ORIGINAL ARTICLE

Analysis of existence of multidrug-resistant H58 gene in *Salmonella enterica* serovar Typhi isolated from typhoid fever patients in Makassar, Indonesia

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Abstract

The surveillance of multidrug-resistant (MDR) H58 typhoid is highly important, especially in endemic areas. MDR strain detection is needed by using a simple PCR technique that only uses a pair of primers. This is conducted considering the detection of *Salmonella* Typhi strains that have been carried out so far are only using antimicrobial sensitivity tests to determine microbial resistance phenotypically and to determine genotypically using complex molecular techniques. We aimed to analyse the existence of *Salmonella* Typhi MDR H58 in patients with typhoid fever in Makassar, Indonesia. A total of 367 blood samples of typhoid fever patients were collected from April 2018 until April 2019. The blood sample was cultured, then confirmed via simple PCR. All of the confirmed samples were tested for susceptibility against antibiotics and molecularly analysed for MDR H58 existence using a simple PCR technique. We found 7% (27/367) of the samples to be positive by both blood culture and PCR. All 27 isolates were found to be sensitive to sulfamethoxazole/trimethoprim. The lowest drug sensitivities were to amoxicillin, at one (3.7%) of 27 isolates, and ampicillin, at 13 (48.1%) of 27 isolates. *Salmonella* Typhi H58 PCR results showed that one (3.7%) of 27 isolates carried a positive fragment of 993 bp that led to the H58 strain, since the deletion flanks this fragment. The isolate was also found to be resistant to amoxicillin and fluoroquinolone according to a sensitivity test. Further molecular analysis needs to be conducted to examine the single isolate that carried the 933 bp fragment.

Keywords: Antibiotic resistance, Antimicrobial sensitivity test, MDR, *Salmonella enterica* serovar Typhi H58, simple PCR
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Introduction

Typhoid fever is an infectious disease common in developing countries. Globally, typhoid fever was reported in 26.9 million cases in 2010 [1]. In Indonesia, typhoid fever is an endemic disease, with 81.7 cases per 100 000 people per year [2]. South Sulawesi, one of the five largest islands in Indonesia, is reported to be one of the islands with the highest incidence of typhoid fever. The cases detected in 1991 reached 257 cases per 100 000 population and increased to 386 cases per 100 000 population in 2007 [3]. In 2014, in the health profile of South Sulawesi province, it was reported there were 23 271 suspected cases of typhoid disease occurring in 11 723 men and 11 548 women, while 16 743 patients had typhoid fever. The report also mentioned that Makassar had one of the highest case loads, with up to 2325 cases [4].

In developing countries, the antibiotics often used to treat typhoid fever are chloramphenicol, ampicillin and cotrimoxazole [5]. Chloramphenicol resistance was first reported in 1950. In the early 1970s, it was found that *Salmonella enterica* serovar Typhi was resistant to chloramphenicol and ampicillin, and soon there was resistance to these three types of antibiotics, resulting in multidrug resistance (MDR) [6]. In Indonesia, cases of typhoid fever associated with MDR in chloramphenicol,

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ampicillin and cotrimoxazole tend to increase every year. In South Sulawesi, *Salmonella* Typhi resistance to antibiotics before 2001 was reported to be very low (<1%) and from 2001 to 2007 showed an MDR increase of 1.2% to 6.8% [3].

Antibiotic resistance in microbes can be detected by several methods of antimicrobial susceptibility tests. Each has its advantages and disadvantages [7]. There is considerable interest in the possibility of using molecular genetic methods for the detection of antimicrobial resistance mechanisms with certain genes of antibiotic resistance. The tests give highly sensitive and specific results, although they cannot substitute anytime soon for phenotypic methods in routine antimicrobial susceptibility because the presence of these genes may not be expressed or may not result in clinically relevant levels of resistance [7,8]. Accurate and rapid detection methods are required to detect MDR pathogens or genes. Molecular detection as a complement to conventional phenotypic analysis will enable us to confirm the presence of specific resistance mechanisms that may interfere with infection control; analyse the spread of specific pathogens for molecular epidemiologic purposes; and detect resistance mechanisms in slow-growing organisms and genetic elements. PCR is one of the most frequently used molecular detection tools used to detect determinants of resistance and to conduct surveillance of antimicrobial-resistant bacteria [8].

