

Abundance and antimicrobial susceptibility of *Shigella* spp. isolated from aquatic environments in Banda Aceh, Indonesia

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Abstract. Zahara Y, Suhartono S, Nirliana N. 2022. Abundance and antimicrobial susceptibility of *Shigella* spp. isolated from aquatic environments in Banda Aceh, Indonesia. *Biodiversitas* 23: 6420-6427. Accumulating pathogenic bacteria in aquatic systems increases waterborne infections, including shigellosis. The infection is causatively associated with *Shigella* spp. within the water system. This study aimed to isolate and determine the abundance of *Shigella* spp. collected from three rivers in Banda Aceh, Indonesia, namely Krueng Lamnyong, Krueng Aceh, and Krueng Neng water, and their antibiotic susceptibility. The bacterial enumeration was conducted using the MPN method followed by bacterial isolation using XLD and SSA selective media, whereas antibiotic susceptibility was evaluated using the standards of CLSI. Results showed that there was no significant difference ($p=0.059$) in the bacterial abundance among rivers i.e., 4.67 MPN/mL in Krueng Lamnyong, 27.33 MPN/mL in Krueng Aceh, and 18.33 MPN/mL in Krueng Neng. In total, 19 isolates of *Shigella* spp. were isolated, of which four isolates were from Krueng Lamnyong, nine isolates from Krueng Aceh, and six isolates from Krueng Neng. Antibiotic susceptibility test showed that the isolates had resistance to tetracycline, penicillin, and nalidixic acid. More than half of the isolates were resistant to more than five antibiotic classes. The study indicates that rivers are the main reservoir for the transmission of pathogenic bacteria in aquatic systems and some efforts are needed to reduce potential health risks and ensure water quality in aquatic systems.

Keywords: Antibiotic resistance bacteria, aquatic environment, river, *Shigella*, shigellosis

INTRODUCTION

Shigella spp. are Gram-negative non-motile bacteria associated with shigellosis (bloody diarrhea) leading to significant global morbidity and mortality. Fever, stomach pain, tenesmus, and blood and mucus in the stool are substantial symptoms of shigellosis. *Shigella* infection is commonly spread from person to person through contaminated food, contaminated water, or direct touch (Kato et al. 2020). Globally, *Shigella* infection is estimated to contribute to 212,438 deaths, primarily children aged <5 years with a total of 75 million annual cases (Khalil et al. 2018). In the Netherlands, there are 300 to 500 cases of *Shigella* infection annually and are mainly associated with travelers (Schuster et al. 2018). Meanwhile, in Japan, the Act on the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases designates shigellosis as a category 3 notifiable disease. Most occurrences of shigellosis in Japan were tourists returning from underdeveloped nations as well as domestically acquired (Kato et al. 2020). In Indonesia, the prevalence of diarrhea has reached 7% of the population, with the most significant frequency occurring in children aged 1-4 years (Christmasyanti et al. 2020). In Aceh, there were 2,724 and 2,533 patients treated for diarrhea in 2018 and 2019, respectively (Dinkes Banda Aceh 2020).

In many regions of the world, about half of *Shigella* strains are currently drug-resistant with a variety of

mechanisms for antibiotic resistance (Ranjbar and Farahani 2019). Different bacterial isolates had varying levels of resistance as *S. flexneri* exhibited the highest level of resistance to ampicillin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole (80%-90%); most strains of *S. sonnei* and *S. boydii* exhibited tetracycline and trimethoprim-sulfamethoxazole resistance (75%-100%), and all *S. dysenteriae* exhibited chloramphenicol and trimethoprim-sulfamethoxazole resistance (100%) (Meiyanti et al. 2016). Others found that more than 73% of *S. flexneri* isolates were resistant to all four tested antibiotics: ampicillin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole (Agtini et al. 2007). The extensive use of antibiotics in humans, animals, and agricultural practices raises concerns about the spread of antibiotic resistance. The emergence of antibiotic-resistant bacteria (ARB), especially bacteria that are no longer susceptible to antibiotics, has a significant impact on clinical and non-clinical settings (Manyi-Loh et al. 2018). Furthermore, genetic mutations or acquiring antibiotic resistance genes through horizontal gene transfer also contribute to the dissemination and emergence of ARB, particularly through the aquatic environment including rivers (Suhartono et al. 2017).

