**BIODIVERSITAS**
Volume 23, Number 11, November 2022
Pages: 5806-5813

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d231134

# The diversity and susceptibility against antibiotics of *Salmonella* spp. clinical isolates from Yogyakarta, Indonesia

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Manuscript received: 2 August 2022. Revision accepted: 6 November 2022.

**Abstract.** *Kurniati E, Retnaningrum E, Wijayanti N, Wibawa T. 2022. The diversity and susceptibility against antibiotics of Salmonella spp. clinical isolates from Yogyakarta, Indonesia. Biodiversitas 23: 5806-5813. Salmonella* spp. are bacteria that causes enteric fever. *Salmonella* infection is a serious burden in both developed and developing countries, including Indonesia. Because of infrastructure limitation, *Salmonella* spp. infection treatment mostly based on clinical observation without proper identification of the causative bacteria. Although the clinical manifestation of the diseases is difficult to distinguished, it is believed that *Salmonella* spp. diversity is high in the community. This work aims to analyze the diversity of *Salmonella* spp. and their susceptibility against antibiotics. The *Salmonella* spp clinical isolates obtained from three clinical laboratories were subjected to cultivation, phenotypic characterization, and further biochemical and molecular characterization. Biochemical properties of strains were analyzed, whereas protein profiles were analyzed using the SDS-PAGE method. The molecular characterization was carried out by sequencing of 16S rRNA encoded gene. Numeric-phenetic clustering analysis of the 18 *Salmonella* spp clinical isolates showed that, they were allocated to two large clusters, namely Cluster I and Cluster II. Reconstruction of the phylogeny tree of the 18 clinical isolates and the reference strains showed that all of them belonged to one clade, namely the *Salmonella enterica* subsp. Enterica. However, it was recognized also six sub-clades. The antibiotic susceptibility pattern of *Salmonella* spp. revealed that practically all antibiotics (80%) had a high proportion of susceptibility, except for cefazoline, gentamicin, and amikacin, which have zero susceptibility. Numeric-phenetic clustering analysis combined with phylogeny tree reconstruction showed the diversity of *Salmonella* spp. circulating in Yogyakarta with alarming signal of certain antibiotic resistance.

**Keywords:** Antibiotic resistance, numeric-phenotypic, phylogenetic 16S rRNA, *Salmonella* spp

## INTRODUCTION

*Salmonella* is a genus of pathogenic bacteria that causes enteric fever. This fever includes typhoid fever and paratyphoid fever caused by *Salmonella enterica* subspecies enterica serovar Typhi *S.*, and *paratyphi* A and B (and rarely *S. paratyphi* C), respectively ever is clinically hard to distinguish from typhoid fever (de Kraker et al. 2016; Zghair et al. 2022). It was estimated that globally there are 93.8 million cases of gastroenteritis because of *Salmonella* spp. infection each year with 155,000 deaths (Jiang et al. 2020). *Salmonella* infection is burden in both developing and developed countries (Zha et al. 2019; Jiang et al. 2020) with the estimation of 535,000 (95% uncertainty interval 409000-705000) cases of non-typhoidal *Salmonella* invasive disease occurred in 2017 globally (Stanaway et al. 2019). Thus, understanding and identifying the specific character of *Salmonella* can be the basis for understanding the study of pathogenicity caused by *Salmonella* spp. (Gunn et al. 2014).

Automatic identification tools can be used to characterize the biochemical properties of bacterial species belonging to the Enterobacteriaceae family including enzyme activity and its use of carbon sources (Salaki et al. 2010; Srivastava et al. 2017). Further classification and characterization of Enterobacteriaceae can be done by numerical-phenetic characterization based on biochemical and phenotypic properties of strains (Eng et al. 2015; Britto et al. 2018). These biochemical and protein properties coffering at least 50 parameters can be used to construct a dendrogram for more precise identification of bacteria (Ihsan and Retnaningrum 2020).

Biochemical properties of bacteria can also be analyzed through its protein profile. Numerical analysis of cellular protein profiles obtained by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was reported previously (Aksakal 2010; Sapalina and Retnaningrum 2020). Several studies have shown that there are high similarity values between the DNA hybridization analysis results with the protein profiling by using the SDS-PAGE method (Aksakal 2010; Kumar et al. 2019).