Haplotype 58, or H58, is a multidrug-resistant (MDR) strain of *Salmonella* Typhi that is also resistant to nalidixic acid, leading to reduced sensitivity against fluoroquinolone antibiotics [9]. A previous study has noted that H58 is a single genotype of *Salmonella* Typhi which dominates the global spread [10].

Previous studies have used various methods to identify H58, including whole genome sequencing [11–14], genotyping [15], single nucleotide polymorphism typing [6,16,17] and multiplex ligation-dependent probe amplification [10]. These examination methods are expensive and require laboratory facilities and molecular biology laboratories, which is inconvenient, especially for developing countries like Indonesia. Simple PCR examination should permit a molecular examination with high sensitivity and specificity.

Materials and methods

A total of 367 blood samples from typhoid fever patients were collected from April 2018 to April 2019; 50 were from a public hospital and 317 were from primary health centres in Makassar, Indonesia.

Culture and identification

Blood was placed into a medium of bile salts (Oxoid, Basingstoke, UK), then incubated at 35°C to 37°C for 24 hours. The growing colonies were then inoculated into *Salmonella–Shigella* agar medium (Merck, Darmstadt, Germany) and incubated at 37°C for 24 hours. After incubation, colonies were observed and tested biochemically using triple sugar iron medium (Merck), methyl red–Voges-Proskauer medium (Merck), solid indol motility medium (Oxoid), Simon citrate agar medium (Oxoid), urea medium (Merck) and carbohydrate fermentation medium of lactose, sucrose, mannitol and glucose (Merck), then incubated at 35°C to 37°C for 18 to 24 hours.

Disc diffusion test

Dilution was carried out from bacterial suspensions obtained from *Salmonella* Typhi cultures to determine the turbidity level using McFarland 0.5 turbidity standards containing 1.5×10^8/mL bacteria. Bacteria were then plated on the surface by swabbing them evenly on Müller-Hinton agar medium (Oxoid), then left for 10 minutes so that the bacteria could stick to the surface of the media. Each disc containing antibiotics was then placed on Müller-Hinton agar and incubated at 37°C for 24 hours. The antibiotic disks (Oxoid) contained ampicillin (10 μg), amoxicillin (30 μg), sulfamethoxazole/trimethoprim (SXT; 25 μg), ceftriaxone (30 μg), cefepime (30 μg), cefixime (5 μg), ofloxacin (5 μg) and chloramphenicol (30 μg). The diameter of the inhibition zone was measured and interpreted on the basis of Clinical and Laboratory Standards Institute criteria. Specifically, organisms were considered resistant if the diameter of the zone of inhibition was $\leq$13 mm for ampicillin, 13 mm for amoxicillin, 10 mm for SXT, 13 mm for ceftriaxone, 18 mm for cefepime, 15 mm for cefixime, 12 mm for ofloxacin and 12 mm for chloramphenicol [18].

DNA isolation

DNA isolation was performed using guanidium thiocyanate solution (Fluka, Buchs, Switzerland), where as much as 1 mL of *Salmonella* Typhi culture was centrifuged and the supernatant discarded. For the cell lysis step, 600 μL of nuclei lysis solution (Promega, Madison, WI, USA) was added into the precipitate by pipetting gently until well mixed. It was incubated for 5 minutes at 80°C, then cooled to room temperature. We added 3 μL RNase solution (Promega), mixed well and incubated it at 37°C for 15 to 60 minutes, then cooled to room temperature. Furthermore, protein precipitation was carried out by adding 200 μL protein precipitation solution (Promega), which was vortexed and incubated on ice for 5 minutes, then centrifuged at 13 000 to 16 000 × *g* for 3 minutes. DNA precipitation and rehydration were carried out by transferring the supernatant to a new tube containing 600 μL isopropanol (Merck) at room temperature. After being evenly mixed, centrifugation was performed and the supernatant separated. Into the sediment was added 600 mL of 70% ethanol (Merck) at room

