGGAACGCTTTG
STY2513	408	64.1	CCTGTCATGGCGAAGACAC/TTCACAGACCAGGCTACGGT
STY2575	439	61.5	ACAAGAGGAGAAGCTGCTCG/CAGCTCATCCGATACGCA
STY2621	458	64.1	GCGGCACATAGGTCGTCA/TGTGCTGGGTACGGGAAT
STY2629	435	55.6	GGAGAACTACTCCGTGGAGG/CGGGACTGGTCCAATGAA
STY2711	407	62.1	CCCGGAGATTGGCTATACG/CTTTTACGCTCTGGGCTTCC
STY2785	474	63.4	AGTACATGCAGGCCTGCC/GGGCATCGACTTAGGCACT
STY2834	479	61.3	GTTTCATCCCACTGCCCT/CGGACCAGCCGTACACTAAG
STY2877	434	62.3	AATGGCTAACCTGTGCGG/GCGTCAGGTTCTCTCGTAGG
STY2948	423	63.2	AGACGTCCGCCGTTAGATC/GCTCCACCGATTTGGGTA
STY2971	468	63.2	ATATTCGGCGTGCTCAGC/GTTCTACGTCCACCGTGGTA
STY3033	409	62.3	ATCGGCAGTTACGTTCCG/CATAGCTCTTACTTGCCGCG
STY3082	413	62.9	CGGAGTGCTGAGTCGGTT/CGGGGAAGTGTTCGTTACTG
STY3196	410	61.0	CCAGACCCCAGCTCTTCTC/GGACATGTACCTGCGTATCG
STY3444	413	61.0	ACGTCCCTTACCACAACTGC/GAAAGGCGTGTAAGCCCAC
STY3499	444	62.1	CAGGTTGAGCTAACGCCC/CGGGATAACGTACTCCGG
STY3507	448	60.0	CTGTTCGTAGGTCAGGGAGA/AGAGTCAAGGCAGCGACTG
STY3614	458	61.3	GATGGACTATCACGTTCCGG/GAGCCTCGCAATGTATAGCC
STY3622	567	63.8	GTGGCCTGGAGTTTTCCACT/GACCAATAGCCGACAGCGTAG
STY3876	418	62.1	GCGAGCGATTAGCCACTATC/CACGCGTAACAGACGAGTCT
STY3940	404	60.1	CGTGTATATGCACTCCGAGC/CGTTAGAGCGTAGACGCTTG
STY4417	431	61.8	CGAGTTCTGGGACTGGGTC/CGATAAAGTCACACGGCACC
STY4499	436	63.2	CGCGACCTACGATAATGG/AGCACCCACTATTACGACGC
STY4545	467	62.1	GTGACCGAACCCCAGTTCTA/TCGAGACGTAACGCTGAGGT
STY4562	499	58.5	CAACAGGCTCCGGAGTTGT/GTAACCGCTGGCTACTCCCT
STY4793	444	61.1	TGGATGCCACTCCTGACAC/GGCATGACCAACCCACAC
STY4851	404	58.3	AACATACCCCCAGCTCTCG/CATCCTAAGTGGCGTGACAG

cording to the DHPLC profiles, representative PCR products showing evidence of mutations were purified and sequenced from both strands by Agowa (Berlin, Germany).

RESULTS

Origin and antimicrobial susceptibility of Typhi isolates. A total of 90 Typhi isolates (49 from blood culture only, 8 from both blood and stool culture, and 33 from stool culture only) were recovered from 90 patients with confirmed TF among the 617 patients who were admitted with a suspected TF in Son La province hospitals during the time of the study.[22] None of the patients infected with Typhi reported any travel outside the province during the incubation period. The isolates were recovered from patients living in four different areas: Thuan Chau district ($N = 28$), Phu Yen district ($N = 32$), Quynh Nhai district ($N = 23$), and Son La town ($N = 7$). Isolates from Thuan Chau district were recovered from patients living in two villages: Lai Le Phong Lai ($N = 9$) and Lai Cang Phong Lai ($N = 19$) during an outbreak that occurred during weeks 40 and 41 (September–October). Isolates from Phu Yen district were recovered in patients from 11 communities of Yen Ha village during an outbreak that occurred in week 46 in November ($N = 31$) or afterward in December

