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Original Article

Molecular Subtyping of *Salmonella enterica* Serovar Typhi by Pulsed-Field Gel Electrophoresis and Multiple-Locus Variable-Number Tandem-Repeat Analysis in India: Their Association with Antimicrobial Resistance Profiles

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SUMMARY: Molecular subtyping and DNA sequencing-based methods, which are commonly used for discriminating *Salmonella enterica* serovar Typhi (*S*. Typhi) isolates, lead to improved molecular epidemiological investigations for prevention and control of typhoid fever. We obtained *S*. Typhi blood isolates (n = 66) from India during 2007–14 for molecular subtyping by pulsed-field gel electrophoresis (PFGE) and multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) in association with antibiotic resistance profiles. Genotypic diversity was observed more by MLVA (Simpson's index of diversity, D value = 0.997) than PFGE (D value = 0.864). Two prevalent pulsotypes containing nalidixic acid-resistant (NAL^R) and NAL^R-ciprofloxacin-resistant (CIP^R) *S*. Typhi isolates circulated in India. Multidrug-resistant (MDR), NAL^R-CIP^R, and most NAL^R isolates were found to be clonal by PFGE. MLVA could differentiate the clonal isolates. Most of the MDR and NAL^R-CIP^R isolates showed variation in single or double VNTR loci, whereas NAL^R isolates varied in more than 2 loci, reflecting higher genetic diversity among the NAL^R isolates. Of the 6 VNTR loci, TR4,699 (D value = 0.838) and Sal02 (D value = 0.890) loci played important roles as MLVA cluster-supporting alleles. The rapid turnaround time and high-level discriminatory power of MLVA may be useful for tracking and controlling the transmission of *S*. Typhi isolates during epidemiological investigations.

INTRODUCTION

Typhoid fever caused by *Salmonella enterica* serovar Typhi (*S*. Typhi) continues to be a major public health problem in developing countries such as India because of inadequate sanitation and poor hygiene. The estimated burden of typhoid fever was 20.6 million cases and 222,000 deaths in 2010 (1). Antimicrobial therapy is the mainstay of management of typhoid fever; mortality is as high as 30% in untreated cases, but falls to $<$ 1% with appropriate antimicrobial therapy (2). The emergence of multidrug resistant (MDR) *S*. Typhi (resistance to first-line antimicrobials such as ampicillin, chloramphenicol, and co-trimoxazole) led to ciprofloxacin becoming the treatment of choice for typhoid (3). However, *S*. Typhi isolates with decreased ciprofloxacin susceptibility (DCS) (ciprofloxacin MIC, 0.12-0.5 μg/ml) associated with treatment failure have become common, and ciprofloxacin resistance is increasing (4, 5, 6). *S*. Typhi is a genetically monomorphic human-restricted pathogen, which evolved from a recent origin approximately 50,000 years ago (7).

Epidemiological investigations on pathogens based on various genotyping methods provide useful information to assess genetic relatedness for short and long-term periods and to control their dissemination. Highly discriminatory typing methods for the differentiation of closely related isolates, such as those derived from a very recent common ancestor over a period of months or years, are suitable for the investigation of short-term epidemiology (8). Multilocus sequence typing (MLST) and single-nucleotide polymorphism (SNP) analysis, which can distinguish between clones among isolates evolved over a period of decades, are appropriate for long-term epidemiological studies, but poorly suited for subtyping of *S*. Typhi (7, 9, 10). Pulsed-field gel electrophoresis (PFGE) was adopted as a molecular sub-typing tool for national Salmonella surveillance and outbreak research in the 1990s (11). Although this technique is considered the gold standard for Salmonella molecular typing, it does not display equal sensitivity with different serovars (12).

Multiple-locus variable-number tandem-repeats (VNTR) analysis (MLVA) is a well-known molecular typing method for many bacteria that involves the determination of the number of repeats at multiple VNTR

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loci, and the number of loci required varies depending on the diversity of the organism studied (13). The polymorphisms in VNTRs are believed to be a result of slippage-strand-misalignment during DNA replication (14). MLVA has been particularly effective in typing homogenous clones, including *Yersinia pestis* (15), *Escherichia coli* O157:H7 (16), *Clostridium difficile* (17), and *Mycobacterium tuberculosis* (18). In Salmonella enterica (*S. enterica*), a few serovars such as Enteritidis (19), Typhimurium (20), and Typhi (10) have been studied by MLVA. Moreover, MLVA is cost-effective, accessible to any laboratory equipped with minimum molecular biology equipment, and suitable for large-scale standardization. PFGE has been shown to be useful in detection of different clones of MDR, nalidixic acid-resistant (NAL^R), and ciprofloxacin-resistant (CIP^R) *S*. Typhi isolates (4, 21). MLVA based on 8 VNTR loci showed higher discriminatory power than PFGE in *S*. Typhi (22, 23). In addition, MLVA is more discriminatory than PFGE among MDR and susceptible Salmonella isolates (20); however, no report is available on genetic relatedness, based on PFGE and MLVA in association with antibiotic resistance of *S*. Typhi. Inclusion of antimicrobial resistance data provides additional information in epidemiological investigations for *S*. Typhi. In the context of epidemiological follow-up in less developed countries, the present study emphasizes the comparative assessment of genetic diversity by PFGE and MLVA in *S*. Typhi isolates collected from different regions in India.