The acute effect of *Salmonella* is known as salmonellosis with symptoms ranging from mild illness to slight fever. Salmonellosis is a food-borne and zoonotic disease that causes serious health problems in humans and animals (Tarazi et al. 2021). The common manifestations

of *Salmonella* infection are gastroenteritis followed by bacteremia and enteric fever (Eng et al. 2015). Antibiotic intervention is needed for the treatment of this diseases. However, many report showed that antibiotic resistance is commonly found in *Salmonella* spp. It was reported in Bangladesh that 64.28% of *Salmonella typhi* was multidrug resistant (Reed et al. 2019) as well as 47.06% in China (Gong et al. 2022). This condition may need extra concern about antibiotic use to eradicate *Salmonella*.

Salmonellosis in Indonesia was frequently misdiagnosed with other infectious diseases because the laboratory confirmatory diagnosis was not properly available in clinical setting (Gasem et al. 2020). Serological diagnostic method has several limitations because of its debatable accuracy, which need to be implemented with geographical consideration Direct detection by using molecular methods has not been a routine laboratory procedure. Culture from blood and other body fluids did not yield good results because of this procedure's limitation to obtain positive results (Arora et al. 2019). Meanwhile, culture is important to isolate the bacteria and perform antimicrobial susceptibility testing for *Salmonella* spp clinical isolates, but the positivity rate of blood culture is limited. This is the reasons of empirical antibiotics treatment for the typhoid and non-typhoid patients are commonly prescribed based on the clinical findings (Vollaard et al. 2005; Gasem et al. 2020).

Historically, well known antibiotics such as chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole were the first-line drugs for typhoid fever therapy (Marchello et al. 2020). However, the emergence of multidrug-resistant (MDR) in the 1980s-1990s globally including Southeast Asia led to intensive study regarding antibiotic resistance in *Salmonella* (Rowe et al. 1997; Marchello et al. 2020). The most common pattern of antibiotic resistance (45%) was found in *S. typhimurium* (Britto et al. 2018). This resistance pattern includes the ASSuT (ampicillin, streptomycin, sulfonamides, and tetracycline) pattern and the Penta-resistant pattern ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (Wang et al. 2019). This phenomenon causes the need for new specific identification of *Salmonella* and updated treatment strategies for these pathogens (Mellon et al. 2001; Sneeringer et al. 2015).

The aim of this work is to analyze the correlation between the diversity of *Salmonella spp* circulating in Yogyakarta with its susceptibility against antibiotics commonly used in the clinics for patients suffering from *Salmonella* spp infections. Our work showed that the diversity of *Salmonella* spp. in Yogyakarta, one of the provinces of Indonesia, was not correlated with the susceptibility against antibiotics. The numerical-phenetics combined with genetics approach may be applicable to show the diversity of *Salmonella* spp. circulating in Yogyakarta. However, the application of this method to provide pivotal data for clinical application are still challenging because of practical reason in the clinical setting.

## MATERIALS AND METHODS

### *Salmonella* spp. clinical isolates

The samples of *Salmonella* spp used in this study were obtained from clinical isolates of Dr. Sardjito Hospital, Universitas Gadjah Mada (UGM) Academic Hospital, and Health Laboratory Center at Yogyakarta, Indonesia. The sample groups used were encoded with A2, A3, A4, A6, A7, A9, A10, A11, A12, A13, A14, A15, A17 (from Sardjito Hospital), B1 (from UGM Academic Hospital), K+ as the positive control, C1, C2 and C3 (from Yogyakarta Health Laboratory Center).

### Bacterial cultivation

Samples from different locations were inoculated on nutrient broth (Merck, Catalog 1.05443.0500) and incubated for 24 hours at 37°C. After colonies were growth, some were transferred to blood agar media (BAP) (Merck, *Catalog* 1.10886.0500) and MacConkey agar media (MCA) (Merck, *Catalog* 1.05465.0500). All samples were incubated for 24 hours at 37°C. Colony morphology and Gram staining characterization were performed. The rest of the colonies were stored in the refrigerator at 4°C.

