

Identification and characterization of *Salmonella typhi* isolates from Southwest Sumba District, East Nusa Tenggara based on 16S rRNA gene sequences

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ABSTRACT

Amarantini C, Sembiring L, Kushadiwijaya H, Asmara W (2011) Identification and characterization of *Salmonella typhi* isolates from Southwest Sumba District, East Nusa Tenggara based on 16S rRNA gene sequences. *Biodiversitas* 12: 1-6. The incidence rate of typhoid fever in the Southwest Sumba District, East Nusa Tenggara was approximately about 725/100,000. In spite of such rate, there was not much known-yet about the molecular epidemiology of the disease. Thus, having accurate data and a strong discriminatory ability was crucial to scrutinize the molecular epidemiology of *S. typhi* with a molecular phylogenetic approach based on 16S rRNA gene sequences. Sixteen isolates representative of *S. typhi* from different geographical regions in Southwest Sumba District along with the reference strain *S. typhi* NCTC 786 had been identified and characterized based on 16S rRNA gene sequences using PCR amplification and sequencing. The 16S rRNA sequences data were aligned with the corresponding available *S. typhi* sequences retrieved from the NCBI database by using CLUSTAL X software. Phylogenetic trees were generated with PHYLIP software package. Molecular phylogenetic analysis indicated that all the isolates belong to *S. typhi* species were suggested by their relativity with the type strain of *S. typhi* ATCC19430^T. It was also found that the isolates which belong to *S. typhi* species formed several different centers of diversity within the 16S rRNA gene tree. Each clade consisted of the strains from different geographical places in the District. Thus, to conclude the inquiry, there was evident inter-geographical spread of the strains and it tended to spread further into more remote areas in the District.

Key words: *Salmonella typhi* strains, typhoid fever, 16S rRNA gene sequences, molecular phylogenetic analysis.

INTRODUCTION

There are 17 to 22 million cases of typhoid fever worldwide per year and it causes 216,000 to 600,000 deaths annually (Steele 2008). Based on a preliminary survey, the fever was highly endemic in Southwest Sumba District, East Nusa Tenggara and it infected 725/ 100,000 people per year in the District according to the report made by Karitas Hospital in 2006. Until recently however it has not been under controlled because the limitations of health service units to diagnose the typhoid through laboratory studies. The incidence rate in the area was higher than the incidence in rural areas (358/100,000) or semi rural areas (157/100,000 inhabitants). It was nearly the same as the total number of people infected in urban areas (810/100,000) annually in Indonesia (WHO 2003).

Typhoid fever is an acute systemic infection caused by *Salmonella enterica* subsp. *enterica* serotype *typhi* (*Salmonella typhi*). The acute systemic infection causes *S. typhi* get into the blood stream after passing through several organs in the host. This disease shows clinical

symptoms ranging from mild illness with slight fever, the body feels uncomfortable, coughing and the clinical circumstances such as severe abdominal pain and complications. This condition could often cause difficulties to diagnose it when merely based only on clinical symptom (Muliawan and Surjawidjaja 1999). In addition to such high rate, information about the molecular epidemiology of the disease was unknown. Thus, it was indispensable to have accurate data and a strong discriminative ability to distinguish the types of the strains of the pathogenic bacteria needed in the epidemiological study.

The application of the molecular detection methods for the epidemiological study was advantageous because the genetic profile of the bacteria is a source of information to map the spread of the bacteria in the community. Henceforth it is beneficial to determine best-fitted control strategies over the disease. With the given rationale, this study was aimed to unravel the strain diversities belonging to the *S. typhi* species isolated from typhoid fever patients using a molecular phylogenetic approach based on 16S rRNA gene sequences, and also to understand the spread of *S. typhi* based on its varieties and interrelations among the

strains isolated from the infected patients in Southwest Sumba District, East Nusa Tenggara.

MATERIALS AND METHODS

Bacterial strains

In this inquiry, there were 16 representative isolates of *S. typhi* from different geographical regions in Southwest Sumba District. They were from infected patients in Karitas Hospital in Weetabula, a private clinic in Elopada Subdistrict in Southwest Sumba District, and Lende Moripa Hospital in Waikabubak, West Sumba District. Specimen collection and microbiological identification methods were described in the journal article published previously (Amarantini et al. 2009).

