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IS*200* and multilocus sequence typing for the identification of *Salmonella enterica* serovar Typhi strains from Indonesia

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Summary. In this work, IS*200* and multi-locus sequence typing (MLST) were used to analyze 19 strains previously serotyped as *Salmonella enterica* serovar Typhi and isolated in Indonesia (16 strains), Mexico (2 strains), and Switzerland (1 strain). Most of the strains showed the most common Typhi sequence types, ST1 and ST2, and a new Typhi genotype (ST1856) was described. However, one isolate from Mexico and another from Indonesia were of the ST365 and ST426 sequence types, indicating that they belonged to serovars Weltevreden and Aberdeen, respectively. These results were supported by the amplification of IS*200* fragments, which rapidly distinguish Typhi from other serovars. Our results demonstrate the utility of IS*200* and MLST in the classification of *Salmonella* strains into serovars. These methods provide information on the clonal relatedness of strains isolated worldwide. [**Int Microbiol** 2015; 18(2):99-104]

Keywords: *Salmonella* Typhi · bacterial molecular typing · multilocus sequence typing (MLST) · clonal complex · insertion sequence IS200

Introduction

Typhoid fever, a systemic febrile illness in humans, is caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (Typhi). The disease is transmitted by the fecal-oral route, mainly via contaminated food and water. It is a global health problem, especially in the developing world, with more than 27 million cases determined each year, resulting in an estimated 217,000 deaths [6].

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The classification of *Salmonella* has been traditionally based on serotyping, which depends on agglutination reactions using anti-sera specific for epitopes of the lipopolysaccharide antigens (O antigens) and one of two alternative flagellar antigens (phases 1 and 2 of the H antigen) [12]. Although the designation of serovars is widely used in epidemiology, serotyping has the disadvantage that, because it depends on numerous antibodies obtained by the immunization of rabbits, its implementation is expensive and laborious, such that it is performed by only a few reference laboratories. Moreover, serotyping does not necessarily reflect evolutionary relationships [15,17]. In fact, previous studies have shown that strains with the same serovar can be distantly related at the chromosomal level whereas strains of different serovars may be closely related [3,20].

Several molecular methods have been proposed as alternatives to serotyping in the classification of *Salmonella* [22]. For example, variations in the nucleotide sequences of multiple gene regions with cellular housekeeping functions have been used to determine relationships among bacterial strains [15,18]. This technique, referred to as multilocus sequence typing (MLST), consists of the amplification and sequencing of defined, internal, 400- to 500-bp fragments of selected housekeeping genes. The different gene sequences are assigned as alleles; for each strain, the combination of alleles for each gene locus defines the allelic profile, or sequence type (ST). MLST is increasingly being used as a typing method for many different pathogenic organisms [17].

In 2002, Kidgell and colleagues designed an MLST scheme for Typhi strains [13] that makes use of the partial sequences of seven housekeeping loci (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) and thus reveals the possible variation within 3,336 bp. This scheme has been applied in the publicly available on-line MLST database for *Salmonella enterica* [http://mlst.warwick.ac.uk/mlst/dbs/Senterica]. It is another of the advantages of MLST: comparisons of the allelic profiles of any strains reported worldwide.