### Biochemical and antibiotic susceptibility test

A bacterial suspension was prepared by transferring a bacterial colony into NaCl solution (0.85 %) with turbidity equal to 0.5 McFarland standard. The bacterial suspension was inserted into the first card for bacterial identification and the second card for sensitivity testing. Then enter the sample data with the barcode system and insert the card into the VITEK® 2 Version: 08.01 (Biomerieux, USA). The whole process of inoculation, incubation, reading, validation, and interpretation of the results will be carried out automatically by the machine.

### Protein profile analysis

Proteins were isolated from the bacterial culture and then quantified using the Bradford method and subjected to protein separation using acrylamide gel (SDS-PAGE). The separated protein band pattern in SDS-PAGE was then stained with silver stain, scanned for the protein band pattern formed. The molecular weight of the protein was determined by calculated the Rf values and plotted on a logarithmic graph of the Rf marker protein whose molecular weight is known.

### Molecular analysis

*DNA Extraction and Amplification of 16S rRNA*

*Salmonella* sp. clinical isolates were grown in nutrient broth media (Merck *Catalog* 1.05443.0500). The DNA extraction was carried out using PurelinkTM Genomic DNA Mini Kit (Invitrogen *TM Catalog number* K1820-02) according to the procedure.

*The 16S rRNA Gene PCR Amplification*

The extracted DNA was used as a DNA template for amplification using PCR. The composition of each reaction consisted of 0.5 uL of DNA template with 2 uL of forward and reverse primer, 25 uL of MyTaqTM HS Red Mix

(Bioline: BIO21127), and UltraPureTM ddH2O (Invitrogen) until the total volume reaches 50 uL. The primers used were 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3'. Amplification was carried out at 94°C for 2 minutes (initial denaturation) and then 35 cycles at 95°C for 1 minute (denaturation), 55°C for 1 minute (annealing), 72°C for 2 minutes (extension) and extra extension at 72°C for 5 minutes followed by hold temperature at 4°C. The amplicons were analyzed using electrophoresis in 1.5% agarose gel stained with SYBR Safe DNA in TBE solution.

*The 16S rRNA sequence gene analysis and construction of the phylogenetic tree*

The 1,500 bp PCR amplified gene was used for further analysis using MEGA software. The 16S rRNA nucleotide sequences of *Salmonella* found in GenBank included: *S. enterica* LT2, *S. diarizonae* DSM 14847, *S. salamae* DSM 9220, *S. houtenae* DSM 9221, *S. indica* DSM 14848, *S. arizonae* ATCC 13314, *S. bongori* BR 1859, *S. subterranea* FRCl, *S. enterica* NBRC, *S. typhimurium* ATCC 13311, *S. choleraesuis* DSM 14846, *S. typhi* ATCC 19430, *S. enteritidis* ATCC 13076, *S. bongori*_DSM_13772, *S. arizonae* DSM and *B. subtilis* IAM 12118 which is used as an outgroup. Based on the 16S rRNA nucleotide sequences, the phylogenetic tree of *Salmonella* was constructed using the neighbor Joining Algorithm method with 1000x replication and the Kimura method for genetic distance using the MEGA-X program. The resulting phylogenetic tree is then used as the basis for determining the relationship between bacteria samples.

**Analysis**

The data were analyzed using MVSP Plus software version 3.1 (Kovach 2007) aimed to determine the similarity between strains. The similarity value is determined using a simple matching coefficient (SSM), the classification done by using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithm (Gould 1974). The results of the analysis are presented as a dendrogram using Corel Draw X5. Therefore, dendrogram results are used as a basis for determining the similarity between strains.

## RESULTS AND DISCUSSION

**Identification and biochemical test**

We analyzed 18 *Salmonella* spp which were isolated from patients from three different hospitals with one control *Salmonella sp*. The characterization of colony morphology was carried out on the blood agar plates (BAP) and MacConkey Agar (MCA). On the BAP medium, the colonies were observed cloudy white, medium in size, and nonhemolytic. On MCA media, bacterial colonies were clear, shaped in medium-size and non-lactose fermenters. The results of gram staining of colonies on BAP media were rod-shaped bacteria and gram-negative.