Extraction of bacterial DNA, PCR amplification and DNA sequencing

Bacterial DNA was extracted in accordance with the protocol's instructions using a Purelink™ Genomic DNA Mini Kit (Invitrogen K1820-00). The bacterial DNA and control were amplified with 0.2 μM primers (Invitrogen™) and PCR SuperMix (Invitrogen™ 11306-016). PCR amplification for the 16S rRNA sequences showed bands of 428 bp, 484 bp, and 483 bp. These fragments were amplified using primer SR1/SR2, SR3/SR4, and SR5/SR6 respectively. The primers which were used to amplify these fragments showed at Table 1 (Massi et al. 2005). The PCR mixtures were amplified for 40 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, with a final extension at 72°C for 10 minutes in automated Applied Biosystems GeneAmp PCR System 2400. An aliquot of 10 μL of each amplified product was electrophoresed in 1.5% (wt/vol) agarose gel, with a DNA Molecular Weight Marker (Gel Pilot 100bp Ladder 100 Lanes, Qiagen, Germany) in parallel.

The PCR product was gel purified with a QIAquick PCR purification kit (QIAGEN, Germany). The purified PCR product was sequenced with ABI Prism 3100-Avant Genetic Analyzer in accordance with the manufacturer's instructions (Applied Biosystems, USA) using PCR primers (Massi et al. 2005).

Analysis and alignment of 16S rRNA nucleotide sequences

The 16S rRNA nucleotide sequences were analyzed,

edited and assembled with Finch TV 1.4.0 and DNA Baser sequence analysis software. Complete assembled sequences were aligned with the corresponding *S. typhi* sequences retrieved from the NCBI database with CUSTAL X software (Thompson et al. 1997).

Construction of phylogenetic tree

Based on 16S rRNA nucleotide sequences, a phylogenetic tree was constructed with PHYLIP software package (Felsenstein 1993) with the neighbor-joining algorithm (Saito and Nei 1987). The evolutionary distance matrix for the neighbor-joining method was generated in accordance with the description introduced by Jukes and Cantor (1969). The phylogenetic distances were obtained by adding only the values of the horizontal components. Eventually, the matrix of the nucleotide similarity and difference was generated with PHYDIT software (Chun 1999).

Mapping the spread of *S. typhi* strains in Southwest Sumba District, East Nusa Tenggara

The spread of *S. typhi* strains was isolated from the endemic regions in Southwest Sumba District was mapped according to the infected patients' residences using a satellite navigation system (Global Positioning System /GPS). The spread of the strain types and the phylogenetic relationships among the strains, as the results of the 16S rRNA gene sequencing, were also mapped according to the geographical distribution of the diseases.

RESULTS AND DISCUSSION

Molecular characterization of *S. typhi* strains using phylogenetic approach based on 16S rRNA gene sequences

Based on 16S rRNA gene, the phylogenetic analysis of the 16 isolates of *S. typhi* from different geographical origins in Southwest Sumba District is shown in Figure 1. There were 29 isolates of 16S rRNA gene sequences used in constructing the phylogeny tree. It consisted of 18 *S. typhi* strains, including the type strain of *S. typhi* ATCC 19430^T (accession no. Z47544) which belonged to *S. enterica*, one strain belonged to *S. bongori* BR 1859 (AF029227), and 11 strains from Enterobacteriaceae family [*Escherichia coli* ATCC 25922 (X80724.1), *Citrobacter freundii* ATCC 29935 (M59291.1), *Serratia marcescens* (M59160.1), *Erwinia carotovora* ATCC 15713 (M59149.1), *Yersinia ruckeri* ATCC 29473 (X75275.1), *Yersinia intermedia* ER-3854 (X75279.1), *Hafnia alvei* ATCC 13337 (M59155.1), *Photobacter luminescens* DSM 3368 (X82248.1), *Xenorhabdus nematophilus* DSM 3370 (X82251.1), *Proteus vulgaris* IFAM 1731 (X07652.1), and *Plesiomonas shigelloides* (M59159)]. *Plesiomonas shigelloides* was used as the out group strain in the root position since this species belongs to

Table 1. The primers used for PCR amplification of 16S rRNA gene sequence of *S. typhi* (Massi et al. 2005).