($N = 1$). Isolates from Quynh Nhai district were recovered from patients living in the village of Pac Ma during an outbreak that occurred in weeks 31–34 (July–August, $N = 19$) or afterward in weeks 38 and 39 (September, $N = 4$). Isolates from Son La town were sporadically recovered from patients living in three villages—Nam Hua La ($N = 2$), Ne Nua ($N = 3$), and Hia Hua La ($N = 2$)—during weeks 36–40 (September–October).

Antimicrobial susceptibility testing revealed that 94.5% (85/90) of the Typhi isolates collected in 2002 were fully susceptible to amoxicillin, chloramphenicol, trimethoprim, tetracycline, and nalidixic acid (Table 2). Four isolates (4.4%) displayed single resistance to amoxicillin. One isolate displayed single resistance to sulfamethoxazole (1.1%). When compared with the data of 116 Typhi strains collected during routine surveillance in Son La province from 1995 to 1999, the results clearly indicate a decline in the occurrence of MDR and MDR-Nal^R Typhi isolates collected in this province in recent years (Table 2).

Molecular and phage typing of Typhi isolates. Vi-phage typing was done for the 90 Typhi isolates from Son La province, 2002. All but five (94.4%) were of phage type A. Four isolates were degraded Vi-strain (DVS) and one was Vi-negative (Table 2).

For the molecular typing study, 33 Typhi isolates from Son La province were selected: 9 from Phu Yen ($N = 32$), 10 from Thuan Chau ($N = 28$), 7 from Quynh Nhai ($N = 23$), and 7 from Son La town ($N = 7$). We used four molecular methods to study the genotypic relationship among these isolates: *Pst*I-IS*200* typing, *Pst*I-ribotyping, *Xba*I-PFGE (only 29 isolates were typed by PFGE), and the newly described haplotyping by DHPLC.

To compare the Typhi genotypes circulating in Son La province in 2002 to other genotypes previously observed in northern provinces or currently observed in southern Vietnam, we have also tested seven CS recovered from 1995 to 2002 in different provinces of Vietnam and displaying various antimicrobial-resistance phenotypes (Table 3).

Only two *Pst*I-IS*200* profiles were observed in our study: profile IS1 was present in the 33 selected Typhi isolates from Son La province, whereas profile IS2 was observed in the 8 CS (including reference strain Ty-2) (Table 3, Figure 1A).

Fifteen *Pst*I-ribotypes were found: 10 (S007, 177, 340, 343-349) in Son La province isolates and 5 (03a, 26a, 187, 236 and 73/34/8) in CS (Table 3, Figure 1B). Ribotype 344 was the most frequently observed ribotype in Son La province isolates (19/33, 57.6%), whereas it was not found in CS.

TABLE 2
Antibiotic susceptibility of Typhi strains isolated from Son La province, Vietnam, 1995–2002

Antibiotic	% Resistance					
	1995 ($n = 15$)	1996 ($n = 27$)	1997 ($n = 28$)	1998 ($n = 33$)	1999 ($n = 13$)	2002 ($n = 90$)
Amoxicillin	100	63.0	92.9	72.7	30.8	4.4
Ceftriaxone	0	0	0	0	0	0
Tetracycline	100	66.7	92.9	63.6	30.8	0
Cotrimoxazole	93.3	63.0	92.9	72.7	30.8	0
Chloramphenicol	0	66.7	92.9	72.7	30.8	0
Nalidixic acid	0	0	3.6	27.3	7.7	0
Norfloxacin	0	0	0	0	0	0
Ciprofloxacin	ND	ND	ND	ND	ND	0

ND, not determined.