The identification of the clinical isolate based on biochemical test was performed by using Viteks2 automated system are shown in Table 1. Most of the clinical isolates showed positive reactions in the D-glucose test, $H_2S$ production, D-glucose fermentation, positive for D-mannose, D-maltose, D-mannitol, D-trehalose, alpha-galactosidase, Citrate/Sodium (CIT), and a positive test for Lysine Decarboxylase and Coumarate (CMT) (Figure 1). However, the biochemical tests showed huge varieties of result among the isolates. This is indicative that the biochemical test results are not homogenous among *Salmonella* spp. identified using routine biochemicals test performed in the clinical laboratories.

**Table 1.** The identification of Clinical Isolate based on biochemical test

| Sample code | Identification result | Probability (%) | Confidence |
|---|---|---|---|
| A2 | *Salmonella spp* | 98 | Excellent identification |
| A3 | *Salmonella typhi* | 89 | Good identification |
| A4 | *Salmonella typhi* | 90 | Good identification |
| A6 | *Salmonella spp* | 94 | Very good identification |
| A7 | *Salmonella spp* | 99 | Excellent identification |
| A9 | *Salmonella typhi* | 90 | Low discrimination |
| A10 | *Salmonella spp* | 98 | Excellent identification |
| A11 | *Salmonella spp* | 98 | Excellent identification |
| A12 | *Salmonella spp* | 98 | Excellent identification |
| A13 | *Salmonella spp* | 94 | Very good identification |
| A14 | *Salmonella spp* | 98 | Excellent identification |
| A15 | *Salmonella spp* | 98 | Excellent identification |
| A17 | *Salmonella paratyphi* A | 95 | Very good identification |
| B1 | *Salmonella typhi* | 98 | Exellent identification |
| K+ | *Salmonella spp* | 99 | Exellent identification |
| C1 | *Salmonella typhi* | 90 | Good identification |
| C2 | *Salmonella spp* | 99 | Exellent identification |
| C3 | *Salmonella paratyphi* B | 88 | Low discrimination |

Note: the biochemical test were performed by VITEK® 2 Version: 08.01(Biomerieux, USA).