Rivers are means of spreading antibiotic-resistant bacteria among human and animal populations. Incidences of shigellosis due to poor water quality have been reported worldwide, including in Indonesia. In most developing

countries, *S. flexneri* continues to be the predominant contributor to shigellosis as a waterborne illness with a high fatality rate. The last cases of *S. dysenteriae* diarrhea noted in Jakarta in 1985 can be attributed to this aquatic disease (Subekti et al. 2001). Flowing surface water such as rivers and estuaries in areas affected by anthropogenic activities is a means of spreading antibiotic resistance through its flow. Some rivers receive their inputs from wastewater treatment plants, which may contain antibiotics or ARB (Ju et al. 2019).

Rivers in Banda Aceh, such as Krueng Aceh, Krueng Lamnyong, and Krueng Neng collected waste from settlements, hotels, markets and home industries, causing a substantial increase in domestic pollutants. In addition, rivers also receive large amounts of floating inorganic waste, such as plastic waste, due to improper land plastic waste management (Hadi et al. 2018). Due to the lack of knowledge regarding the risks and dangers of using appropriate antibiotics, the drugs accumulate in the aquatic systems increasing the number of antibiotic-resistant microorganisms. Previous studies by Suhartono et al. (2021) showed a number of *Salmonella* spp. isolates recovered from the aquatic environment in Banda Aceh with relatively high bacterial densities. Both *Shigella* spp. and *Salmonella* spp. species were detected in the sediments and river water (Ekwanzala et al. 2017) and other previous studies found the relationship between *Salmonella* and *Shigella* was significant (Shahryari et al. 2017). This implies that isolates from river water and riverbed silt are

also closely genetically connected. Therefore, the aim of the present study was to enumerate, isolate, and determine the bacterial densities and antibiotic susceptibility of *Shigella* spp. collected from the aquatic systems in Banda Aceh, Indonesia.

MATERIALS AND METHODS

Study area and water sampling

The purposive sampling method was applied to determine the water sampling sites in this study. The water samples were collected from three major rivers in Banda Aceh, Indonesia, namely the Krueng Lamnyong, Krueng Aceh, and Krueng Neng (Figure 1). The water samples were placed aseptically in 250 mL sterile Schott Duran bottles kept in ice-cool boxes and then processed in the laboratory for further analysis. Physical conditions, i.e., water temperature, pH, and turbidity measurements in each sampling site were also measured.

Bacterial enumeration and isolation

The bacterial enumeration of *Shigella* spp. of river water samples was performed using a three-tube series MPN method. As a pre-enrichment, a 25 mL water sample was added to an Erlenmeyer flask containing 225 mL of BPW media for making a total sample mixture of 1:10 dilution.