Table 1. Description of the sources of the studied strains and the MLST results

Strain	Country	Laboratory	Year	Serovar	H antigen	*aroC*	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	ST	Complex
2	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
12	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
13	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
84405	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
97457	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
98531	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
98864	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
99282	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	1	1	1	1	5	ST1	Cx13
99319	Jakarta	USNAMRU-2	1989	Typhi	d	1	1	1	1	1	1	5	ST1	Cx13
99155	Jakarta	USNAMRU-2	1989	Typhi	j	1	1	2	1	1	1	5	ST2	Cx13
2218	Yogyakarta	USNAMRU-2	1989	Typhi	d	1	1	1	1	1	1	5	ST1	Cx13
2219	Yogyakarta	USNAMRU-2	1989	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
2233	Yogyakarta	USNAMRU-2	1989	Typhi	d	1	1	1	1	1	1	5	ST1	Cx13
3042	Yogyakarta	USNAMRU-2	1989	Typhi	d	1	1	**340**	1	1	1	5	**ST1856**	Cx13
TY404	Indonesia	IP	1981	Typhi	d:Z_{66}	1	1	2	1	1	1	5	ST2	Cx13
MM160	Switzerland	UZ	1995	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
IMSS1	Mexico	IMSS	1980	Typhi	d	1	1	2	1	1	1	5	ST2	Cx13
MK28	Mexico	INCMNSZ	1987	Weltevreden		130	97	25	125	84	9	101	ST365	Cx 205
11	Jakarta	USNAMRU-2	1989	Aberdeen		46	124	112	12	36	19	18	ST426	Cx165

USNMRU-2, United States Naval Medical Research Unit-2; IP, Institute Pasteur; UZ, Universität Zürich; IMSS, Instituto Mexicano del Seguro Social; INCMNSZ, Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán". The new *hemD* allele and ST reported in this study are highlighted in boldface.

In a previous study [4], we determined that Typhi strains possess a copy of the IS*200* insertion element between the *gyrA* and *rcsC* genes. By PCR amplification, this region renders a band of ~1.5 kb for Typhi strains and of ~0.8 kb for other *Salmonella* strains. Accordingly, the method can be used in the rapid differentiation of Typhi from other serovars, and therefore perhaps also for taxonomic and epidemiologic purposes [4].

In the present work, 19 strains previously serotyped as Typhi were analyzed using the IS*200* PCR typing method and the *Salmonella enterica* MLST scheme in order to confirm their serovar assignments and detect genetic diversity. Most of the strains displayed the more common genotypes in Typhi, ST1 and ST2, but a new genotype, ST1856,was also discovered. Two of the strains originally serotyped as Typhi instead corresponded to the serovars Weltevreden and Aberdeen (ST101 and ST426, respectively). The IS*200* typing data and the MLST results were in good agreement. Our results demonstrated the utility of IS*200* and MLST as molecular tools to distinguish among the serovars of *Salmonella* strains and to establish the genetic relationships of these isolates with those previously reported worldwide.

Materials and methods

***Salmonella* strains.** Nineteen strains isolated from blood cultures of human infections and identified serologically as Typhi were included in this study (Table 1). Fifteen strains were isolated from Jakarta and Yogyakarta in Indonesia, at the United States Naval Medical Research Unit-2 (USNAMRU-2) laboratory, and donated by Dr. Gary K. Schoolnik (Stanford University (Stanford, CA, USA). Strain Ty404 was also from Indonesia and was described by Guinee and colleagues in their report of H antigen type Z66 [9]. Strain MM160 was from the collection of Dr. Martin Altwegg (Universität Zürich, Switzerland). The remaining two strains were from Mexico: strain MK28, provided by Dr. Juan Sierra-Madero (Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán" (Mexico City), and strain IMSS1, provided by Dr. Armando Isibasi (Instituto Mexicano del Seguro Social (Mexico City). All of the strains were generously donated to the laboratory of one of us (EC). Strains IMSS-1 and MM160 were included in a previous study that identified a distinctive feature of the IS*200* element that allows the rapid identification of Typhi strains [4].

DNA extraction and PCR amplifications. The lyophilized strains were resuspended in 5 ml of LB broth. Genomic DNA was extracted using standard laboratory protocols [19]. PCR amplifications were performed using Taq DNA polymerase (Invitrogen, Brazil). The products were purified with a purification kit from Qiagen (Valencia, CA, USA) and submitted for sequencing at Macrogen (Seoul, South Korea). The strains were analyzed using the seven loci of the *S. enterica* MLST scheme (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) [13]. The PCR primers and conditions were those reported on the MLST web site [http://mlst.warwick.ac.uk/mlst/dbs/Senterica]. The sequences at each locus were submitted to the MLST database for allele assignment, and the combination of alleles was assigned to a ST.