temperature, which was mixed evenly and then centrifugated for 2 minutes at 13 000 to 16 000 × *g*. Ethanol aspiration was conducted and the feculence then dried for 10 to 15 minutes. Rehydration of DNA deposits was performed by placing it in a rehydration solution for 1 hour at 65°C [19–21].

Detection of *Salmonella* Typhi DNA

A total of 2 µL of DNA extract was placed into a PCR tube containing 22.5 µL PCR mix (Kapa2G Fast ReadyMix; Sigma-Aldrich, St Louis, MO, USA) consisting of 2.5 µL 10 × buffer, 0.5 µL Taq DNA polymerase, 2 µL $MgCl_2$, 2.5 µL dNTP and 15 µL double-distilled water. Primers (each containing 1 µL) were as follows (Macrogen, Seoul, Korea): forward, 5′-ACTGC-TAAAACCACTACT-3′ and reverse, 5′-TTAACGCAGTAAA-GAGAG-3′. The primer for regular PCR, used to amplify a 458 bp fragment, corresponded to nucleotides 1063 to 1530 of the VI region of the flagellin gene [21]. Amplification was carried out using a PCR machine (Applied Biosystems 2720 Thermal cycler; Applied Biosystems; Thermo Fisher Scientific, Waltham, MA) in stages, with an initial denaturation at 95°C for 10 minutes; 35 cycles were then carried out, each cycle comprising denaturation at 96°C for 30 seconds, annealing at 56°C for 30 seconds, then extension at 70°C for 1 minute followed by 72°C for 1 minute.

Detection of *Salmonella* Typhi H58 MDR

This process was carried out on isolated DNA samples. First, a PCR mix was made which would be amplified as much as 22.5 µL, which comprised 2.5 µL 10 × PCR buffer, 0.5 µL Taq polymerase, 2 µL $MgCl_2$, 2 µL dNTPs and 13.5 µL distilled water (Kapa2G Fast ReadyMix; Sigma-Aldrich), to which was added the forward and reverse primers (Macrogen), each containing 1 µL. As much as 2.5 µL of DNA extract was added to 22.5 µL of the mixture of forward (5′-AATAGGCCTCATCACGTTCG-3′) and reverse (5′-CAAACCGTTGAATCGGAAGT-3′) primers [11]. This primer produced an amplification product of 993 bp. Then amplification was carried out, with the first stage comprising 94°C for 2 minutes, followed by 40 cycles of 60 seconds each at 94°C, 45 seconds at 57°C and 60 seconds at 72°C. This process was continued at 72°C for 2 minutes using a PCR machine (Applied Biosystems 2720 Thermal cycler). Next, the amplification results from the PCR mixture were taken (2.5 µL each) and placed into the loading buffer mixture for electrophoresis.

Detection of PCR products

Each 2.5 µL PCR amplification product was mixed with a 2 µL loading solution. The mixture was then pipetted into a 2% agarose gel (Vivantis Technologies, Subang Jaya, Malaysia) well submerged in a Tris–borate–EDTA buffer in an electrophoresis tank. Electrophoresis was run for 1 hour at a constant voltage of 75 V. After 1 hour, the electrophoresis was stopped, and the gel was removed for observation under UV light. Electrophoresis results were documented with a camera.

Results

Blood culture results

During the period of study, April 2018 to April 2019, a total of 367 blood samples were collected from patients suspected to have typhoid fever. Among these, we found 32 positive cultures. On the basis of biochemical test results, 30 cultures (8.2%) were positive for *Salmonella* Typhi (two isolates were from a public hospital and other 27 isolates were from various health centres in Makassar), and two cultures (0.5%) were positive for Salmonella Paratyphi.