By using *Xba*I-PFGE, 14 distinct profiles were found: 9 (X1–X9) in Son La province isolates and 5 (X10–X14) in CS (Table 3, Figure 2A). Profile X1 was the most frequently observed PFGE profile in PFGE-typed Son La province isolates (19/29, 65.5%), whereas it was not found in CS. Profile X2 differed from X1 by an additional low-molecular-weight band of ≈ 70 kb, possibly corresponding to a plasmid. Profiles X3–X9 differed from X1 by one to four bands > 100 kb. Clustering analysis performed by UPGMA revealed that Typhi isolates from Son La province clustered together (85% similarity) (Figure 2B). A significant genetic diversity was observed between the Son La province isolates and the CS.

Combination of the three classic molecular typing methods results indicated that IS1-344-X1 was the most frequently encountered combined profile in Son La province isolates (12/29, 41.4%). This combined profile was observed in outbreak or sporadic isolates collected from Son La town and from all the three districts of the study (Table 3).

By using the newly described haplotyping method, only one haplotype, H68 was obtained among the 31 isolates from Son La (Table 3). This haplotype was characterized by two synonymous single-nucleotide polymorphisms (sSNP) located in the genes *Hem*D and *fad*D and by an insertion of 17 nucleotides in the gene STY2629, compared with the haplotype of reference CT18. Four additional haplotypes were found in the CS, the haplotype of 162/95 and 119/96 were unique whereas 230/95, 14/96, 358/98, and 339/98 harbored the same haplotype, H58.

DISCUSSION

The present study revealed that almost all of the Typhi isolates collected from four outbreaks and sporadic cases in Son La province, Vietnam, in 2002 were susceptible to classic first line antibiotics. It is an interesting finding because MDR and MDR-Nal^R Typhi isolates have been established in this province since at least 1995 and 1997, respectively. A decline of MDR Typhi isolates has been noted in India, whereas, in contrast to our study, isolates with a single resistance to nalidixic acid (and a decreased susceptibility to ciprofloxacin) were reported to increase.[16,17,30] Throughout Vietnam, emergence of Typhi isolates that are resistant only to nalidixic acid has also been observed since 2002 (H. Le, unpublished data). The discrepancy between the trends of antimicrobial resistance in Son La province and other regions of Vietnam may be explained by differences in geography and access to health care. In Son La province, TF outbreaks were often restricted to small communities living in mountainous areas. Because of poor road quality (it can take 2 days to reach study sites from Son La town) and a very low average income, access to antibiotics is limited. In richer Vietnamese regions, antibiotics can be bought over the counter and self-medication is common.

The genetic relatedness of the Typhi strains isolated in Son La province in 2002 was assessed using phage typing and four molecular typing methods. All but four isolates from Son La in 2002 were of phage type A. The remaining isolates could not be typed (DVS and Vi negative). In a previous study, E1 ($N = 38$) and E3 ($N = 24$) were the most frequent phage types observed in 81 epidemiologically independent MDR Typhi isolates collected throughout Vietnam during 1995–

TABLE 3
Characteristics of 33 selected Typhi isolates from Son La province, Vietnam, 2002, and of 8 Typhi comparison strains*