Primer	Sequence	Nucleotide position
SR1	5' AGTTTGATCCTGGCTCAG 3'	3-20 (AC: Z47544)
SR2	5' AGTACTTTACAACCCGAAGG 3'	411-430 (AC: Z47544)
SR3	5' AAGTACTTTTCAGCGGGGA 3'	424-441 (AC: Z47544)
SR4	5' TTGAGTTTTAACCTTGCGG 3'	898-916 (AC: Z47544)
SR5	5' AACTCAAATGAATTGACGG 3'	901-919 (AC: Z47544)
SR6	5' AGGCCCGGGAACGTATTAC 3'	1364-1383 (AC: Z47544)

Note: AC: GenBank accession no.

Enterobacteriaceae family and it formed a separate sub-line of descent of the evolution of *Salmonella*, *E. coli*, and *C. freundii* (Chang et al. 1997).

The result of the phylogenetic analysis (Figure 1) showed that 16 representative isolates of *S. typhi* from various regions in Southwest Sumba District, East Nusa Tenggara belonged to *S. typhi* species because of their relativity with the type strain of *S. typhi* ATCC 19430^T. They formed several different centers of diversity within the 16S rRNA gene tree. All the strains fell into four clades and form a clear center of diversity with the reference strain of *S. typhi* ATCC 19430^T.

The first clade consisted of eight strains, namely BPE 121.1-MC isolates from Weedindi in East Wewewa Subdistrict, RSK 32.1-CCA isolates from Pakamutu in Kodi Subdistrict, two isolates (BPE 122.1-CCA and BPE 122.4-CCA) from Durru Lodo in East Wewewa Subdistrict, other two isolates (BPE 127.1-MC and BPE 127.2-MC) from Elopada in East Wewewa Subdistrict, BPE 123.1-CCA isolates from Kongge in East Wewewa, and one isolates (BPE 120.1-MC) from Omba Rade in East Wewewa Subdistrict.

According to the previous research (Amarantini et al. 2009), two isolates (BPE 121.1-MC and BPE 127.1-MC) were categorized as Biotype III (d-xilose +; l-arabinose +). All the others (RSK 32.1-CCA, BPE 122.1-CCA, BPE 122.4-CCA, BPE 127.2-MC, BPE 123.1-CCA and BPE 120.1-MC) were categorized as Biotype I (d-xilose +; l-arabinose -). Both categories were classified as one single clade. In the first clade, it was evident that the resistant strains and the sensitive strains from the same geographical area could be separated into different subclades. The strains were BPE 127.1-MC and BPE 127.2-MC from Elopada in East Wewewa and BPE 122.1-CCA and BPE 122.4-CCA from Durru Lodo in East Wewewa. This research proved that the phylogenetic classification for the first clade could differentiate two strains with different characters in terms of their nalidixic acid resistance in separate subclade even though they were originally from the same geographical origin.

The second clade consisted of four strains, they were BPE74.1-CCA isolates from Weerambo, East Wewewa, RSL 1.3-SSA isolates from Kampung Sawah, Waikabubak, BPE 7.10-MC isolates from Ombawawi, North Wewewa, BPE 1.1-SSA isolates from Wanowitu, East Wewewa. It meant that four isolates in this clade were closely related with *S. typhi* NCTC 786. According to the database, this strain was a culture collection of HPA in United Kingdom deposited by the Lister Institute (accession data: 01.01.1920). It was isolated in 1920 from a single colony of *S. typhi* Rawlings strains, NCTC 160. According to WHO standard, this strain was categorized in the third group as hazardous pathogens. Thus, people should be very cautious to these strains.

The third clade consisted of three strains; they are RSK 5.1-SSA isolates from Watubero in North Kodi Subdistrict and two isolates (RSK 22.2-CCA and RSK 22.4-CCA) from Pala Mata Loko in North Wewewa. In this clade, the strains belong to Biotype I and Biotype III. All these isolates were sensitive to nalidixic acid.