MATERIALS AND METHODS

Bacterial isolates: The 66 *S*. Typhi blood isolates included in the present study were obtained from the Gastrointestinal Tract Pathogens Repository (GTPR), National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. The isolates were collected during 2007 to 2014 from different regions in India: Kolkata, eastern region (n = 28); Delhi, northern region (n = 11); Vellore, southern region (n = 10); Karnataka, southern western region (n = 15) and Nagpur, central region (n = 2). All were single patient isolates. The isolates were identified by standard biochemical tests (24) followed by serotyping using Salmonella O, H, and Vi factor antiserum (Denka Seiken Co Ltd., Tokyo, Japan). The *S*. Typhi isolates were stored at $-70^{\circ}C$ in brain/heart infusion broth with 15% glycerol until they were needed.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing (AST) was performed by Kirby-Bauer disk diffusion method using the following antimicrobial disks: ampicillin (AMP, 10 µg), chloramphenicol (CHL, 30 µg), co-trimoxazole (SXT, 25 µg), tetracycline (TET, 30 µg), nalidixic acid (NAL, 30 µg), ciprofloxacin (CIP, 5 µg), ceftriaxone (CRO, 30 µg), and azithromycin (AZM, 15 µg) (Oxoid, Basingstoke, UK). The minimum inhibitory concentrations (MICs) of ciprofloxacin for the isolates were determined by E-test according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). *E. coli* ATCC 25,922 was used as the control strain. The interpretation of disk diffusion and E-test results were performed following the Clinical and Laboratory Standard Institute (CLSI) guidelines (25).

PFGE: PFGE was performed using the PulseNet one-day standard protocol with *S. enterica* serovar Braenderup H9,812 as a reference strain as described previously (26).

MLVA typing by PCR and DNA sequencing: MLVA typing of the study isolates was performed by PCR amplification of the previously described 6 VNTR loci individually designated as TR1, TR2, TR4,699, Sal16, Sal02, and Sal20 (10). PCR was performed using the GeneAmp PCR system 9,700 (Applied Biosystems, Foster City, CA,USA), and amplicons were electrophoresed on 2% agarose gels, stained in ethidium bromide, and visualized on a GelDoc (Bio-Rad, Hercules, CA, USA). The products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) followed by nucleic acid sequencing using a 3,730 DNA analyzer (Applied Biosystems).

Data analysis: The PFGE patterns were analyzed using FPQuest software version 4.5 (Bio-Rad) by methods described previously (27). Similarity analysis was performed by using Dice coefficient with 1.5% optimization and tolerance levels, and clustering of matched bands was performed using unweighted pair group method with arithmetic means (UPGMA). The isolates with identical PFGE patterns were described as genetically indistinguishable (Dice coefficient of similarity = 100%); isolates with PFGE patterns differing by 3 or less bands were designated as related (Dice coefficient of similarity $>$ 80%). Threshold similarity $>$ 80% was arbitrarily used to define isolates belonging to the same PFGE cluster.

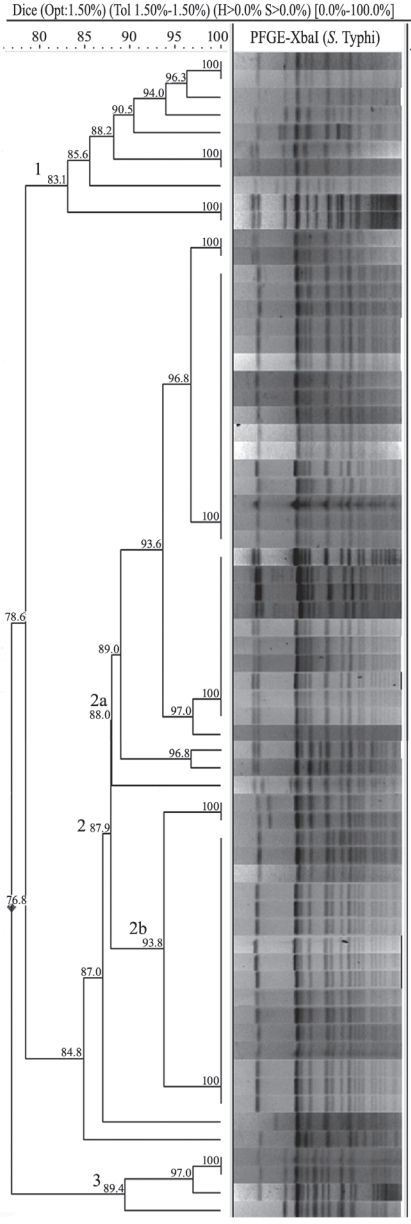
DNA sequences were analyzed with Sequence Scanner software version 1.0 (Applied Biosystems) followed by identification of repeat number of VNTR loci accessible at a public database (http://minisatellites.u-psud.fr). The cluster analysis was performed using the UPGMA algorithm, and a rooted tree was constructed, which is available at http://minisatellites.u-psud.fr (28). The isolates showing distinct MLVA patterns were defined as MLVA types that differed by minimum number of changes in the number of repeats of any locus. To compare the discriminatory power of PFGE, MLVA, and individual VNTR locus, the Simpson's index of diversity (D value) with 95% confidence interval (CIs) was calculated as described previously (29).

RESULTS

Antimicrobial resistance: Five resistance profiles were noted among the isolates (n = 66): NAL-DCS, n = 30; NAL-CIP, n = 16; AMP-CHL-SXT-NAL-DCS, n = 13; TET-SXT-NAL-CIP, n = 2 and DCS, n = 5. Noticeably, 48 (72.7%) isolates were DCS-associated with either NAL^R or MDR. CRO and AZM resistance was not found among the isolates.