Isolate	Date	Origin	Sex/age group†	Geographic origin			Antimicrobial resistance pattern‡	Phage type	IS*200* type	Ribo-type	PFGE type	Haplo-type
				Province	District	Village						
Outbreak 1 (23 cases)												
1	14/08	Blood	F/IV	Son La	Quynh Nhai	Pac Ma	Susceptible	A	IS1	349	X9	H68
3	11/08	Blood	F/III	Son La	Quynh Nhai	Pac Ma	Susceptible	A	IS1	343	X1	H68
7	10/08	Blood	M/IV	Son La	Quynh Nhai	Pac Ma	Susceptible	A	IS1	344	X1	H68
9	10/08	Blood	M/IV	Son La	Quynh Nhai	Pac Ma	Susceptible	A	IS1	S007	X1	H68
13	24/09	Blood	M/IV	Son La	Quynh Nhai	Pac Ma	Susceptible	A	IS1	344	X1	H68
15	11/08	Blood	M/IV	Son La	Quynh Nhai	Pac Ma	Susceptible	A	IS1	344	X1	H68
19	22/09	Stools	M/IV	Son La	Quynh Nhai	Pac Ma	Susceptible	A	IS1	348	X6	H68
Sporadic cases (7 cases)												
23	04/08	Blood	F/IV	Son La	Son La town	Nam	A	A	IS1	S007	X5	H68
24	18/09	Stools	F/IV	Son La	Son La town	Nam	Susceptible	A	IS1	344	X1	H68
25	20/09	Blood	M/IV	Son La	Son La town	Ne Nua	Susceptible	A	IS1	344	X1	H68
26	08/09	Blood	M/IV	Son La	Son La town	Ne Nua	A	DVS	IS1	345	X1	H68
27	09/09	Stools	M/IV	Son La	Son La town	Hia	Susceptible	A	IS1	344	X9	H68
28	23/09	Blood	F/IV	Son La	Son La town	Hia	Susceptible	A	IS1	340	X4	H68
30	10/09	Blood	M/IV	Son La	Son La town	Ne Nua	Susceptible	A	IS1	344	ND	H68
Outbreak 2 (28 cases)												
31	01/10	Stools	M/IV	Son La	Thuan Chau	Lai Le	Susceptible	A	IS1	344	ND	H68
33	01/10	Stools	F/IV	Son La	Thuan Chau	Lai Le	Susceptible	A	IS1	345	X1	H68
36	01/10	Stools	F/IV	Son La	Thuan Chau	Lai Le	Susceptible	A	IS1	344	X1	H68
39	10/10	Blood	F/IV	Son La	Thuan Chau	Lai Le	Susceptible	DVS	IS1	344	X1	H68
40	01/10	Stools	M/IV	Son La	Thuan Chau	Lai Cang	A	A	IS1	344	X2	H68
41	01/10	Blood	F/IV	Son La	Thuan Chau	Lai Cang	Susceptible	A	IS1	344	ND	H68
46	01/10	Stools	F/IV	Son La	Thuan Chau	Lai Cang	Sul	A	IS1	347	X1	H68
48	02/10	Blood	F/IV	Son La	Thuan Chau	Lai Cang	Susceptible	A	IS1	344	X1	H68
52	04/10	Stools	F/IV	Son La	Thuan Chau	Lai Cang	Susceptible	A	IS1	344	X1	H68
55	10/10	Stools	F/IV	Son La	Thuan Chau	Lai Cang	Susceptible	A	IS1	346	X3	H68
Outbreak 3 (32 cases)												
59	13/10	Blood	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	344	X1	H68
61	13/10	Stools	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	S007	X1	H68
65	13/10	Blood	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	344	X1	H68
68	13/10	Blood	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	177	X8	H68
72	13/10	Blood	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	177	X8	H68
74	13/10	Blood	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	344	X7	H68
78	13/10	Stools	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	S007	X1	H68
85	12/10	Stools	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	344	X1	H68
89	12/10	Stools	M/IV	Son La	Phu Yen	Yen Ha	Susceptible	A	IS1	344	ND	H68
Comparison strains												
162/95	Sep 95				Ha Bac§		Susceptible	A	IS2	187	X12	H75
119/96	Jan 96				Ha Tay§		Susceptible	M3	IS2	26a	X13	H50
230/95	Oct 95				Than Hoa§		ACSulTpTe	E1	IS2	236	X10	H58
14/96	Jan 96				Son La		ACSulTpTe	E1	IS2	236	X10	H58
339/98	1998				Ha Tay		ACSulTpTeNal	E1	IS2	03a	X11	H58
358/98	Jan 98				Than Hoa		ACSulTpTeNal	E1	IS2	03a	X11	H58
CM2664	Jun 02				HCMC§		ACSulTpTeNal	E1	IS2	03a	X11	ND
Ty-2	Reference strain						ND	ND	IS2	73/34/8	X14	H10

* Abbreviations: ND, not determined; DVS, degraded Vi strain.
† Age group I, < 1 year; II, 1–5 years; III, 6–14 years; IV, 15–64 years; V, > 65 years.
‡ Antimicrobial resistance patterns: A, amoxicillin; C, chloramphenicol; Sul, sulfonamides, Tp, trimethoprim; Te, tetracycline; Nal, nalidixic acid.
§ Ha Bac, Ha Tay, and Than Hoa are northern provinces of Vietnam. HCM, Ho Chin Minh City (HCMC) is in southern Vietnam.