The last clade was named Clade Palekki since it comprised of the isolates originally from Palekki in North Wewewa Subdistrict. It belonged to Biotype I. This clade was the most different isolates among the sixteen isolates because it was a single-member clade which contained only one isolate (BPE 88.1-CCA).

The 16S rRNA nucleotide similarity values (%) and the number of nucleotide differences among 16 *S. typhi* isolates from the infected patients in different geographical regions in Southwest Sumba District and the reference strain of *S. typhi* ATCC 19430^T are shown in Table 2. All the tested strains possess a 16S rRNA sequence with 99% similarity with the reference strain of *S. typhi* ATCC 19430^T. Based on the similarity scores, these strains were identified as *S. typhi* (Drancourt et al. 2000). They were entirely congruent either between similarity values and nucleotide differences or based on the phylogenetic analysis. Thus, this inquiry concluded that the isolates belong to *S. typhi* species even though they formed several different centers of diversity within the 16S rRNA gene tree.

It was also evident that in terms of similarity and difference of nucleotide (Table 2), BPE 122.4-CCA was identical with BPE 122.1-CCA. BPE 122.1-CCA was also like BPE 121.1-MC and BPE 120.1-MC. The last two strains (BPE 121.1-MC and BPE 120.1-MC) were identical. All these strains were originally from East Wewewa Subdistrict and classified into the first clade. Consequently, the similarity values and the number of nucleotide differences between the strains were completely congruent with the results of the phylogenetic analysis.

Analysis of *S. typhi* isolates according to geographical distribution of the diseases using global positioning system and its public health implications

The map of the *S. typhi* isolates according to the geographical distribution of the diseases using Global Positioning System is shown in Figure 2. It revealed that the phylogenetic relationships of *S. typhi* strains were based on the phylogenetic distance value. In addition, the result of phylogeny tree analysis also shows that each clade was made up of the strains from different places of origin in Southwest Sumba District, East Nusa Tenggara. This fact indirectly answers the hypothesis of inter-geographical spread of the strains.

The spread of the strains within the first clade originated from Durru Lodo, East Wewewa. It reached out Weedindi in East Wewewa Subdistrict and went further out to Pakamutu in Kodi Subdistrict. The transmission was indicated with the brown line. The other three strains of the same clade spread circulatory in the territory of East Wewewa Subdistrict. As indicated by the red line, the strain from Ombarade spread outward to Elopada and Kongge (Figure 2).

In the second clade, the strain from Wanowitu in East Wewewa Subdistrict spread outward to Ombawawi in North Wewewa Subdistrict, and then went further out to Kampung Sawah in Waikabubak Subdistrict and Weerambo in East Wewewa Subdistrict. The spread of these strains is indicated with the purple line (Figure 2).