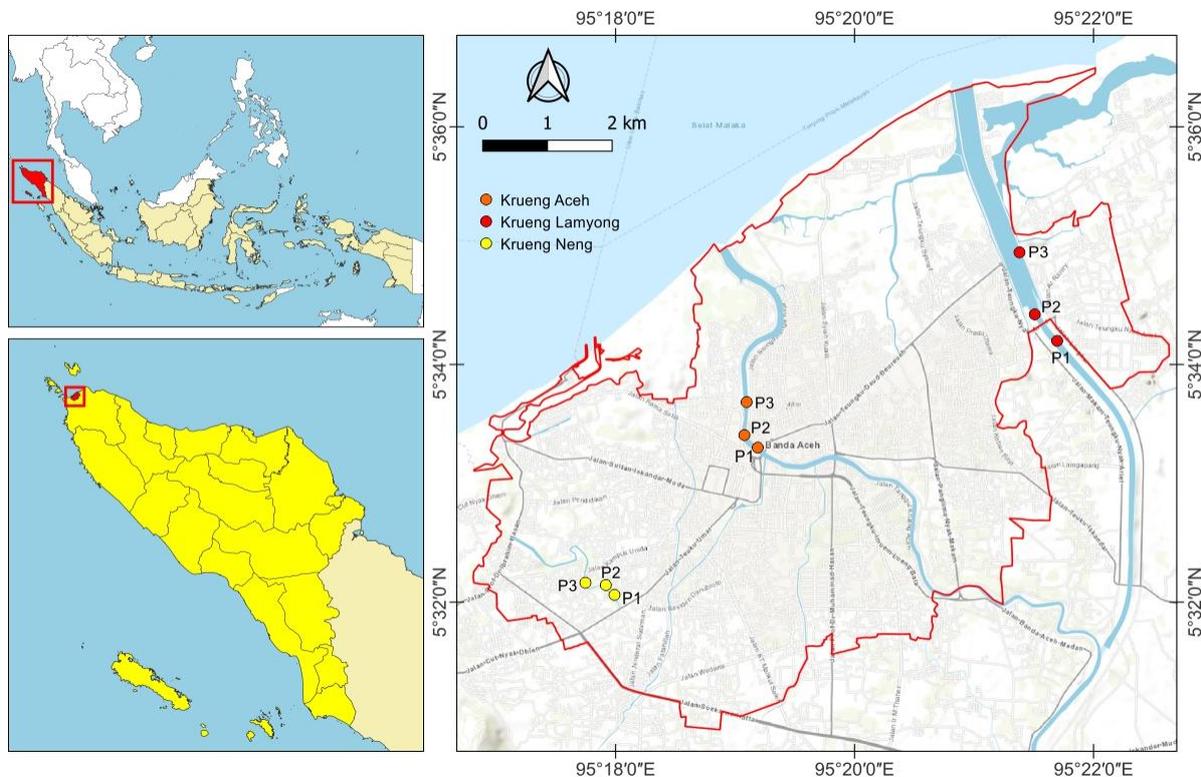


Figure 1. Water sampling location of three rivers in Banda Aceh, Indonesia. Krueng Lamnyong (point 1 (P1): 5°34'15.6"N 95°21'39.6"E; point 2 (P2): 5°34'30.0"N 95°21'28.8"E; and point 3 (P3): 5°35'02.4"N 95°21'21.6"E). Krueng Aceh (point 1 (P1): 5°33'16.7"N 95°19'09.4"E; point 2 (P2): 5°33'22.7"N 95°19'03.7"E; and point 3 (P3): 5°33'40.2"N 95°19'04.5"E). Krueng Neng (point 1 (P1): 5°32'00.0"N 95°18'00.0"E; point 2 (P2): 5°32'05.3"N 95°17'55.9"E; and point 3 (P3): 5°32'06.5"N 95°17'46.3"E)

A 1 mL of the sample mixture was transferred into a sterile test tube containing 9 mL of BPW media for 1:100 dilution, the same process was followed for final concentration of 1:1000 dilution. The tubes were then incubated at 37°C for ± 24 hours. A total of 1 mL of each solution was then transferred into nine test tubes containing 9 mL of LB broth and incubated for ± 24 hours at 37°C. A loopful of each sample in the pre-enrichment was inoculated into a plate containing XLD media and incubated at 37°C for ± 24 hours. Single colonies growing on plates were then inoculated onto SSA media for confirmation.

Gram staining

The Grams stain kit (HiMedia Laboratories, India) was used to perform the staining procedure. Microscopic slides were prepared using fresh cultures of *Shigella* spp. Gram staining was performed according to the protocol of the standard microbiology laboratory manual and slides were observed under oil immersion at 1000x magnification.

Antibiotic susceptibility testing

Shigella spp. isolates were suspended in physiological saline according to the turbidity measurements standard of 0.5 McFarland then spread evenly on MHA (Oxoid, UK) using sterile cotton buds and then incubated at 37°C for 24 hours. The antibiotic sensitivity test was carried out using the Kirby-Bauer disc diffusion method and results were interpreted using the CLSI standards. The antibiotics used in the present study were AMP (10 μ g), P (10 μ g), AMC (20 μ g), CTX (30 μ g), CRO (30 μ g), CN (10 μ g), K (30 μ g), S (10 μ g), TE (30 μ g), CIP (5 μ g), OFX (5 μ g), NA (30 μ g), C (30 μ g) and AZM (15 μ g).

Statistical analysis

All data were tabulated using Microsoft Excel to generate descriptive information for tables. Statistical analysis was performed using IBM Statistical Package for Social Sciences (SPSS) version 26 software with p value less than 0.05 was considered statistically significant. A 2D graphing and statistical data analysis were performed using Prism-GraphPad by Dotmatics version 8.