2002.[8] In another study, untypeable Vi (UVS) and E1 were the most frequent phage types found in four outbreaks caused by MDR or MDR-Nal^R Typhi isolates in Vietnam during 1993–1997.[7]

Haplotyping revealed that the strains isolated in Son La bore a unique haplotype, H68, which was characterized by combination of 2 sSNP located in the genes *Hem*D and *fad*D and a 17-bp insertion located in the gene STY2629 (Figure 3). The *Hem*D SNP was common among Vietnamese strains, whereas the 17-bp insertion was Son La-specific. This insertion was not present among 480 worldwide strains, including 149 susceptible MDR and MDR-Nal^R Typhi strains isolated from several Vietnamese regions.[29] In addition, *Xba*I-PFGE, considered the method of choice for subtyping Typhi, revealed that the Son La isolates were highly related with profiles clustering into the same group with a similarity of 85%.[5,7,9,31–39] The ribotyping results were more difficult to interpret. One ribotype, 344, was found in 57.6% of the isolates. Seven isolates with a predominant PFGE profile, X1, showed ribotypes other than 344. For these isolates, we could have performed PFGE with another restriction enzyme to check if these isolates belonged to the same or to different clones. However, in studies involving Typhi, use of additional enzymes did not significantly enhance the discriminatory power of *Xba*I-PFGE alone.[35,36,39] Rather than suggesting different clones, ribotyping results could be explained by possible homologous recombinations between *rrn* operons of related isolates, as previously described by Echeita and Usera.[40] As these rearrangements can dramatically modify the ribotype and subsequently disturb cluster analysis, ribotyping