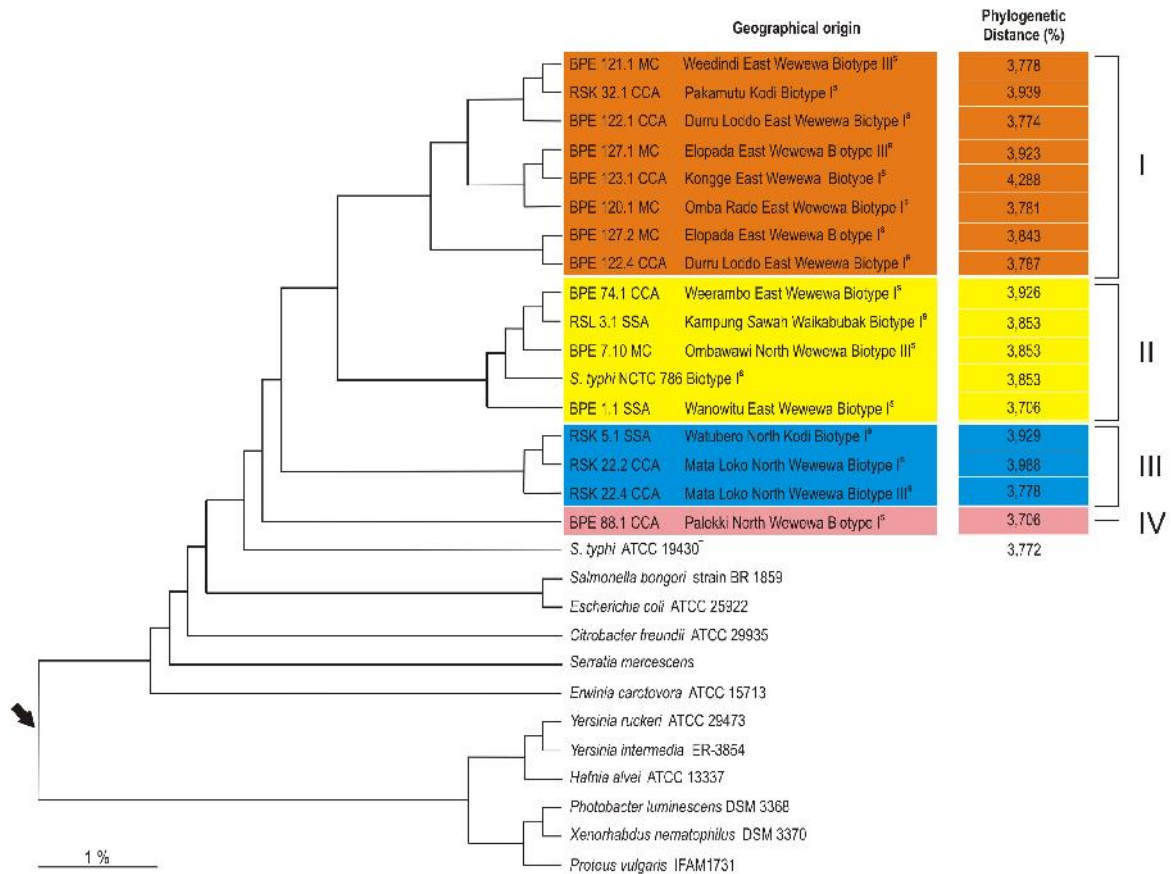


Figure 1. Neighbor-joining (Saitou and Nei 1987) dendrogram represents the phylogenetic relationships of the 16S rRNA gene of *S. typhi* strains from different geographical areas in Southwest Sumba District, East Nusa Tenggara and the representative strains of Enterobacteriaceae family. The arrow indicates estimated root.