PFGE: Three distinct clusters (1 to 3) linked at 76.8% similarity and 21 (P1 to P21) PFGE types (pulsotypes) were generated among 66 *S*. Typhi isolates (Fig. 1). Cluster 1 was composed of 10 isolates at 83.1% similarity, of which 8 (80%) were NAL^R-DCS isolates. Cluster 2 was considered at 87.9% similarity, containing most of the study isolates (48/66, 72.7%), which showed 2 sub-clusters (2a and 2b). Sub-cluster 2a contained NAL^R-DCS (14/32, 43.8%) and MDR (13/32, 40.6%)

isolates, which were linked at 88% similarity. Of the 16 isolates in cluster 2b linked at 93.8% similarity, 15 (93.8%) were NAL^R-CIP^R. Only 4 NAL^R-DCS isolates belonged to cluster 3. Two TET^R-SXT^R-NAL^R-CIP^R isolates were grouped under cluster 2a, whereas 5 DCS isolates distributed to cluster 1 and 2a. The D value of PFGE typing was 0.864. The prevalent pulsotypes P9 (majority of NAL^R-DCS isolates) and P16 (majority of NAL^R- CIP^R isolates) were found in different regions of India, while pulsotype P10 (consisting of MDR isolates) was observed in Kolkata (Fig.1, Table 1). Therefore, a close association was observed between resistance types



Isolate	Year	Location	R profile	PFGE type	MLVA type	MLVA profile
OSS-022	2008	Karnataka	NAL-DCS	P1	M15	05-40-10-12-11-23
OSS-155	2011	Delhi	NAL-DCS	P1	M17	05-43-11-11-11-23
OSS-197	2011	Vellore	NAL-DCS	P2	M16	05-28-12-11-11-23
OSS-011	2007	Nagpur	NAL-DCS	P3	M13	08-08-11-11-07-10
BCR-232	2011	Kolkata	NAL-DCS	P4	M10	08-05-15-10-07-16
OSS-140	2011	Karnataka	DCS	P5	M3	11-17-14-07-10-14
OSS-152	2011	Karnataka	DCS	P5	M4	11-17-15-07-08-14
OSS-010	2007	Nagpur	NAL-DCS	P6	M13	08-08-11-11-07-10
BCR-052	2009	Kolkata	NAL-DCS	P7	M1	11-34-10-07-10-19
BCR-089	2009	Kolkata	NAL-DCS	P7	M2	11-33-08-07-10-21
OSS-156	2011	Delhi	AMP-CHL-SXT-NAL-DCS	P8	M24	05-34-12-11-11-21
OSS-157	2011	Delhi	AMP-CHL-SXT-NAL-DCS	P8	M48	05-37-11-11-11-21
OSS-159	2009	Delhi	DCS	P9	M57	05-34-09-12-11-20
OSS-160	2009	Delhi	DCS	P9	M59	13-24-20-08-11-12
OSS-203	2011	Vellore	DCS	P9	M11	08-12-16-10-07-16
OSS-205	2011	Vellore	NAL-DCS	P9	M8	08-15-11-09-10-13
OSS-198	2011	Vellore	NAL-DCS	P9	M53	05-28-11-11-09-11
OSS-202	2011	Vellore	NAL-DCS	P9	M56	05-18-11-12-08-21
OSS-163	2009	Delhi	TET-SXT-NAL-CIP	P9	M26	05-37-12-11-11-20
OSS-164	2009	Delhi	TET-SXT-NAL-CIP	P9	M19	05-31-10-11-11-18
OSS-047	2009	Karnataka	NAL-DCS	P9	M9	08-10-11-06-10-16
OSS-146	2011	Karnataka	NAL-DCS	P9	M1	08-10-13-11-11-17
OSS-051	2009	Karnataka	NAL-DCS	P9	M54	05-17-11-13-11-16
BCR-162	2011	Kolkata	NAL-DCS	P9	M51	05-29-11-11-10-20
BCR-048	2009	Kolkata	NAL-DCS	P9	M14	05-18-10-11-11-23
OSS-151	2010	Karnataka	NAL-DCS	P9	M31	05-22-14-11-11-21
OSS-267	2014	Karnataka	NAL-DCS	P9	M58	05-13-10-12-11-20
OSS-154	2011	Delhi	NAL-DCS	P9	M55	05-29-11-13-11-12
BCR-305	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M36	05-16-12-10-11-28
KOL-017	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M44	05-30-11-11-11-21
KOL-018	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M23	05-18-12-11-11-21
KOL-038	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M46	05-38-11-11-11-21
BCR-177	2011	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M20	05-43-07-11-11-22
KOL-061	2013	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M47	05-36-11-11-11-21
KOL-062	2013	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M47	05-36-11-11-11-21
BCR-342	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M21	05-45-07-11-11-22
BCR-049	2009	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M43	05-28-11-11-11-21
KOL-034	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	P10	M45	05-20-11-11-11-21
OSS-257	2014	Karnataka	NAL-DCS	P11	M49	05-28-11-11-11-12
OSS-199	2011	Vellore	NAL-DCS	P12	M5	11-08-10-08-09-08
OSS-200	2011	Vellore	NAL-DCS	P13	M16	05-28-12-11-11-23
OSS-158	2011	Delhi	AMP-CHL-SXT-NAL-DCS	P14	M18	05-45-10-11-11-13
BCR-211	2011	Kolkata	NAL-CIP	P15	M39	05-36-13-11-11-19
OSS-132	2011	Karnataka	NAL-CIP	P15	M22	05-26-12-11-11-22
OSS-161	2011	Delhi	NAL-CIP	P16	M29	05-31-14-11-11-20
OSS-162	2009	Delhi	NAL-CIP	P16	M29	05-31-14-11-11-20
OSS-131	2011	Karnataka	NAL-DCS	P16	M34	05-24-09-11-11-22
BCR-062	2009	Kolkata	NAL-CIP	P16	M35	05-35-12-10-11-19
KOL-052	2012	Kolkata	NAL-CIP	P16	M28	05-31-14-11-11-19
BCR-175	2011	Kolkata	NAL-CIP	P16	M37	05-37-13-11-11-19
BCR-108	2010	Kolkata	NAL-DCS	P16	M32	05-10-09-13-11-22
BCR-110	2010	Kolkata	NAL-DCS	P16	M33	05-25-09-13-11-22
RGK-080	2011	Kolkata	NAL-CIP	P16	M38	05-32-13-11-11-19
BCR-191	2011	Kolkata	NAL-CIP	P16	M39	05-36-13-11-11-19
BCR-243	2012	Kolkata	NAL-CIP	P16	M42	05-19-13-13-11-19
OSS-133	2011	Karnataka	NAL-CIP	P16	M30	05-30-14-11-11-20
OSS-248	2014	Karnataka	NAL-CIP	P16	M40	05-10-13-11-11-19
KOL-005	2012	Kolkata	NAL-CIP	P16	M41	05-32-13-12-11-19
KOL-001	2012	Kolkata	NAL-CIP	P16	M27	05-32-14-11-11-19
KOL-045	2012	Kolkata	NAL-CIP	P16	M27	05-32-14-11-11-19
OSS-206	2011	Vellore	NAL-DCS	P17	M50	05-29-11-11-11-24
OSS-204	2011	Vellore	NAL-CIP	P18	M52	05-40-11-11-11-20
OSS-263	2014	Karnataka	NAL-DCS	P19	M6	08-07-06-07-08-08
OSS-264	2014	Karnataka	NAL-DCS	P19	M6	08-07-06-07-08-08
BCR-096	2010	Kolkata	NAL-DCS	P20	M7	08-15-15-06-10-09
OSS-201	2011	Vellore	NAL-DCS	P21	M25	05-12-12-11-11-24