RESULTS AND DISCUSSION

Water quality sampling

The water quality parameters of the three rivers in the study were found to vary. The temperature values showed no significant difference between the three river locations ($p=0.775$), while the pH value ($p=0.020$) and turbidity ($p=0.010$) in the rivers showed very significant differences (Table 1).

Table 1. Water quality parameters of three major rivers

Parameters	Rivers		
	Krueng Lamnyong	Krueng Aceh	Krueng Neng
Temperatures (°C)	27.67 \pm 2.08	27.67 \pm 0.58	28.33 \pm 0.58
pH	7.32 \pm 0.42	8.00 \pm 0.14	7.21 \pm 0.09
Turbidity (cm)	83.33 \pm 20.82	38.67 \pm 3.06	50.00 \pm 0.0

Bacterial enumeration and isolation

The bacterial density of *Shigella* spp. from three rivers is shown in Figure 2. Results showed that there was no significant difference in bacterial density among the three river locations ($p=0.059$). The average bacterial density of *Shigella* spp. was 4.67 MPN/mL in Krueng Lamnyong, 27.33 MPN/mL in Krueng Aceh, and 18.33 MPN/mL in Krueng Neng.

A total of 19 isolates of *Shigella* spp. were collected, namely four isolates collected from Krueng Lamnyong (coded as SHKL), nine isolates from Krueng Aceh (coded as SHKA), and six isolates from Krueng Neng (coded as SHKN) (Figure 3). Morphologically, *Shigella* spp. isolates showed red colonies without any black centers on XLD agar and formed colorless colonies without black centers on SSA (Figure 4). The isolates were typically short bacilli and pinkish-red color Gram-negative bacteria (Figure 5). The size of single colonies of bacteria ranged from 1-2 μ m.

Antibiotic susceptibility testing

The antibiotic sensitivity of *Shigella* spp. isolates in the present study is shown in Table 2. Four isolates collected from Krueng Lamnyong had resistance to azithromycin, penicillin, amoxicillin, and ampicillin. In contrast, isolates from Krueng Aceh river showed resistance to tetracycline, chloramphenicol, kanamycin, azithromycin, ciprofloxacin, cefotaxime, gentamicin, streptomycin, penicillin, amoxicillin, and ampicillin. In comparison, isolates from Krueng Neng had resistance to nalidixic acid, chloramphenicol, azithromycin, cefotaxime, streptomycin, penicillin, amoxicillin, and ampicillin. Almost all isolates, except SHKL2, SHKL3, and SHKL4, were resistant to more than four antibiotics (Table 3).

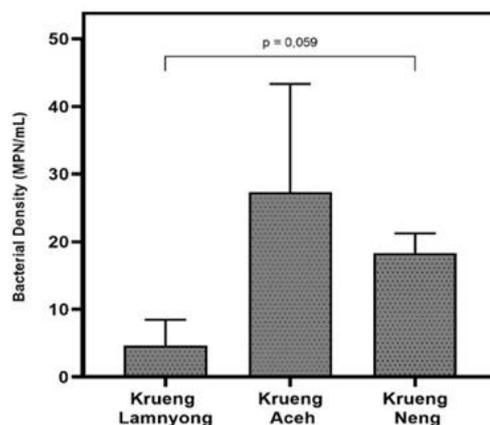


Figure 2. The mean of *Shigella* spp. cell densities (MPN/mL) from each river

Table 2. The number of isolates (% age) of antibiotic susceptibility of *Shigella* spp. isolated from three rivers in Banda Aceh, Indonesia