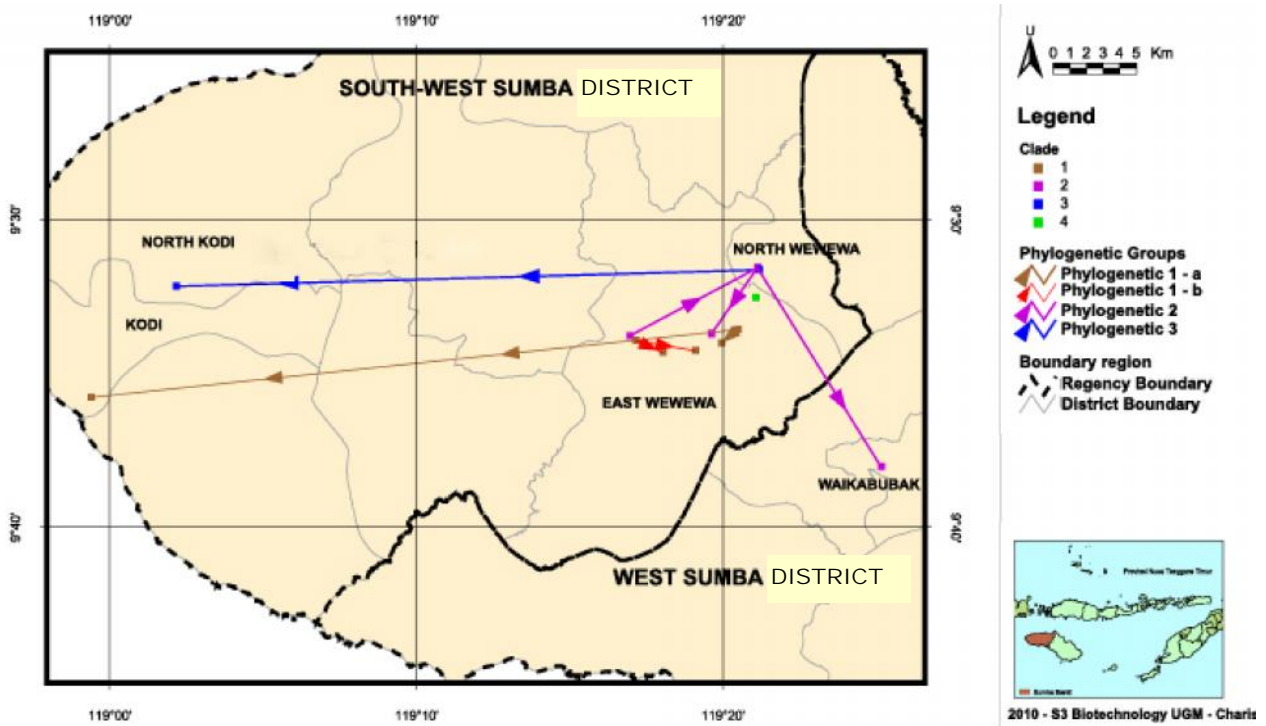


Figure 2. The map of diversity and distribution of *S. typhi* strains in Southwest Sumba District, East Nusa Tenggara based on phylogenetic relationships.

Table 2. 16S rRNA similarities value (%) and the number of nucleotide difference between the sixteen strains of *S. typhi* originated from different geographical places in Southwest Sumba District, East Nusa Tenggara and the reference strain of *S. typhi* ATCC 19430^T.

Strain code	A	P	E	R	K	O	J	B	L	M	D	N	F	H	I	Q	G	Z47544
A	---	5/1384	4/1382	5/1381	4/1382	6/1382	4/1382	6/1382	4/1381	4/1382	5/1380	11/1381	5/1381	3/1381	6/1381	5/1381	4/1381	7/1381
P	99.64	---	3/1382	4/1381	1/1382	3/1382	1/1382	3/1382	1/1381	1/1382	6/1380	8/1381	4/1381	2/1381	5/1381	4/1381	3/1381	6/1381
E	99.71	99.78	---	3/1381	2/1382	4/1382	2/1382	4/1382	2/1381	2/1382	4/1380	9/1381	3/1381	1/1381	4/1381	3/1381	2/1381	5/1381
R	99.64	99.71	99.78	---	3/1381	5/1381	3/1381	5/1381	3/1381	3/1381	6/1380	10/1381	4/1381	2/1381	5/1381	4/1381	3/1381	6/1381
K	99.71	99.93	99.86	99.78	---	2/1383	0/1383	2/1382	0/1381	1/1383	5/1380	7/1381	3/1381	1/1381	4/1381	3/1381	2/1381	5/1381
O	99.57	99.78	99.71	99.64	99.86	---	2/1383	4/1382	2/1381	3/1383	7/1380	8/1381	5/1381	3/1381	6/1381	5/1381	4/1381	7/1381
J	99.71	99.93	99.86	99.78	100	99.86	---	3/1383	0/1381	1/1384	5/1381	7/1382	3/1382	1/1381	4/1381	3/1381	2/1381	5/1381
B	99.57	99.78	99.71	99.64	99.86	99.71	99.78	---	2/1381	3/1383	7/1381	10/1382	6/1382	3/1381	6/1381	5/1381	4/1381	7/1381
L	99.71	99.93	99.86	99.78	100	99.86	100	99.86	---	0/1381	5/1380	7/1381	3/1381	1/1381	4/1381	3/1381	2/1381	5/1381
M	99.71	99.93	99.86	99.78	99.93	99.78	99.93	99.78	100	---	5/1381	7/1382	3/1382	1/1381	4/1381	3/1381	2/1381	5/1381
D	99.64	99.57	99.71	99.57	99.64	99.49	99.64	99.49	99.64	99.64	---	10/1381	6/1381	4/1380	7/1380	6/1380	5/1380	8/1380
N	99.2	99.42	99.35	99.28	99.49	99.42	99.49	99.28	99.49	99.49	99.28	---	10/1382	8/1381	12/1382	11/1382	9/1381	12/1381
F	99.64	99.71	99.78	99.71	99.78	99.64	99.78	99.57	99.78	99.78	99.57	99.28	---	2/1381	5/1381	4/1381	3/1381	6/1381
H	99.78	99.86	99.93	99.86	99.93	99.78	99.93	99.78	99.93	99.93	99.71	99.42	99.86	---	3/1381	2/1381	1/1381	4/1381
I	99.57	99.64	99.71	99.64	99.71	99.57	99.71	99.57	99.71	99.71	99.49	99.13	99.64	99.78	---	1/1382	4/1381	7/1381
Q	99.64	99.71	99.78	99.71	99.78	99.64	99.78	99.64	99.78	99.78	99.57	99.2	99.71	99.86	99.93	---	3/1381	6/1381
G	99.71	99.78	99.86	99.78	99.86	99.71	99.86	99.71	99.86	99.86	99.64	99.35	99.78	99.93	99.71	99.78	---	3/1381
Z47544	99.49	99.57	99.64	99.57	99.64	99.49	99.64	99.49	99.64	99.64	99.42	99.13	99.57	99.71	99.49	99.57	99.78	---