The strains were also tested for amplification of the IS*200* sequence characteristic of Typhi [4], using specific primers (IS200A5 5′- GGTGCGTACCCGAGTGTC-3′ and IS200B3 5′-CTGCCAATCAGGAAAACGCG-3′) and the same conditions as for the PCR used in MLST. The IS*200* products were visualized on 1% agarose gels to determine the sizes of the amplification products, based on a comparison with the O'GeneRuler 1 kb Plus DNA ladder (Thermo Scientific) as a molecular ruler.

Sequence analyses. Multiple alignments were performed for the sequences of each MLST locus using the BioEdit program [10]. The sequences were edited to fit the size reported on the MLST web site and were submitted for the assignment of alleles and STs [http://mlst.warwick.ac.uk/mlst/dbs/Senterica].

The genetic relationships among the multilocus genotypes (STs) of our strains and those reported as Typhi in the database were determined by a clonal complex analysis carried out using the eBURST software [8]. This program divides the MLST data in groups of related strains to identify clonal complexes, predicting the founder genotype (ancestral) for each of the latter. The predicted founder genotype is the ST with the highest number of single-locus variants (SLV) in the group. The visual representation of the descent patterns among the strains shows the diversification of the clones within the clonal complex [8].

Results and Discussion

Combined IS*200* amplification and MLST to discriminate Typhi strains. A collection of 19 strains from Indonesia, Mexico, and Switzerland that had been previously serotyped as Typhi were analyzed in parallel using IS*200* PCR amplification and MLST to confirm their serovar assignments and to detect genetic diversity. Amplification of the IS200 region showed that most of the strains contained the 1.5-kb band characteristic of Typhi strains; however, bands of 2.8 kb and 0.5 kb were detected in strains MK28 and 11, respectively (Fig. 1). In 1997, Calva et al. [4] showed that a 0.5-kb band was displayed by most non-Typhi serovars, and that a 2.8-kb band was characteristic of Weltevreden strains [4]. Therefore, our results suggested that strain MK28 was a Weltevreden strain.

The MLST analysis supported the IS*200* typing results. The nucleotide sequences of the genes amplified in the 19 strains (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) corresponded to alleles already described in the *S. enterica* MLST database, with the exception of a new allele, *hemD*340 (Table 1). The allelic combination for 12 strains was that of ST2, and that of 4 strains ST1. Both STs have been described for other Typhi strains and are the most abundant (93%) in the database for Typhi. Strain 3042, carrying the new allele *hemD*340, was assigned the new sequence type, ST1856, which is a SLV of both ST1 and ST2 (Table 1). This is shown in Fig. 2, where in ST1 a T replaces a C at position 129 in the alignment of the partial sequence of the *hemD*1 allele, with