Antibiotics	Krueng Lamnyong river (n=4)			Krueng Aceh river (n=9)			Krueng Neng river (n=6)		
	S	I	R	S	I	R	S	I	R
Nalidixic Acid	4 (100)	0 (0)	0 (0)	7 (77.8)	2 (22.2)	0 (0)	2 (33.3)	3 (50)	1 (16.7)
Tetracycline	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)	9 (100)	6 (100)	0 (0)	0 (0)
Chloramphenicol	3 (75)	1 (25)	0 (0)	2 (22.2)	0 (0)	7 (77.8)	5 (83.3)	0 (0)	1 (16.7)
Kanamycin	4 (100)	0 (0)	0 (0)	5 (55.6)	2 (22.2)	2 (22.2)	6 (100)	0 (0)	0 (0)
Ofloxacin	4 (100)	0 (0)	0 (0)	9 (100)	0 (0)	0 (0)	6 (100)	0 (0)	0 (0)
Azithromycin	2 (50)	0 (0)	2 (50)	0 (0)	0 (0)	9 (100)	1 (16.7)	1 (16.7)	4 (66.7)
Ciprofloxacin	4 (100)	0 (0)	0 (0)	6 (66.7)	1 (11.1)	2 (22.2)	6 (100)	0 (0)	0 (0)
Cefotaxime	4 (100)	0 (0)	0 (0)	3 (33.3)	0 (0)	6 (66.7)	2 (33.3)	1 (16.7)	3 (50)
Gentamicin	4 (100)	0 (0)	0 (0)	5 (55.6)	1 (11.1)	3 (33.3)	6 (100)	0 (0)	0 (0)
Streptomycin	4 (100)	0 (0)	0 (0)	1 (11.1)	0 (0)	8 (88.9)	0 (0)	3 (50)	3 (50)
Penicillin	2 (50)	0 (0)	2 (50)	0 (0)	0 (0)	9 (100)	0 (0)	0 (0)	6 (100)
Amoxicillin	2 (50)	0 (0)	2 (50)	4 (44.4)	0 (0)	5 (55.6)	0 (0)	0 (0)	6 (100)
Ampicillin	2 (50)	0 (0)	2 (50)	5 (55.6)	0 (0)	4 (44.4)	0 (0)	0 (0)	6 (100)
Ceftriaxone	4 (100)	0 (0)	0 (0)	7 (77.8)	2 (22.2)	0 (0)	6 (100)	0 (0)	0 (0)

Note: S: sensitive, I: intermediate, R: resistan.

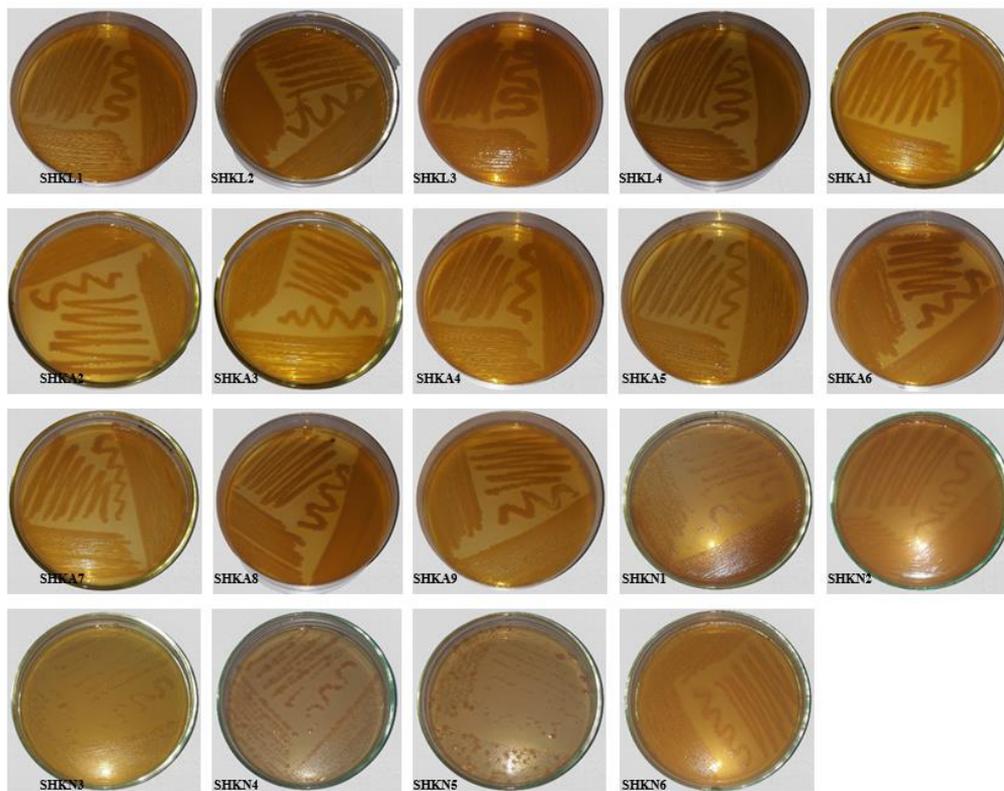


Figure 3. Isolates of *Shigella* spp. grown on SSA selective media, four isolates of Lamnyong (coded as SHKL), nine isolates of Krueng Aceh (coded as SHKA) and six isolates of Krueng Neng (coded as SHKN)