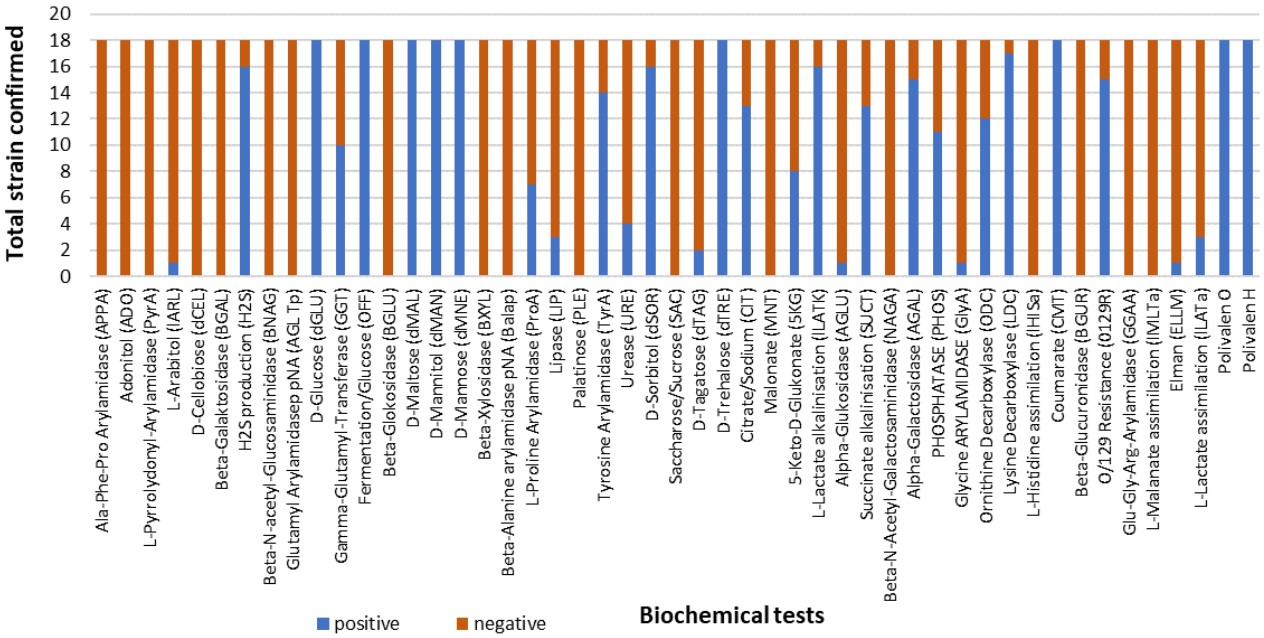


**Figure 1.** The proportion of positive and negative result of biochemical tests performed to 18 *Salmonella* spp clinical isolates

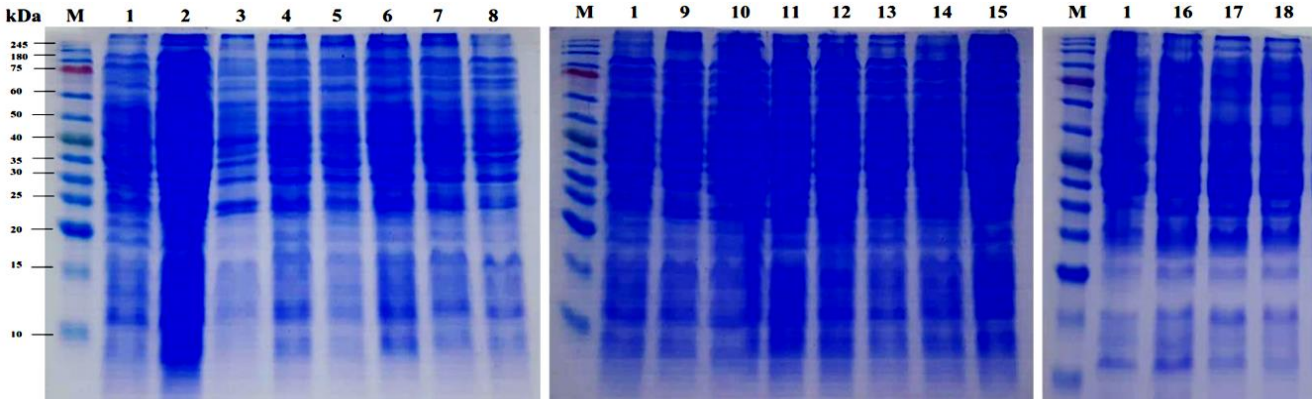


**Figure 2.** The protein banding pattern on SDS-PAGE for 20 isolates with M, 1-18 refers to *Salmonella typhimurium* strain with code of Marker protein (Tris-Glycine), positive control (K+), A2, A3, A4, A6, A7, C1, A9, A10, A11, A12, A17, C2, A13, A14, A15, B1, and C3, respectively

**Proteins profile**

The protein profile of clinical isolates was required to determine their diversity and correlated with the pathogenicity of *Salmonella*. *Salmonella*'s outer proteins (sops) (SPI effector protein) encoded by sop gene have relevance to *Salmonella* virulence (Thung et al. 2018). The type III protein secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) also delivers effector proteins required for intestinal invasion and the production of enteritis. The T3SS is regarded as the most important virulence factor of *Salmonella* (Lou et al. 2019). The diversity of protein leads to the different pathogenicity of *Salmonella*. The SDS page electrophoresis results were clearly shown the diversities on the visual profile (Figure 2). Most of the strains showed multiple bands between 245-10 kDa.

The results of the biochemical analysis and protein profiles were further analyzed using the Simple Matching Coefficient (SMC) and the UPGMA algorithm, then displayed in the form of a dendrogram (Figure 3). Numeric-phenetic clustering analysis of the 18 clinical isolates and control of *Salmonella* spp showed that they were allocated to two large clusters, namely Cluster I and Cluster II. Cluster I consisted of only B1 clinical isolate with a similarity index of 84.6%. While Cluster II consists of the rest of other clinical isolates, which has a similarity value of 87.4%.