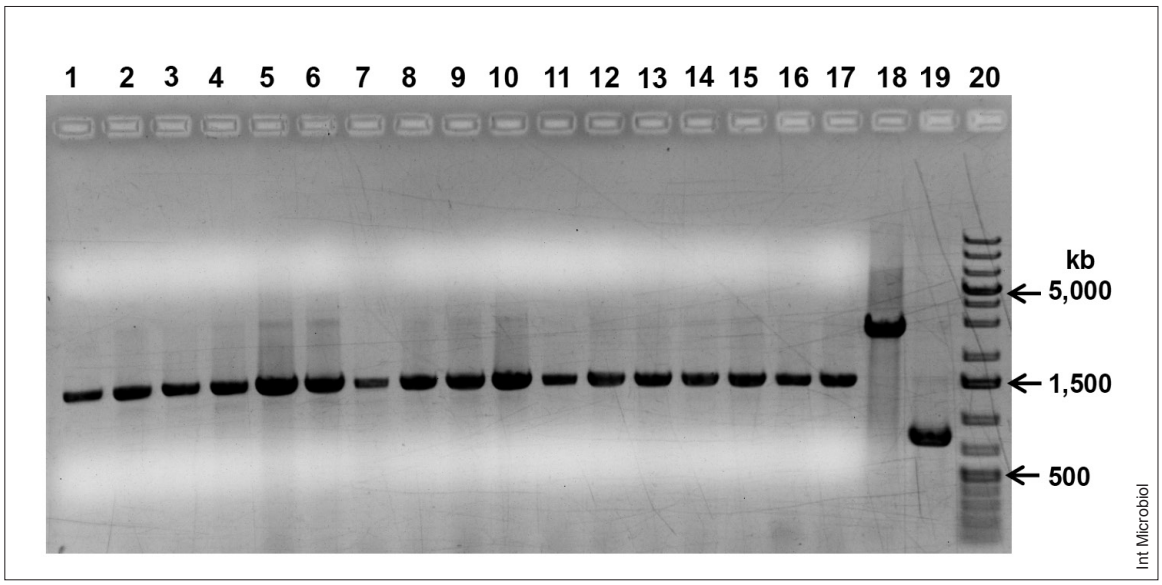

Fig. 1. Agarose gel showing the amplification products of the IS*200* region for the 19 *Salmonella* strains. Lanes: 1, 2; 2, 12; 3, 13; 4, 84405; 5, 97457; 6, 98531; 7, 98864; 8, 99282; 9, 99319; 10, 99155; 11, 2218; 12, 2219; 13, 2233; 14, 3042; 15, TY404; 16, MM160; 17, IMSS1; 18, MK28; 19, 11; and 20, molecular ruler.

respect to the sequence of ST2 (*hemD*2 allele). In the *hemD*340 allele from ST1856, a T replaces a C at position 54. Both substitutions are synonymous and do not alter the protein product. After *hisD*, which has four alleles, the hemD locus, with three alleles (including *hemD*340), is the second most variable gene for Typhi in the MLST database.

For strains MK28 and 11, the sequences of the seven loci differed completely from those of Typhi strains, although they corresponded to alleles already reported in the database. In accordance with their allelic combinations, strain MK28 was ST365, which is characteristic of strains of the Weltevreden serovar, and strain 11 was ST426, characteristic of Aberdeen serovars.

According to the classification of *Salmonella* strains reported worldwide and entered in the database [1], all Typhi strains (ST1, ST2, and ST1856) belonged to clonal complex Cx13; whereas strain MK28, with ST365, grouped with clonal complex Cx205 of the Weltevreden strains and strain 11, with ST426, grouped with clonal complex Cx165 of the Aberdeen strains (Table 1). These results suggest that the initial serotyping of strains MK28 and 11 was incorrect and demonstrate the advantages of combining IS*200* and MLST approaches to distinguish Typhi strains.

Low-variability MLST clonal complex of Typhi strains. Seventeen of the Typhi strains were submitted to the *Salmonella enterica* MLST database: They are the first strains from Mexico, Indonesia, and Switzerland entered in the database. In addition, we contributed a new Typhi sequence type, ST1856. Thus, the *Salmonella enterica* MLST

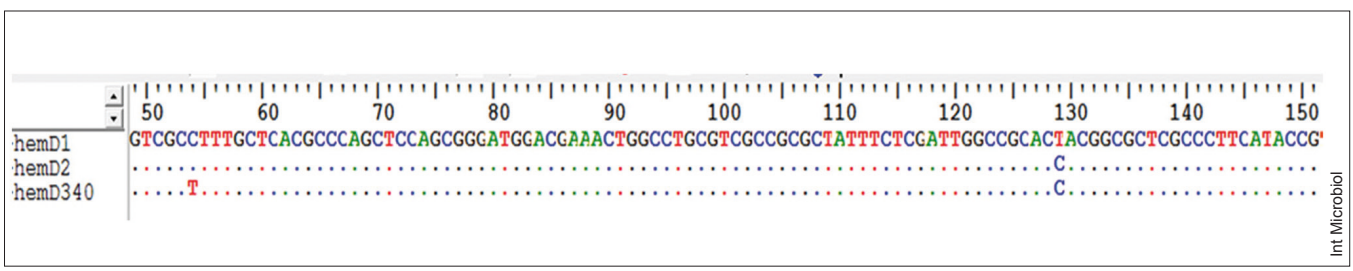

Fig. 2. Multiple alignment of the three hemD alleles found in Typhi. The region displaying the three nucleotide substitutions that distinguish *hemD*1, *hemD*2, and *hemD*340 is shown. The dots indicate that the sequence is the same as that in the first row.