Figure 4. A. Growth of *Shigella* spp. isolates on (A) xylose lysine deoxycholate agar media (B) *Salmonella*-*Shigella* agar

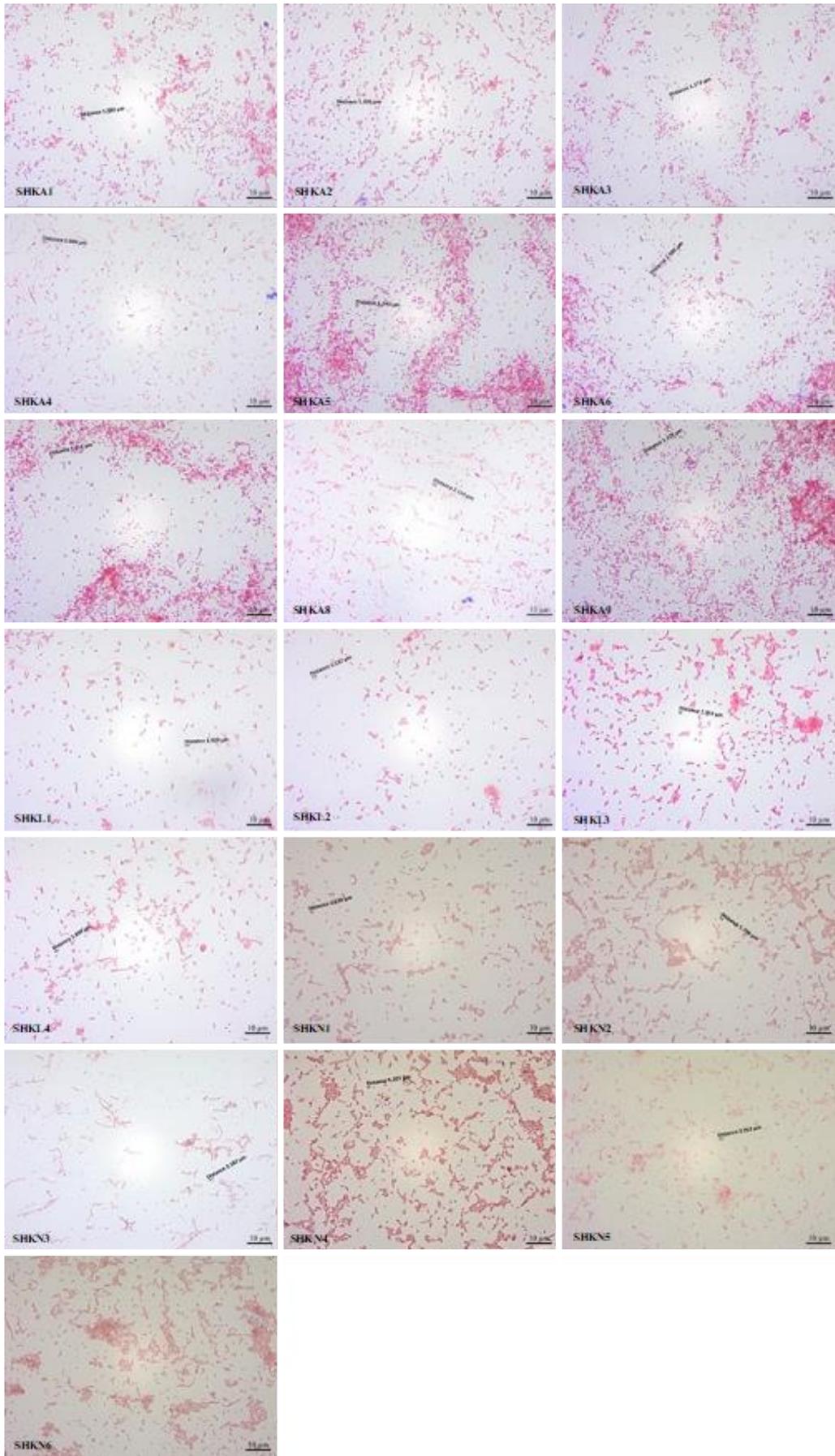


Figure 5. Gram staining results of 19 isolates of *Shigella* spp. collected from three rivers in Banda Aceh, Indonesia

