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 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.

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,
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 testing. Accurate and rapid detection methods are required 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 × 10⁹/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 ≤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 × 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
Antibiotic sensitivity of Typhi isolates. On the basis of this examination, 27 (96%) of 27 isolates showed the existence of resistance to one or more antibiotics. Twenty (74%) of 27 isolates were mono-resistant and six (2%) of 22 were polyanionic (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                 | (3.7)             | 26 (96.3)         |
| Chloramphenicol             | (3.7)             | 26 (96.3)         |
| Cefepime                    | (3.7)             | 26 (96.3)         |
| Ofloxacin                   | (3.7)             | 26 (96.3)         |
| Cefixime                    | (11)              | 24 (88.9)         |
| Ampicillin                  | (52)              | 13 (48.1)         |
| Amoxicillin                 | 26 (96)           | 1 (3.7)           |

Data are presented as n (%).

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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

| Antibiotic                        | N (%)  |
|-----------------------------------|--------|
| Polyresistant, amoxicillin, 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) |

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

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.

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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 cefoxime 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 blaTEM-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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