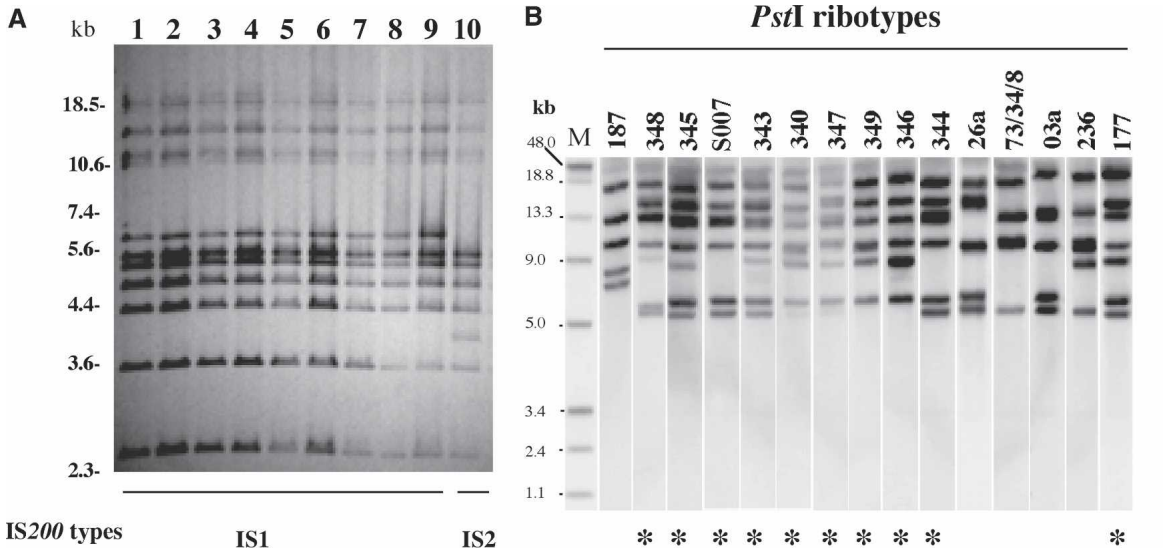

FIGURE 1. Representative IS*200* profiles **A**, and *Pst*I-ribotypes **B**, obtained from the 33 selected Typhi isolates and the 8 comparison strains under study. **A**, Lanes 1–9, IS1 type; lane 10, IS2 type. **B**, Image generated by BioNumerics. M, Riboprinter marker size (band sizes in kilobase pairs). Ribotype numbering according to BBPE-Unit database is indicated. Asterisks indicate the ribotypes observed in isolates from Son La province, 2002.

should be used with caution and concomitantly with other methods like PFGE during epidemiologic investigations.

It remains difficult to define whether the Son La clone had emerged from a local strain or had been imported. Only a single earlier isolate from Son La province was available for the present study (other isolates were not stored). This MDR-Nal^R isolate (14/96) collected in 1996 was characterized by a different haplotype, IS*200*-type, and phage type in comparison with the 2002 clone. This 14/96 isolate was similar to 230/95, an MDR isolate collected in 1995 in Than Hoa province, located near Son La province. They both belong to the H58 haplotype, which is now found predominantly in Vietnam and South Asia in MDR and MDR-Nal^R Typhi strains.[29] This suggests that the Son La susceptible clone did not emerge from a plasmid-purged MDR Typhi strain.

In the absence of earlier isolates collected in Son La province, we have no information on when exactly the clone emerged in this province. Molecular analysis performed in the past showed that, in contrast to MDR, susceptible Typhi strains displayed extensive genetic heterogeneity.[35,41] The very weak genetic diversity of the Son La clone suggests that it might have emerged rather recently in Son La province.