*Salmonella* nomenclature system, which has been recommended by the World Health Organization (WHO) Collaborating Centre (WHO 2011; WHO: Standards 2018) having the genus classification into two species, namely *Salmonella enterica* (species type) and *Salmonella bongori*. Phenotypically, all *Salmonella* sp. has morphological characteristics in the form of rods, Gram-negative, and facultative anaerobes. This genus classification (*S. enterica* and *S. bongori*) was also conducted based on its difference in the 16S rRNA sequence analysis (Taşkale Karatuğ et al. 2018; Jajere 2019).

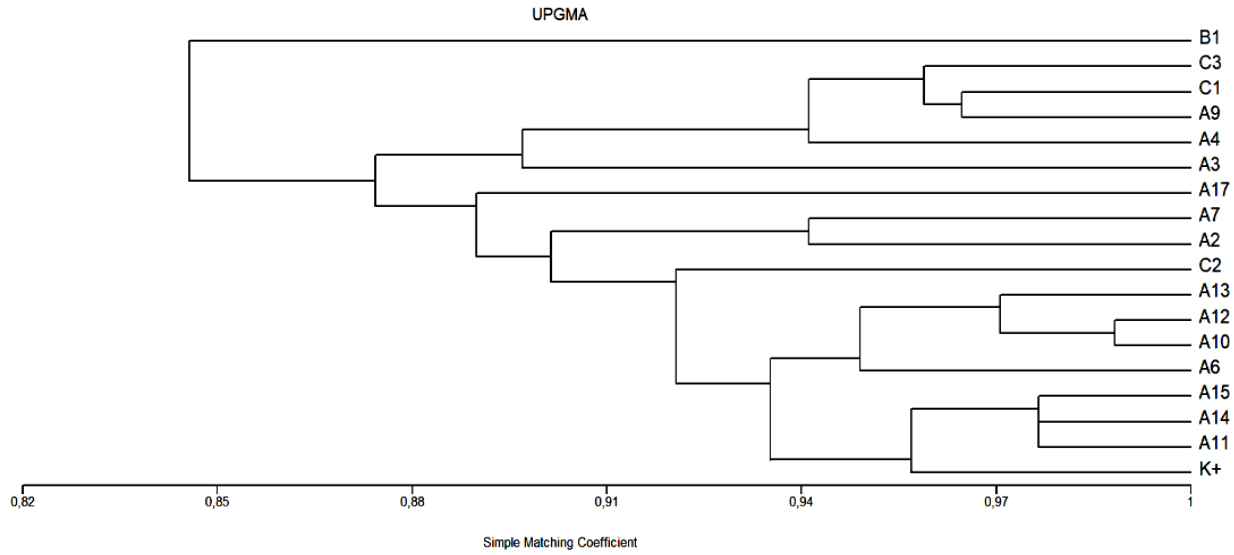


**Figure 3.** Dendrogram of phenotypic biochemical and protein character based on Simple matching Coefficient similarities between 18 strains of *Salmonella* spp. bacteria from 3 different locations in Yogyakarta, Indonesia based on Simple Matching Coefficient (SMC) analysis and UPGMA algorithm. *Salmonella* spp clinical isolates and K+ as a positive control