Note: A (RSK 5.1-SSA); B (RSK 32.1-CCA); C (RSL 2.1-CCA); D (RSK 22.2-CCA); E (RSK 22.4-CCA); F (BPE 7.10-MC); G (BPE 88.1-CCA); H (BPE 1.1-SSA); I (BPE74.1-CCA); J (BPE 120.1-MC); K (BPE 121.1-MC); L (BPE 122.1-CCA); M (BPE 122.4-CCA); N (BPE 123.1-CCA); O (BPE 127.1-MC); P (BPE 127.2-MC); Q (RSL 3.1-SSA); R (*S. typhi* NCTC 786); Z47544 (*S. typhi* ATCC 19430^T).

In the second clade, the strain from Wanowitu in East Wewewa Subdistrict spread outward to Ombawawi in North Wewewa Subdistrict, and then went further out to Kampung Sawah in Waikabubak Subdistrict and Weerambo in East Wewewa Subdistrict. The spread of these strains is indicated with the purple line (Figure 2).

In the third clade, the strain from Mataloko in North Wewewa Subdistrict spread outward to Watubero in North Kodi Subdistrict (blue line). There was one strain (RSK 22.4-CCA) from North Wewewa Mataloko, which was originally able to use l-arabinose (biotype III). Later on it shifted into a strain of RSK 22.2-CCA which has no ability to use carbon sources (biotype I). The phylogenetic distance showed that the horizontal line of the strain was longer than that of the strains capable of using arabinose as the source of carbon. This fact proved that the strain of *S. typhi* Biotype I strains came up after the strain of *S. typhi* biotype III.

Based on the distribution of *S. typhi* strains in Southwest Sumba District NTT, it was evident to conclude that the diversity and the spread of the strains were high only in East Wewewa Subdistrict. From the region, it spread outward to nearby subdistricts such as North Wewewa and Waikabubak, and then went further out to Kodi and North Kodi. The phylogenetic relationships of *S. typhi* strains based on the phylogenetic distance value significantly showed that the spread of the diseases as indicated with the blue line and the brown line tends to widen to more remote areas.

This tendency should get attention because the strains which were originally from East Wewewa were very diverse, some of which were known to be resistant to nalidixic acid. Moreover, the people who lived in the remote rural areas have difficult lives, not only poor in economy but also poor in sanitation, food, water, commodities, and limited medical care. Thus, it was necessary to prevent the spread of the disease from East Wewewa Subdistrict and to cut off all related factors supporting its transmission.

CONCLUSION

All *S. typhi* isolates from the infected patients in Karitas Hospital in Southwest Sumba District, East Nusa Tenggara belonged to *S. typhi* species because of their relativity with the type strain of *S. typhi* ATCC 19430^T. The genetic diversity of the strains within *S. typhi* species could be disclosed using a molecular genetic approach based on 16S rRNA gene sequences. It was evident that the isolates belonging to *S. typhi* species form several different centers of diversity within the 16S rRNA gene tree. Each clade consisted of the strains from different places of origin in

Southwest Sumba District, East Nusa Tenggara. Therefore, it was reasonable to conclude that there was inter-geographical spread of the strains and it tended to spread outward to more remote areas.

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