Fig. 1. Cluster analysis of 66 *S*. Typhi isolates by XbaI-PFGE. Band comparison has been performed by using the Dice coefficient with 1.5% optimization (Opt) and 1.5% position tolerance (Tol). The related results of resistance profiles and MLVA types are provided for direct comparison. AMP, ampicillin; CHL, chloramphenicol; SXT, co-trimoxazole; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; DCS, decreased CIP susceptibility.

Table 1. Distribution of different resistance profiles of *S*. Typhi isolates (n = 66) based on their PFGE types

Resistance profile (n)	PFGE type (n)[1)]	Total PFGE types
NAL-DCS (30)	P1 (2), P2 (1), P3 (1), P4 (1), P6 (1), P7 (2), **P9 (11)**, P11 (1), P12 (1), P13 (1), P16 (3), P17 (1), P19 (2), P20 (1), P21 (1)	15
NAL-CIP (16)	P15 (2), **P16 (14)**	2
AMP-CHL-SXT-NAL-DCS (13)	P8 (2), **P10 (10)**, P14 (1)	3
TET-SXT-NAL-CIP (2)	P9 (2)	1
DCS (5)	P5 (2), P9 (3)	2

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; DCS, decreased ciprofloxacin susceptibility; NAL, nalidixic acid; TET, tetracycline; SXT, co-trimoxazole.
1): Major pulsotypes are shown in bold.

and PFGE clusters/types irrespective of the isolation location.

MLVA: The number of repeats for each VNTR was estimated from the DNA sequence data. The different number of repeats was assigned to the alleles. Overall, 4 to 30 alleles were observed, and the repeat units ranged from 5 to 45 repeats among the 6 VNTR loci (Table 2). The D value of the 6 individual VNTR loci ranged from 0.402 for TR1 to 0.957 for TR2, whereas the D value of MLVA typing was 0.997. Predominant alleles in TR1, TR4,699, Sal16, and Sal20 loci had repeat numbers of 5 (50/66, 75.8%), 11 (20/66, 30.3%), 11 (41/66, 62.1%), and 11 (49/66, 74.2%), respectively (Fig. 2).

Nine clusters (A to I) and 59 MLVA types (M1

Table 2. Comparison of individual VNTR locus, MLVA and PFGE methods in *S*. Typhi isolates (n = 66)

VNTR locus/Method	Consensus Sequence	Unit length (bp)	No. of alleles/ genotypes	Range of no. of repeats	D value (95% CI)[1)]
TR1	AGAAGAA	7	4	5, 8, 11, 13	0.402 (0.269-0.535)
TR2	CCAGTTCC	8	30	5, 7, 8, 10, 12, 13, 15-20, 22, 24-26, 28-38, 40, 43, 45	0.957 (0.948-0.966)
TR4,699	TGTTGG	6	12	6-16, 20	0.838 (0.788-0.888)
Sal16	GACCAT	6	8	6-13	0.595 (0.465-0.724)
Sal20	CAG	3	5	7-11	0.435 (0.292-0.576)
Sal02	TACCAG	6	17	8-14, 16-24, 28	0.890 (0.856-0.924)
MLVA	NA	NA	59	NA	0.997 (0.994-1.000)
PFGE	NA	NA	21	NA	0.864 (0.815-0.914)

NA, not applicable; MLVA, multiple-locus variable-number tandem-repeat (VNTR) analysis; PFGE, pulsed-field gel electrophoresis.
[1)]: Simpson's index of diversity, D value; CI, confidence interval.