Table 3. Number (percentage) of multidrug resistant *Shigella* spp. isolates isolated from three rivers in Banda Aceh, Indonesia

Number of resistances	Number (percentage) of resistant isolates			Total (n= 19)
	Krueng Lamnyong (n=4)	Krueng Aceh (n=9)	Krueng Neng (n=6)	
1-4	3 (75)	0 (0.00)	2 (33.33)	5 (26.31)
5-9	0 (0.00)	9 (100)	4 (66.67)	13 (68.42)
≥2	2 (50)	9 (100)	6 (100)	17 (89.47)
≥4	0 (0.00)	9 (100)	6 (100)	15 (78.94)
≥6	0 (0.00)	7 (77.78)	2 (33.33)	9 (47.37)
≥8	0 (0.00)	5 (55.56)	(0.00)	5 (26.31)

Discussion

In the present study, despite there was no significant bacterial density of *Shigella* spp. collected from the three rivers, these results signified quite high load of *Shigella* spp. densities in the water body of the rivers, especially in the Krueng Aceh. The high bacterial density of *Shigella* spp. might be affected by physical factors in the current location study. The pH is an essential limiting factor in aquatic environments as growth and survival of microbes within the aquatic system are dependent to changes in pH values (Jin and Kirk 2018). The pH of the river water was generally neutral to slightly alkaline in each of the samples taken from the different rivers (6.5-8.0). Washing dishes and laundry in strongly alkaline water, as well as the customary ritual washing along the river channel, can also lead to an increase in pH (Adewoyin and Okoh 2020). Additionally, exposure to wastewater effluent from agricultural and animal rearing may increase the pH since agricultural activities involving application of alkaline fertilizers may cause the water body's pH to rise due to runoff from farms. The result showed that only in Krueng Aceh, where pH values appeared to be favorable for the survival of organisms, the impact of modest variations in pH on the bacteria density was discernible, but Krueng Lamnyong and Krueng Neng rivers displayed the opposite. The effect of pH on bacterial growth might also be related to enzyme activity. Bacteria require enzymes to catalyze reactions associated with bacterial growth. If the pH in a medium or environment is not optimal, it interferes the bacterial growth (Sipriyadi et al. 2021). However, the link between pH and bacterial density in the three rivers in present study was shown to be statistically significant. As a result, the stability of pH around neutral in freshwater resources is still crucial for the survival of living things and affects the bacterial density in the environment. In addition to nutritional considerations, temperature has a significant impact on the density of bacteria as some microorganisms can survive in a wide temperature range, while others can only do so in a smaller range. When bacteria are at their optimal temperature for growth, temperature significantly impacts how quickly they multiply. Enzyme activity ceases if the surrounding temperature falls below the minimum temperature or rises over the maximum temperature for growth (Suriani et al. 2013).

In addition to pH and temperature, turbidity is also likely to affect the total bacterial density in the present study. The weather during sample collection may have affected the differences in turbidity readings of the three rivers. While water samples in Krueng Aceh and Krueng

Neng were collected during the rainy season, water samples from Krueng Lamnyong were collected during the dry season. Seasonal fluctuations in bacterial abundance and turbidity would mostly be due to seasonal precipitation and significant physical surface water dilution because of river runoff, compared to dry and typical seasons. Because the sewage treatment facility and agricultural area were far from the dam reservoir, there was probably an excellent mechanism to eliminate microbial pollutants before they reached the reservoir, which could account for a reduced bacterial density in the dry season (Luo et al. 2019). The clarity of some turbid events and the use of common bacterial indicators for microbial contamination could result in an incorrect assessment of the treatment barrier efficacy, underestimating the health risk for populations. Since the amounts of minerals, organics, and biological particles can change significantly over time and in response to water treatment, as well as the possibility that pathogen concentrations are unrelated to total particle counts or turbidity, the nature of turbidity particles should also be taken into consideration (Cira et al. 2022).