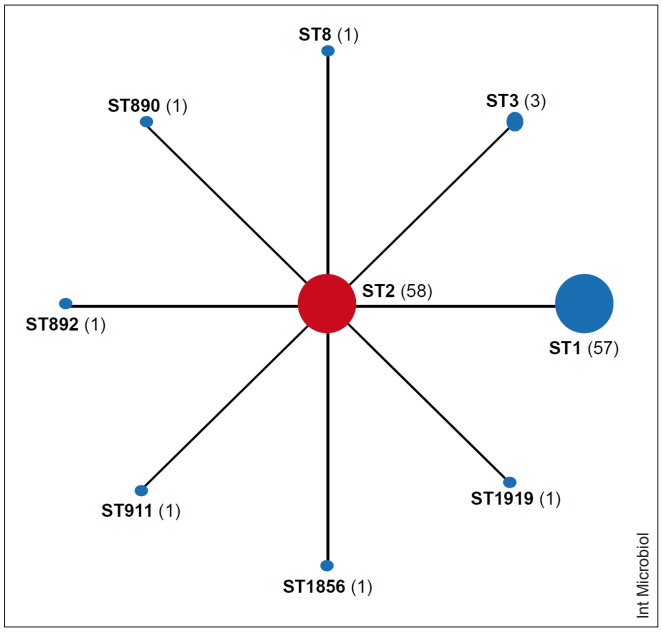

Fig. 3. eBURST clonal complex analysis of the 124 Typhi strains available in the MLST database [http://mlst.warwick.ac.uk/mlst/dbs/Senterica]. ST2 was established as the founder genotype, from which the other STs diversify. The other STs are single-locus variants of ST2. The size of the circles is proportional to the number of strains (in parentheses).

database now lists 124 Typhi strains distributed in nine STs (ST1, ST2, ST3, ST8, ST890, ST892, ST911, ST1856, and ST1919). A clonal complex analysis was performed with eBURST to determine the genetic relationships of the worldwide Typhi strains. The results showed that there is little genetic variation in the Typhi population and that ST2 was the founder genotype from which the remaining STs were derived as SLVs (Fig. 3). A low variability of Typhi strains was reported in previous studies using several molecular techniques [11,13,18,20] . However, greater genetic diversity within the Typhi population of Indonesia was determined using either pulsed-field gel electrophoresis (PFGE) or denaturing high-performance liquid chromatography and DNA sequencing of gene fragments to identify rare single nucleotide polymorphisms (SNPs) that define specific haplotypes, some of them related to specific geographic regions in Indonesia [2,16].

Our study demonstrates the utility of IS200 amplification combined with MLST in the assignment of Typhi serovars. IS*200* amplification enables rapid screening to support, or not, a Typhi affiliation. MLST sequence analysis can then be conducted for further characterization of the strains of interest. As whole-genome sequencing becomes cheaper, it will be possible to use the data to extract the MLST of the strains and integrate the genomic sequences into the MLST framework [1,17]. However, the MLST data lack sufficient resolution to distinguish endemic clones among geographic regions. For example, ST1 and ST2 include most of the Typhi isolates reported worldwide, which does not allow the discernment of a geographic pattern of dissemination. Instead, evaluation of the genetic structure of Typhi populations requires other subtyping tools, such as PFGE [14,16], SNPs [2,18], the analysis of tandem repeated regions (variable number of tandem repeats, VNTR) [21], or clustered regularly interspaced short palindromic repeats (CRISPR) regions [7].

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Competing interests. None declared.

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