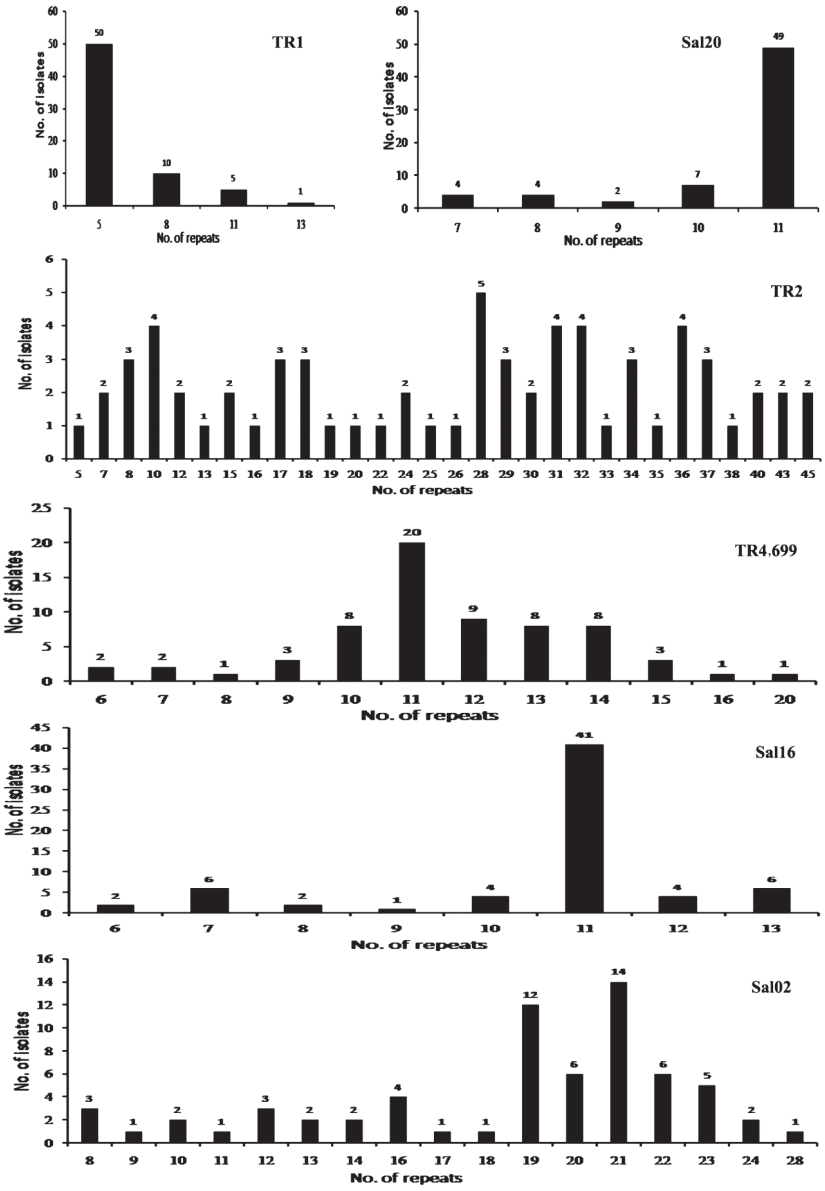
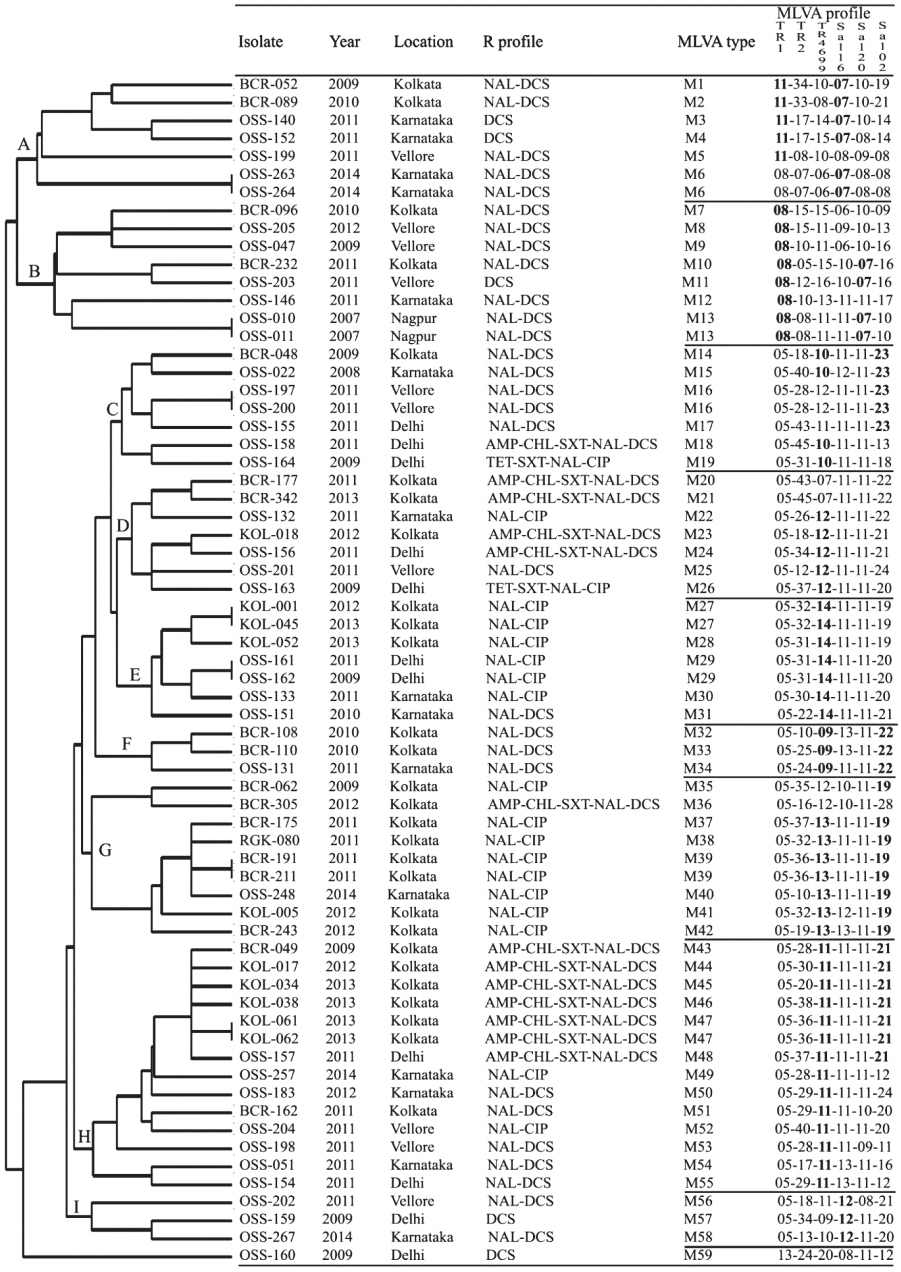


Fig. 2. Allele distribution among 66 *S*. Typhi isolates at VNTR loci TR1, TR2, TR4,699, Sal16, Sal20 and Sal02.