PCR inspection results

We conducted confirmation via PCR of 30 culture-positive *Salmonella* Typhi isolates. On the basis of this examination, 27 (90%) of 30 isolates confirmed *Salmonella* Typhi and three (10%) of 30 isolates confirmed negative *Salmonella* Typhi.

Antibiotic sensitivity test

All 27 isolates were sensitive to only one antibiotic: SXT. The most common resistance was to amoxicillin (Table 1). Twenty-six (96%) of 27 isolates were resistant to amoxicillin, 14 (52%) to ampicillin and three (11%) to cefixime, with one isolate (3.7%) resistant to each of the rest: ceftriaxone, cefepime, ofloxacin and chloramphenicol. The results of the antibiotic sensitivity tests against *Salmonella* Typhi showed the existence of resistance to one or more antibiotics. Twenty (74%) of 27 isolates were monoresistant and six (2%) of 22 were poly-resistant (Table 2).

PCR examination of *Salmonella* Typhi H58

Salmonella Typhi H58 PCR results showed that one (3.7%) of 27 isolates carried a positive fragment 993 bp in size with deletions

TABLE 1. Antibiotic sensitivity of *Salmonella enterica* serovar Typhi isolates

Antibiotic	Resistant (n = 27)	Sensitive (n = 27)
Sulfamethoxazole/trimethoprim	0	27 (100)
Ceftriaxone	1 (3.7)	26 (96.3)
Chloramphenicol	1 (3.7)	26 (96.3)
Cefepime	1 (3.7)	26 (96.3)
Ofloxacin	1 (3.7)	26 (96.3)
Cefixime	3 (11)	24 (88.9)
Ampicillin	14 (52)	13 (48.1)
Amoxicillin	26 (96)	1 (3.7)

Data are presented as *n* (%).

TABLE 2. Types of monoresistant and polyresistant antibiotics of 27 *Salmonella enterica* serovar Typhi isolates

Antibiotic	*N* (%)
Polyresistant	
Amoxicillin, ampicillin, cefixime	1 (3.7)
Amoxicillin, cefixime	1 (3.7)
Amoxicillin, cefixime, ofloxacin	1 (3.7)
Amoxicillin, chloramphenicol	1 (3.7)
Amoxicillin, ceftriaxone	1 (3.7)
Amoxicillin, cefepime	1 (3.7)
Monoresistant amoxicillin	7 (26)
Amoxicillin, ampicillin	13 (48)
Nonresistant	1 (3.7)
Total	27 (100)

enclosed in these fragments (Fig. 1). The presence of a 993 bp fragment leads to the H58 strain because the deletion flanks this fragment. Among 27 *Salmonella* Typhi isolates, only one isolate (slot 6) was positive for this fragment (Fig. 1).

Discussion

Salmonella Typhi identification via blood culture and PCR

We conducted blood culture to obtain *Salmonella* Typhi isolates. Only 7% (27/367) of samples were positive via both blood culture and PCR. The percentage culture in this study was not in line with previous reports, which reached 40% and above. The low number of positive cultures can be caused by the time of sampling or the antibiotic provided before visiting healthcare facilities [21–23].

Antibiotic sensitivity in *Salmonella* Typhi from typhoid fever based on antimicrobial sensitivity tests

According to the 2006 typhoid fever control guidelines, the first-line antimicrobial groups for typhoid are chloramphenicol, ampicillin or amoxicillin and SXT, while the second-line groups are ceftriaxone, cefixime and quinolone [24]. The results showed that the most sensitive antibiotic is SXT. All 27 isolates were found to be sensitive to SXT in this study. One (96.3%) of 27 isolates was sensitive to each antibiotic (ceftriaxone, chloramphenicol, cefepime and ofloxacin). Twenty-four (88.9%) of 27 isolates were sensitive to cefixime. The lowest drug sensitivity was to amoxicillin, which occurred in one (3.7%) of 27 isolates, and ampicillin, which occurred in 13 (48.1%) of 27 isolates (Table 1). A previous study conducted during 2001–3 in Makassar noted that drug resistance towards *Salmonella* Typhi was still at low, without resistance to SXT, ceftriaxone or ciprofloxacin [25]. The same data were obtained during 2011–5, at which time *Salmonella* Typhi and Salmonella Paratyphi resistance to several antibiotics such as ampicillin, SXT, ceftriaxone, ciprofloxacin and levofloxacin were still low [26]. These reports are in line with our results, which found no resistance to SXT, one isolate resistant to ceftriaxone and one isolate resistant to fluoroquinolones.