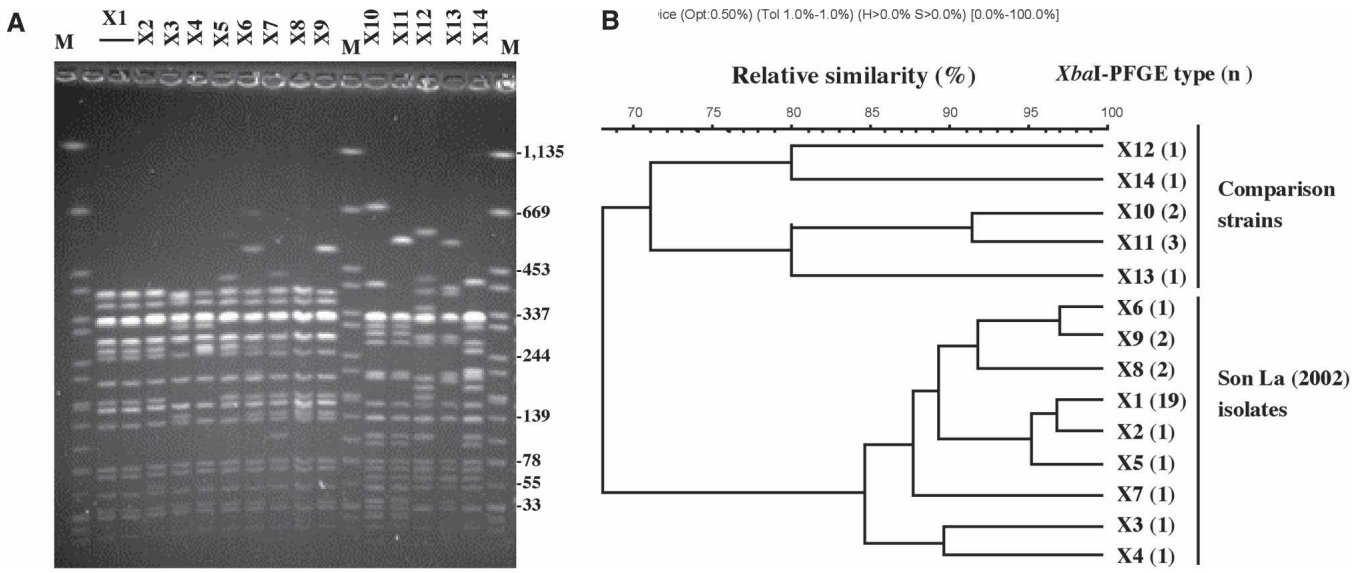

FIGURE 2. **A**, Representative *Xba*I-PFGE profiles obtained from the 29 typed Typhi isolates and the 8 comparison strains under study. M, *S. enterica* serotype Braenderup H9812 used as the molecular size marker (band sizes in kilobase pairs). PFGE profile numbering is indicated. **B**, Dendrogram generated by BioNumerics showing the results of cluster analysis on the basis of PFGE fingerprinting. Similarity analysis was performed using the Dice coefficient, and clustering was by UPGMA. Numbers in parentheses refer to the number of isolates with the indicated PFGE profile.

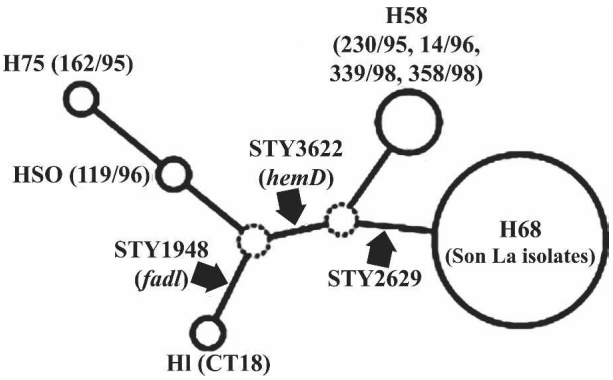

FIGURE 3. Minimal spanning tree of 38 strains based on DHPLC analysis of 52 polymorphic genes (Table 1). The tree shows 5 haplotypes (nodes) plus two hypothetical nodes (missing links) indicated by dashed lines. Size of circles reflects numbers of isolates. Each edge reflects a single mutation. The genes that bore the mutations, which also separate H68 from H1, are annotated (arrows) along three edges.

This susceptible clone could have been established in Son La province as early as 1997, when MDR isolates were reported to decrease, or as late as 2002. It could have been acquired before circulation of MDR or MDR-Nal^R Typhi isolates and maintained in chronic carrier(s) until favorable epidemiologic conditions associated with the disappearance of antibiotic selective pressure, leading to its dissemination in Son La province. In the first hypothesis, the circulation of this clone during the last decade might have resulted in a network of carriers harboring the same or a closely derived strain and being the source of small independent outbreaks in distinct communities. In the second hypothesis, the three outbreaks and the sporadic cases of 2002 were related. The previous epidemiologic study did not support this hypothesis. However, the possibility of a village-to-village dissemination during the 10-week period by healthy carriers was not investigated (only TF cases were investigated). Healthy carriers could have contaminated people through point source contaminated food or more probably through water from wells or streams. Drinking untreated water and poor hygiene habits were among the risk factors found to be associated with TF in the province (83/90 cases and 149/180 controls).[22] Nevertheless, in the absence of earlier isolates, the cause(s) that should explain the Son La province scale replacement of the MDR/MDR-Nal^R Typhi strains by a single susceptible clone described herein remain(s) unclear. The Son La paradox sheds light on the possible shift from multiresistant populations to a susceptible population in one region where TF is already endemic. Understanding the epidemiologic situation that has led to this shift of populations in Son La would be very important for TF control.

The finding that four amoxicillin-resistant isolates have been detected among isolates belonging to the emerging or re-emerging clone(s) indicates that acquisition of resistance determinants is already started. Reasons for emergence of resistance are well documented and are most probably preventable. A resurgence of MDR and MDR-Nal^R Typhi isolates in Son La province, whatever their clonal lineage, should be prevented by the use of classic first-line antibiotics adapted to the results of the monitoring of antimicrobial susceptibility when feasible. Education of health professionals to ensure appropriate antibiotic prescriptions and education of patients to avoid self-medication should be emphasized.

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