Isolate	Year	Location	R profile	MLVA type	MLVA profile TR1	TR2	TR4699	Sal16	Sal20	Sal02
BCR-052	2009	Kolkata	NAL-DCS	M1	**11**-34-10-**07**-10-19					
BCR-089	2010	Kolkata	NAL-DCS	M2	**11**-33-08-**07**-10-21					
OSS-140	2011	Karnataka	DCS	M3	**11**-17-14-**07**-10-14					
OSS-152	2011	Karnataka	DCS	M4	**11**-17-15-**07**-08-14					
OSS-199	2011	Vellore	NAL-DCS	M5	**11**-08-10-08-09-08					
OSS-263	2014	Karnataka	NAL-DCS	M6	08-07-06-**07**-08-08					
OSS-264	2014	Karnataka	NAL-DCS	M6	08-07-06-**07**-08-08					
BCR-096	2010	Kolkata	NAL-DCS	M7	**08**-15-15-06-10-09					
OSS-205	2012	Vellore	NAL-DCS	M8	**08**-15-11-09-10-13					
OSS-047	2009	Vellore	NAL-DCS	M9	**08**-10-11-06-10-16					
BCR-232	2011	Kolkata	NAL-DCS	M10	**08**-05-15-10-**07**-16					
OSS-203	2011	Vellore	DCS	M11	**08**-12-16-10-**07**-16					
OSS-146	2011	Karnataka	NAL-DCS	M12	**08**-10-13-11-11-17					
OSS-010	2007	Nagpur	NAL-DCS	M13	**08**-08-11-11-**07**-10					
OSS-011	2007	Nagpur	NAL-DCS	M13	**08**-08-11-11-**07**-10					
BCR-048	2009	Kolkata	NAL-DCS	M14	05-18-**10**-11-11-**23**					
OSS-022	2008	Karnataka	NAL-DCS	M15	05-40-**10**-12-11-**23**					
OSS-197	2011	Vellore	NAL-DCS	M16	05-28-12-11-11-**23**					
OSS-200	2011	Vellore	NAL-DCS	M16	05-28-12-11-11-**23**					
OSS-155	2011	Delhi	NAL-DCS	M17	05-43-11-11-11-**23**					
OSS-158	2011	Delhi	AMP-CHL-SXT-NAL-DCS	M18	05-45-**10**-11-11-13					
OSS-164	2009	Delhi	TET-SXT-NAL-CIP	M19	05-31-**10**-11-11-18					
BCR-177	2011	Kolkata	AMP-CHL-SXT-NAL-DCS	M20	05-43-07-11-11-22					
BCR-342	2013	Kolkata	AMP-CHL-SXT-NAL-DCS	M21	05-45-07-11-11-22					
OSS-132	2011	Karnataka	NAL-CIP	M22	05-26-**12**-11-11-22					
KOL-018	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	M23	05-18-**12**-11-11-21					
OSS-156	2011	Delhi	AMP-CHL-SXT-NAL-DCS	M24	05-34-**12**-11-11-21					
OSS-201	2011	Vellore	NAL-DCS	M25	05-12-**12**-11-11-24					
OSS-163	2009	Delhi	TET-SXT-NAL-CIP	M26	05-37-**12**-11-11-20					
KOL-001	2012	Kolkata	NAL-CIP	M27	05-32-**14**-11-11-19					
KOL-045	2013	Kolkata	NAL-CIP	M27	05-32-**14**-11-11-19					
KOL-052	2013	Kolkata	NAL-CIP	M28	05-31-**14**-11-11-19					
OSS-161	2011	Delhi	NAL-CIP	M29	05-31-**14**-11-11-20					
OSS-162	2009	Delhi	NAL-CIP	M29	05-31-**14**-11-11-20					
OSS-133	2011	Karnataka	NAL-CIP	M30	05-30-**14**-11-11-20					
OSS-151	2010	Karnataka	NAL-DCS	M31	05-22-**14**-11-11-21					
BCR-108	2010	Kolkata	NAL-DCS	M32	05-10-**09**-13-11-**22**					
BCR-110	2010	Kolkata	NAL-DCS	M33	05-25-**09**-13-11-**22**					
OSS-131	2011	Karnataka	NAL-DCS	M34	05-24-**09**-11-11-**22**					
BCR-062	2009	Kolkata	NAL-CIP	M35	05-35-12-10-11-**19**					
BCR-305	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	M36	05-16-12-10-11-28					
BCR-175	2011	Kolkata	NAL-CIP	M37	05-37-**13**-11-11-**19**					
RGK-080	2011	Kolkata	NAL-CIP	M38	05-32-**13**-11-11-**19**					
BCR-191	2011	Kolkata	NAL-CIP	M39	05-36-**13**-11-11-**19**					
BCR-211	2011	Kolkata	NAL-CIP	M39	05-36-**13**-11-11-**19**					
OSS-248	2014	Karnataka	NAL-CIP	M40	05-10-**13**-11-11-**19**					
KOL-005	2012	Kolkata	NAL-CIP	M41	05-32-**13**-12-11-**19**					
BCR-243	2012	Kolkata	NAL-CIP	M42	05-19-**13**-13-11-**19**					
BCR-049	2009	Kolkata	AMP-CHL-SXT-NAL-DCS	M43	05-28-**11**-11-11-**21**					
KOL-017	2012	Kolkata	AMP-CHL-SXT-NAL-DCS	M44	05-30-**11**-11-11-**21**					
KOL-034	2013	Kolkata	AMP-CHL-SXT-NAL-DCS	M45	05-20-**11**-11-11-**21**					
KOL-038	2013	Kolkata	AMP-CHL-SXT-NAL-DCS	M46	05-38-**11**-11-11-**21**					
KOL-061	2013	Kolkata	AMP-CHL-SXT-NAL-DCS	M47	05-36-**11**-11-11-**21**					
KOL-062	2013	Kolkata	AMP-CHL-SXT-NAL-DCS	M47	05-36-**11**-11-11-**21**					
OSS-157	2011	Delhi	AMP-CHL-SXT-NAL-DCS	M48	05-37-**11**-11-11-**21**					
OSS-257	2014	Karnataka	NAL-CIP	M49	05-28-**11**-11-11-12					
OSS-183	2012	Karnataka	NAL-DCS	M50	05-29-**11**-11-11-24					
BCR-162	2011	Kolkata	NAL-DCS	M51	05-29-**11**-11-10-20					
OSS-204	2011	Vellore	NAL-CIP	M52	05-40-**11**-11-11-20					
OSS-198	2011	Vellore	NAL-DCS	M53	05-28-**11**-11-09-11					
OSS-051	2011	Karnataka	NAL-DCS	M54	05-17-**11**-13-11-16					
OSS-154	2011	Delhi	NAL-DCS	M55	05-29-**11**-13-11-12					
OSS-202	2011	Vellore	NAL-DCS	M56	05-18-11-**12**-08-21					
OSS-159	2009	Delhi	DCS	M57	05-34-09-**12**-11-20					
OSS-267	2014	Karnataka	NAL-DCS	M58	05-13-10-**12**-11-20					
OSS-160	2009	Delhi	DCS	M59	13-24-20-08-11-12					