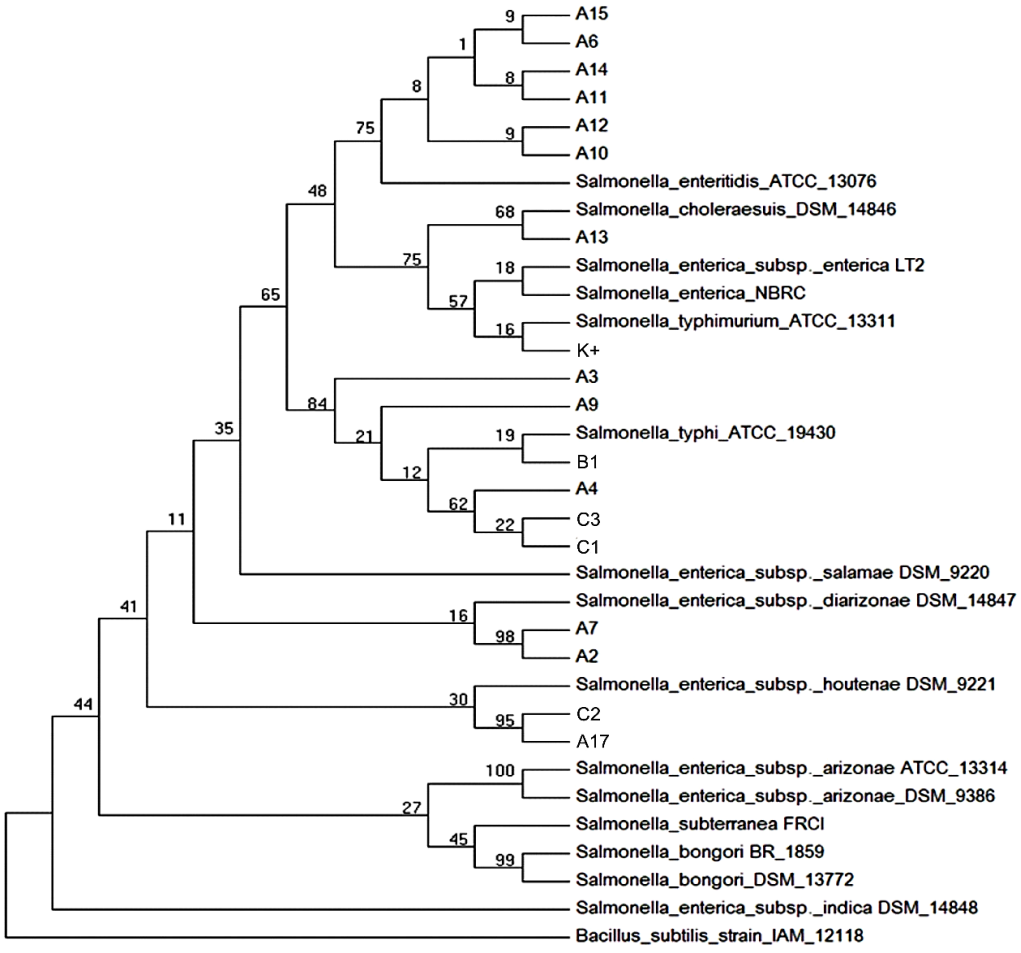


**Figure 4.** Phylogenetic tree among eighteen isolates and reference sequence, based on full 16S rRNA sequences of *Salmonella* spp. All of them belonged to one clade, namely the *Salmonella enterica* subsp. Enterica

**Table 2.** The results of Antibiotic Susceptibility Test (AST) of *Salmonella* spp.

| Antibiotics | Sensitivity (%) | | |
|---|---|---|---|
| | Sensitive | Intermediate | Resistant |
| Amoxicillin | 88.9 | 0 | 11.1 |
| Ampicillin | 72.2 | 0 | 27.8 |
| Sulbactam | 72.2 | 11.1 | 16.7 |
| Piperacillin | 94.4 | 0 | 5.6 |
| Cefazolin | 0 | 0 | 100 |
| Cefotaxime | 94.4 | 0 | 5.6 |
| Ceftazidime | 94.4 | 0 | 5.6 |
| Ceftriaxone | 94.4 | 0 | 5.6 |
| Cefepime | 94.4 | 0 | 5.6 |
| Aztreonam | 88.8 | 5.6 | 5.6 |
| Ertapenem | 94.4 | 0 | 5.6 |
| Meropenem | 94.4 | 0 | 5.6 |
| Amikacin | 0 | 0 | 100 |
| Gentamicin | 0 | 0 | 100 |
| Ciprofloxacin | 0 | 88.9 | 11.1 |
| Tigecycline | 100 | 0 | 0 |
| Nitrofurantoin | 77.8 | 0 | 22.2 |
| Trimethoprim | 94.4 | 0 | 5.6 |
| Chloramfenicol | 94.4 | 0 | 5.6 |

Morphological phenotypic analysis and biochemical activity can provide information on isolates, it has not been able to provide sufficient information, so other characters are needed in the form of analysis based on protein profiles. Except for deteriorating genomes of some parasitic bacteria, the prokaryotic genomes are highly compact, with densely packed protein-coding genes and a low fraction of noncoding sequences of the clinical isolates (Sela et al. 2016; Thung et al. 2018), and it is associated with determining the total protein component that has an important role in the classification, identification, typing, and comparative studies of bacteria. The use of SDS-PAGE is also an important tool for identification at the species level. In this analysis, protein profile data is important to provide more detailed information, regarding the identification and characterization of organisms (Aksakal 2010).