We found *Salmonella* Typhi resistance to several antibiotics by disc diffusion test. The highest antibiotic resistance was to amoxicillin, at 96.3%, then ampicillin, at 48.1%, in 27 *Salmonella* Typhi isolates using the disc diffusion test (Table 1). On the basis of these results, the administration of amoxicillin and ampicillin in patients with typhoid fever needs to be carefully considered, given the high resistance to both antibiotics. A previous study in

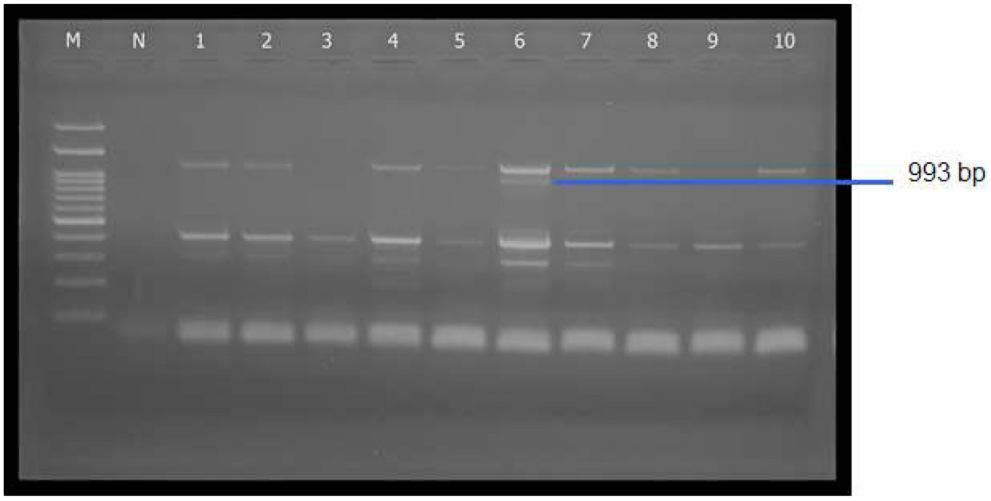

FIG. 1. Agarose electrophoresis of *Salmonella enterica* serovar Typhi H58. Slot 1–5, 7–27, negative *Salmonella* Typhi; slot 6, positive *Salmonella* Typhi H58, with 993 bp of *Salmonella* Typhi. Slot 6 is positive for PCR product. Presence of 993 bp leads to H58 because deletion is conserved in this sequence. M indicates 100 bp ladder marker; N, negative control.

Indonesia noted the existence of resistance to amoxicillin and ampicillin caused by empirically provided antibiotics; penicillin is most often provided in this context because this antibiotic group has broad-spectrum properties and low toxicity [27].

We encountered several problems in the typhoid control programme in Indonesia related to antibiotic resistance, namely the free use of antibiotics by the general public (without a prescription), inappropriate choice of first-line antibiotics, incorrect dosages, inappropriate duration of administration, presence of other diseases that decrease immunity and existence of abnormalities that predispose carriers to typhoid [28].