Fig. 3. Dendogram showing genetic relatedness among 66 *S*. Typhi Indian isolates in MLVA typing. Based on the variation observed at 6 VNTR loci a rooted tree has been constructed by the UPGMA algorithm. The allelic pattern for each MLVA profile and resistance profile are shown. The bold numbers are the alleles that supported the clustering of the MLVA profiles. AMP, ampicillin; CHL, chloramphenicol; SXT, co-trimoxazole; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; DCS, decreased CIP susceptibility.

to M59) were formed among 66 isolates using the 6 VNTR loci (Fig. 3). The MLVA clusters were supported by certain VNTR alleles. However, all these cluster-supporting alleles were not unique for that particular cluster. Allele 11 of TR1 and allele 7 of Sal16, allele 7 of Sal20, allele 23 of Sal02, and allele 9 of TR4,699 were unique for cluster A, B, C, and F, respectively. The association between resistance types and MLVA clusters/types was not close as observed in PFGE. Cluster A, B, C, F, and I contained 29 isolates, of which 22 were (75.9%) NAL^R-DCS isolates. Of 21 isolates in cluster D and H, 11 (52.4%) were MDR. Cluster E and G were composed of 87.5% (14/16) NAL^R-CIP^R isolates. This suggests that MLVA typing was too diverse to draw a robust relationship.

MLVA in discrimination of clonal isolates: Three prevalent pulsotypes were noted in the drug resistant S. Typhi isolates: P9 for most NAL^R-DCS, P10 for MDR, and P16 for most of the NAL^R-CIP^R isolates (Fig. 1). However, the clonal *S*. Typhi isolates of each pulsotypes were differentiated further by MLVA typing. Sixteen and 14 MLVA types were noted in isolates of P9 and P16 pulsotypes, respectively, whereas 9 MLVA types were found in P10 isolates. The rare duplicate MLVA types (M6, M13, M16, M27, M29, M39, and M47) of *S*. Typhi isolates were also found clonal or clonally related by PFGE.

DISCUSSION

Molecular subtyping of *S*. Typhi isolates remains very important in epidemiological investigations because of the increasing incidence of typhoid fever (1) and emergence of MDR, NAL^R, and CIP^R strains (3, 5, 6). Emergence of antimicrobial-resistant strains poses a great concern for typhoid treatment and influences reshaping of the current *S*. Typhi population. Compared to PFGE, MLVA has been adopted by several European countries for epidemiological investigations of non-typhoidal *S. enterica* serovars, including Typhimurium and Enteritidis, due to its cost-effectiveness, rapid turnaround time, and ease of inter-laboratory comparison of results (30). Nevertheless, for *S*. Typhi, the MLVA typing method is still under evaluation (10, 28, 23). The present study examined the discriminatory ability of MLVA typing with respect to PFGE and antimicrobial resistance profiles of *S*. Typhi (n = 66) circulating in India during 2007-14.

Twenty-one pulsotypes were observed by PFGE (D value = 0.864) among the study isolates, of which 15 were in NAL^R-DCS, 3 in NAL^R-CIP^R, 3 in MDR, and the rest were other resistance profiles (Fig. 1). This PFGE results showed less diversity in MDR and NAL^R-CIP^R isolates than in NAL^R-DCS isolates (Table 1). Le *et al*. have suggested that there was a clonal expansion of MDR *S*. Typhi, but the replacement of classical first-line antibiotics with fluoroquinolones (FQs) for typhoid treatment resulted in loss of the MDR phenotype and an increase in NAL^R strains from Vietnam (21). The current global increase in NAL^R-CIP^R isolates is likely due to increased use of FQs in the 1990s, leading to selection of resistant isolates (9). Our findings were in agreement with the above statement of clonal selection of resistant isolates under specific antibiotic pressure.