Result showed that different types of *Shigella* spp. isolates were found in each river site. These findings are in line with previous studies (Suhartono et al. 2021) that confirmed the prevalence of surface waterborne pathogens suggesting that surfaces can facilitate bacteria and that there may be a continuous source of contamination in the water bodies. Additional factors might be attributed to the availability of other physical and hydrological requirements which strongly influence the growth of the pathogen (Cavicchioli et al. 2019). The higher prevalence in summer indicates warmer water temperature meet the requirement of the pathogenic bacteria. Conversely, rainfall is also an important factor causing the decline in the quality of river water levels (Mahagamage et al. 2020).

In this study, *Shigella* spp. isolates from the three rivers were resistant to at least three of the tested antibiotics, namely tetracycline, penicillin, and nalidixic acid. The *Shigella* spp. isolates collected from Krueng Aceh as well as Krueng Neng isolates were resistant to at least five antibiotics and more than half of the isolates were resistant to more than five classes of antibiotics so that isolates could be classified as multidrug-resistant (MDR). This finding is in line with other studies showing that *Shigella* spp. isolates obtained from the waters of Afikpo, Nigeria were resistant to penicillin, tetracycline, cefotaxime and nalidixic acid (Onuoha 2017). Another study showed that *Shigella* spp. was identified multidrug resistance (52.5 %) from surface water Narmada River (India), i.e., ampicillin,

amoxicillin, tetracycline, nalidixic acid, and ceftazidime. The high level of antibiotic resistance in most isolates to amoxicillin and ampicillin suggests that the β -lactamase gene may be widely present in the microbial gene pool (Shahin et al. 2019). *Shigella* spp. can develop drug resistance by a variety of methods, including a reduction in cellular permeability, the expulsion of medications by active efflux pumps, the overexpression of enzymes that modify and inactivate pharmaceuticals, or target modification through mutation. *Shigella* infections, therefore, require more attention, and there is a growing demand for the development of alternative therapeutic procedures that offer alternative defenses against them (Ranjbar and Farahani 2019). Additional investigation, focusing on screening for mobile genetic elements, i.e., plasmids and antibiotic-resistant genes, revealed a sizable number in aquatic environments, was required.

Generally, river water contains most of the enteropathogens originating from municipal sewage, rainfall runoff from livestock and faecal waste from humans, pets, livestock, and wildlife, because river water is not well-treated and maintained (Alegbeleye and Sant'Ana 2020). The main source of the emergence of pathogenic bacteria (such as *Shigella* spp.) in river water is caused by contamination of the fecal-water environment (septic tank leaks, illegal dumping of waste into ditches and the environment). A residential area of Krueng Aceh river is frequently used as a supply of water for washing, domestic wastewater disposal, and other small industrial wastes. The Krueng Aceh river may begin to accumulate heavy metals posing detrimental risks if they infiltrate aquatic ecosystems (Afwanudin et al. 2019). Change in the function of the Krueng Lamnyong River in Banda Aceh may lead to a decline in the riversides function. There have been changes in how the Krueng Lamnyong riverside is used, including the use of agricultural land, cattle, and physical structures, all of which influence the contamination of water sources and the population of microorganisms in them (Dahlan et al. 2021). This situation indicates an urgent need for the development of a domestic wastewater management system (DWMS) in Banda Aceh, Indonesia, which aims to control the level of pollution of independent water bodies at river mouths that accumulate high-resistance bacteria in river water. This development leads to the availability of off-site urban scale domestic wastewater services in accordance with government programs (Harahap et al. 2021). Eventually, an understanding of how perilous *Shigella* spp. as well as factors affecting the total density in aquatic systems is highly necessary to reduce the potential health risks caused by *Shigella* spp. In addition, proper maintenance and procedures are needed to determine water quality to prevent the accumulation of pathogenic bacteria in the aquatic environment.

In conclusion, the present study revealed the presence of MDR *Shigella* spp. in the aquatic systems suggesting that rivers continue to serve as the principal reservoirs for the transmission of the disease in aquatic environments. To reduce the potential health risk posed by these enteric pathogens, it is crucial to comprehend the parameters

influencing the *Shigella* spp. density and diversity in aquatic systems. An appropriate assessment and monitoring is required for the purpose of ensuring water quality and predicting and preventing the transmission of waterborne pathogens in the aquatic environment.

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