Existence of MDR H58 *Salmonella* Typhi from antibiotic-resistant typhoid fever

In this study, we applied a simple PCR method. The primer pair we used amplified *Salmonella* Typhi DNA to 993 bp. The 993 bp location was in the region 1466586 to 1467578 on CT18 (accession no. NC_003198.1). In that sequence, there were deletions that are present in H58. Murgia *et al.* [29] in 2016 validated the 993 bp location and stated that its specificity reached 100%. Deletion at 993 bp was detected in all *Salmonella* Typhi H58 strains tested; no such deletion was present in non-H58 strains. This reinforces the notion that this deletion is strongly conserved in *Salmonella* Typhi H58 [30].

A study in 2008 found nine serovar strains of haplotypes in Indonesia: H1, H8, H42, H45, H50, H52, H59, H84 and H85, with H59 and H8 dominating. Haplotype H59, which is associated with j and z66 bacteria expression, is a specific phenotype in Indonesia [26]. Another report found one H58 strain resistant to fluoroquinolone (obtained from a French traveler returning from Indonesia). That report indicated that there was no recent clonal expansion of H58 in Indonesia because DNA gyrase was not found, regardless of the fact that H58 strains may have been introduced to this country from near neighbors, such as Vietnam, where such strains are common [15]. Population mobility is the main factor behind the distribution of resistant organisms [31]. In our study, only one of 27 *Salmonella* Typhi isolates was detected as H58 using simple PCR, which means that the circulation of the H58 strain is uncommon in Indonesia. Further study needs to be done regarding the presence of the H58 strain in Indonesia using a molecular approach as surveillance for MDR occurrence.

We found one isolate (3.7%) from a sample positive for typhoid fever which carried 993 bp fragments (slot 6) (Fig. 1). Phenotypically (based on disc diffusion test), a *Salmonella* Typhi isolate in slot 6 is known to be resistant to ofloxacin, quinolone antibiotics and cefixime as well as amoxicillin and broad-spectrum penicillin antibiotics (Table 2). Although the isolate was found to be phenotypically resistant to these antibiotics, in the future, it is necessary to confirm the existence of genes related to antibiotic resistance, especially to first-line antimicrobial groups for typhoid and fluoroquinolone. Resistant genes detected in H58 were bla_{TEM-1} (resistant to ampicillin), dfrA7, sul1 and sul2 (resistant to SXT), catA1 (resistant to chloramphenicol) and strAB (resistant to ampicillin/streptomycin). In addition to being MDR, the H58 strain is also always associated with the presence of the gyrA gene, which is the main cause of reduced bacterial sensitivity to fluoroquinolone antibiotics [9,12,30,32]. Quinolone is a bactericidal antibiotic used to treat bacterial infections in humans; it is especially active against Gram-negative bacteria. Quinolone targets DNA gyrase and topoisomerase IV enzymes. These play an important role in DNA replication and transcription processes [29]. Resistance to fluoroquinolones is generally caused by mutations in the gyrA gene [33,34], especially codons that encode serine at position 83 and aspartate at position 87 [27]. Previous research found a mutation in the gyrA gene of 17 *Salmonella* Typhi strain isolates from Surabaya. Eight isolates were known to be resistant to nalidixic acid and ampicillin, which carry the gyrA gene mutation in codon 87; such findings were reported as the first resistance to fluoroquinolone of *Salmonella* Typhi with gyrA mutations in Indonesia [28]. Further work needs to be done to identify mutations in the gyrA gene on the isolate found to be positive with 993 bp in this study. The presence of the gyrA gene mutation corroborates the suspicion of quinolone resistance because the disc diffusion test phenotypically shows resistance to ofloxacin.

Conclusion

On the basis of antibiotic sensitivity testing on *Salmonella* Typhi samples from typhoid fever patients in Makassar, Indonesia, antibiotic resistance to *Salmonella* Typhi is still low except for ampicillin and amoxicillin. By simple PCR examination based on 993 bp DNA fragments, only one isolate of *Salmonella* Typhi H58 was detected. The strain was also phenotypically known to be resistant to ofloxacin, quinolone antibiotics and amoxicillin, as well as the broad-spectrum penicillin antibiotic group. Further work is needed to obtain more information regarding the strain's resistance using a molecular approach.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2020.100793.

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