The 6 VNTR loci used in this study have been reported to be highly variable with discriminatory power of > 0.8 (10, 31, 32). Higher diversity (D value > 0.8) was observed in TR2, TR4,699, and Sal02 loci, whereas TR1 and Sal20 loci showed relatively low variation (D value = 0.4) among the study isolates (Table 2). The diversities in TR1 and TR2 loci were found in tandem with another study from India (33). It was suggested that rapidly evolving VNTR markers are suitable for investigating genetic relationships among closely related isolates in short-term epidemiological surveillance studies, whereas slowly evolving VNTRs are more useful for establishing clonal relationships among isolates in long-term epidemiological investigations, such as the global transmission of important clones (34). The predominant alleles of TR1 loci revealed a repeat number of 5 among the study isolates, which was also shown in earlier studies among *S*. Typhi Indian isolates (31, 33). The range of number of repeats and prevalent alleles in VNTR loci was found to vary in *S*. Typhi isolates from different geographical locations (10, 23, 28). None of the 6 VNTR loci was found to contain zero-repeat copy in any of the study strains (Table 2). In contrast, zero-repeat copy of TR1 and TR2 loci were reported earlier in 15% and 39% of *S*. Typhi isolates from Indonesia and India, respectively (28, 33). Octavia and Lan reported that MLVA clusters based on an UPGMA dendrogram were represented by certain alleles of TR4,500, TR4,699, Sal02, Sal16, and TR1 loci (10). In this study, TR4,699 and Sal02 loci played important roles as MLVA cluster supporting alleles (Fig. 3).

Fragment analysis using fluorescence-labeled primers has been widely used in MLVA of different bacteria including *S*. Typhi (35), but researchers confirmed the repeat numbers using DNA sequencing, as performed in the present study (17, 28, 31). The size difference in a VNTR locus using fragment analysis may not always reflect the real number of tandem repeats because insertions and/or deletions in the amplified region can also give rise to the same size difference. Therefore, sequencing of the amplicons is a more precise method for determining the number of repeats, although we did not find any insertions and/or deletions in any of the VNTR loci. Recently Wang et al. developed a set of endogenous DNA ladder markers for improving the accuracy and reproducibility of VNTR analysis among *S*. Typhi strains using microfluidic chip-based electrophoresis (23).

A high level of genomic diversity (D value = 0.997) in the study isolates was indicated by MLVA typing, which was also observed in earlier studies from different geographical locations (10, 22, 23, 28, 31, 32). Presence of typhoid carriers or indiscriminate use of antimicrobials may cause large diversity in *S*. Typhi genotypes circulating in India, thus confirming the presence of typhoid carriers or indiscriminate use of antimicrobials as reported in earlier studies from various countries (5, 28, 36). MLVA based on 6 VNTR loci discriminated the resistant study isolates into different genotypes, which were found clonal by PFGE over the span of 8 years (2007-14) (Fig. 1). Similar observations have been documented by earlier studies, which showed significance of MLVA with higher discriminatory power of 8-loci MLVA over PFGE in discerning epidemiologically unlinked *S*. Typhi strains isolated from different Asian countries (22, 23). It was observed that PFGE types were associated with resistance profiles among the study isolates, but MLVA typing was too diverse to draw any robust relationship. Lindstedt et al. also found that MLVA discriminated the clonal (by PFGE) multi-resistant and non-resistant *S*. Typhimurium DT104 isolates, but MLVA types had no association with antimicrobial resistance due to the presence of VNTR loci in genes other than antibiotic resistance (20). The VNTR loci investigated in this study also do not reside in resistance genes. AST is a typing method used in public health surveillance, but MLVA typing is useful in epidemiological surveillance of *S*. Enteritidis due to low prevalence of antimicrobial resistance in those isolates (19). In this study, 5 resistance profiles also did not represent the complete diversity among them as represented by 59 MLVA types (Fig. 3). Most of the MDR and NAL^R-CIP^R study isolates showed variation in single or double VNTR locus, whereas NAL^R isolates varied in more than 2 loci reflecting higher genetic diversity among NAL^R isolates. To accommodate such variation for epidemiological typing, it has been suggested that double locus variants may be classified as belonging to the same outbreak (16, 37). The MLVA typing scheme based on rapidly evolving VNTR loci was shown to be more useful than PFGE in discerning resistant clonal study isolates, which might intensify the

extent of epidemiological surveillance of typhoid fever.

In most of the published studies on MLVA typing of *S*. Typhi, including the present study, sporadic strains were included from different geographical locations for analysis (10, 22, 23, 28, 31, 33). To document the stability of VNTR loci, outbreak strains needed to be analyzed; however, we could not collect these during the study period. Chiou et al. documented considerable genetic diversity in outbreak-causing *S*. Typhi strains by MLVA (36). In outbreaks caused by *S*. Typhimurium and *S*. Enteritidis, VNTR loci of the isolates were found to be generally stable, except for small changes (37, 38). VNTR loci tend to evolve too quickly to provide reliable phylogenetic relationships among closely related strains. Despite the drawback mentioned, MLVA seems to be a more promising tool than PFGE for deciphering the potential sources of infections, especially in outbreak situations as the second-line typing method for bacterial isolates (39, 40, 41).

In conclusion, the results of the present study suggest that MLVA provides rapid turnaround time and high-level discrimination among clonal MDR, NAL^R, and NAL^R-CIP^R *S*. Typhi Indian isolates observed by PFGE. This information may be useful for tracing and controlling the transmission of resistant *S*. Typhi isolates during epidemiological investigations. The method can be adapted to automation and will give a relatively rapid response in an outbreak situation involving genetically homogeneous *S*. Typhi isolates. The VNTR data expressed as alleles based on number of repeats will allow comparison of inter-laboratory data. We have deposited our data online, available at http://minisatellites.u-psud.fr/MLVAnet. This resource may be useful for rapid computerized identification of *S*. Typhi isolates for better understanding of the epidemiology of this organism.

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Conflict of interest None to declare.

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