The information gathered from biochemical tests and protein profiles was then used to conduct clustering analysis called numerical phenetic. The numerical-phenetic clustering process can also be carried out based on the protein profile of the tested bacterial strain (VT Nair et al. 2018; Nguyen Thi et al. 2020)

Several studies have been conducted to analyze the diversity of *Salmonella* by using the SDS-PAGE profile (Hatta and Ratnawati 2008; Beig et al. 2010). In a previous study (Darmawati et al. 2011), the *S. typhi* bacteria group can be classified based on micro-morphology, colony morphology, carbon intake source, and enzyme production. Research-based on simple matching coefficient (SSM) shows that there are four clusters with different biochemical characters in Central Java and Yogyakarta. Research (de Carli et al. 2018) showed that S. typhi can be classified into 2 biotypes based on its ability to ferment D-Xylose and L-Arabinose. Biotype I is capable of fermenting D-Xylose and not fermenting L-Arabinose, and biotype III is capable of fermenting D. -Xylose, and L-Arabinose (de Kraker et al. 2016).

**16S rRNA Gene Sequencing Analysis**

The 16S rRNA genes were amplified and subjected for DNA sequencing. The 1,500 bp full segment of 16S rRNA DNA sequences was retrieved and subjected for phylogenetics tree construction. Reconstruction of the phylogeny tree of the 18 clinical isolates and the reference strains showed that all of them belonged to one clade, namely the *Salmonella enterica* subsp. Enterica. However, it was recognized also six sub-clades as indicated in Figure 4.

**Antibiotic Susceptibility Test**

The susceptibility test of the clinical isolates against antibiotic was performed with antibiotics recommended for *Salmonella* spp. The antibiotic susceptibility pattern showed that almost all antibiotics have good percentage of susceptibility (≥ 80%) except cefazoline, gentamicin, and amikacin, which is zero (Table 2).

We report *Salmonella* spp clinical isolates included in this study showed 100% resistant to cefazoline, gentamicin, and amikacin, despite there are limited report regarding to the antibiotic susceptibility report of *Salmonella* spp isolated from human (Heithoff et al. 2008; Elkenany et al. 2019). The group of aminoglycoside enzymes, including aminoglycoside acetyltransferases, can modify amino groups in aminoglycoside antibiotics, including gentamicin and kanamycin. The gene encoding this enzyme is found in *Salmonella* genomic islands, integrons, and plasmids (Shaikh et al. 2015; VT Nair et al. 2018).

Our work showed that the numerical-phenetic analysis able to improve the identification of *Salmonella* spp clinical isolates. The diversity of *Salmonella* spp circulating in Yogyakarta was successfully described by numerical-phenetic analysis, though because practical reason it might not be applicable for daily service in the hospital setting. Table 1 showed the usual identification result which reported to the clinician by clinical laboratory in the hospitals. The limitation of numerical-phenetic analysis are the need of longer time and extensive laboratory work compared to culture and automated identification applied in the clinical microbiology laboratory in the hospital.

Limitations are identified in our study; the sample size is considered small to capture the whole community of our study population. There was no time frame of the clinical isolates collection which make it impossible to analysis the data according to the time which is important for antimicrobial resistant data reporting. The patients' clinical data were not collected, resulting to the lack of information regarding to the illness or carrier status of the subjects. Based on this study, it can be concluded that numeric-phenetic clustering analysis combined with phylogeny tree reconstruction showed the diversity of *Salmonella* spp. circulating in Yogyakarta with alarming signal of certain antibiotic resistance.

## ACKNOWLEDGEMENTS

The authors thank the Department of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia for the generous support in this research, and the Directorate of Research Universitas Gadjah Mada for financial support through Final Project Recognition 2021.

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