Antimicrobial Resistance in East Surabaya's Sewage Sludge: Identifying an Emerging Health Risk for the Local Communities

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ABSTRACT

Antimicrobial resistance is a prevalent issue in Indonesia, as many current studies reported the high level of antimicrobial resistance in Indonesia (AMR). The main cause of this is the inappropriate usage of antimicrobial compounds, especially in the field of public health services and fishery. This study aims to identify the threats of antimicrobial resistance by analysing sludge in Surabaya's sewages for signs of AMR. The methodology of this study works in three main stages, which are to isolate bacteria from sewage sludge samples, identify the isolate, and then test the antibiotic susceptibility of the bacterial isolates. A sludge sample is obtained from sewages near East Surabaya's populace, with consideration of the sewage size and condition of each location. Isolation is then carried out for every sample which produces several microorganisms suspected as Salmonella, and many organisms that have yet to be identified. Identification used on this research include PCR and biochemical assay. Resistance test is then carried out using the agar well method according to CLSI standards, using 5 types of common antibiotics found in pharmacy clinic. List of antibiotics use includes Ciprofloxacin (CIP), Levofloxacin (LFX), Azithromycin (AZM), Amoxicillin-Clavulanate (AMC), and Sulfamethoxazole-Trimethoprim (SXT). Antibiotics are chosen based on the effect on various pathway, some antibiotics may be skipped because it's not appropriate for usage in certain species of bacteria. Results of the test reveal a possible resistance from one isolate (S-45), with the results of biochemical assay pointing to the results shown for Shigella sp. This isolate has a marginal resistance towards LFX, based on the standards of CLSI M-100. The results shown a resistance with an average diameter of clear zone: 10.02 mm (LFX) which is the indication of resistance towards LFX standards (≤16 mm) for Shigella sp.

Keywords: *Antibiotic-resistance*; *Agar well method; Biochemical assay*; *Diarrhea, Shigella*

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INTRODUCTION

On the recent year, rising number of antimicrobial resistances has been confirmed by numerous amounts of published scientific articles. The exact cause of the resistance may vary between cases, but it is mostly believed that the leading cause are the inappropriate usage (wrong dosage or prescription) and the overuse of antimicrobial substances in daily basis. This is especially true in Indonesia where there has been a confirmed number of overuse antibiotics in fishing industries and the lack of distribution protocol in public health service. This kind of practice may lead to the emergence of novel resistance in certain bacterial species towards common antibiotics. It is then hypothesized that the accumulation of such species can mostly be found near the river where the sewage water from fishing industry and regular household together with other things are unified into one stream. It can be said that riverbed essentially is a breeding ground for antimicrobial resistance species to occur, especially with no wastewater management. Trace amount of antibiotics

over a long period of time will be enough to make antimicrobial-resistance trait appear among the microorganism in the wastewater (1). The microorganisms will be most likely to be nested in the sludge part which is often found near the side or the bottom of the river stream, indicated by the amount of biofilm (2)

Sewage sludge is an environmental contaminant, it may also carry possible drug-resistant organism. Certain usage of the sewage sludge as a fertilizer, possess a risk of contaminating the soil area for planting, which may result in serious health risk to the consumer.

The main aim of this study is to find, identify, isolate and characterizing antibiotic's resistance profile of the isolates. For this study, researchers select a few antibiotics that are common to be used on home remedy. The antibiotics are outsourced from public health service pharmacy, that provide antibiotics without a prescription.

MATERIAL AND METHODS

Sample Collection

The sample first was taken from river sites of the four districts in the city of Surabaya, East Java which contain industry waste and other outputs. The four districts are Gubeng, Gunung Anyar, Rungkut and Tenggilis. Also, the sample was taken from sewage sludge deposit near the bottom of the river, with consideration of choosing at least two sampling sites with different streams in each district.

For sludge samples use a sterile tube approx. 50 mL attached to a pipe. Then collect sludge from riverbeds with as little water as possible. If it's necessary, conduct the sampling twice to get an appropriate amount of sludge content. Store samples in cool conditions (12°C) and transport to the lab within 3 hours.

Isolation of Microorganisms

Inoculate samples on general-purpose media for enrichment purpose (e.g., lactose broth) to obtain a broad spectrum of microbial growth. For this research, we use Buffered Peptone Water (BPW) for enrichment purpose. After incubation with shaker for 24 hours, in 37ºC, inoculation is mixed and then transferred into selective enrichment using Selenite Cystine Broth (SCB). Incubation follows the previous step, with a ratio of 1 mL sample per 9 mL selective media. Transfer the enriched samples on selective media Xylose Lysine Deoxycholate (XLD) and MacConkey (MC) using streak method to acquire isolated colony of bacteria. At this stage, select appropriate bacteria colony with corresponding physiology characteristic towards *Salmonella* sp. in accordance with each selective media result (3).

Identification of Bacterial Species

A Triple Sugar Sron (TSI) as biochemical tests were conducted for each obtained isolate. Isolates that was identified as similar to *Salmonella* sp. were then followed with molecular identification by using a singleplex PCR method. PCR was carried out after extracting the isolate's DNA using a boiling-lysis method. To detect *Salmonella* sp., a set of primer targeting thh *invA* gene was used in this study. Amplicon was then analyzed on a 2% agarose electrophoresis (4).

Antibiotic Susceptibility Testing

Assay has been performed in accordance with CLSI M-100 (5). The antibiotics used in this assay includes a mixed antibiotics with broad range. Antibiotics were also chosen in

consideration to match the antibiotics often used in public and the ease of its access, which are Ciprofloxacin (CIP), Levofloxacin (LFX), Amoxicillin-Clavulanate (AMC), and Trimethoprim-Sulfamethoxazole (SXT). Antibiotics which were acquired from a local pharmacy, were prepared by grounded up, mixed with sterile water and then stored in cool temperature as a ready-to-use solution.

Before antibiotics were introduced to the isolates, bacterial suspension was made in saline solution (NaCl 0.7%). The bacterial suspension was set to similar with McFarland standard 0.5 based on visual appearance. Isolate suspensions were cultured in Mueller Hinton Agar (MHA) medium by using the swab-spread method. To ensure the precession of the result, a 15 mL MHA was poured by using a sterilized pipette into a 90 mm disposable Petri dish. A total of 5 wells with a diameter of 6mm were made on the MHA and filled with antibiotic solution after the inoculation. The cultured dishes were incubated in 37ºC for 24 hours, then clear zone diameters were observed and measured by using a caliper. The average of 3 measurements was then compared with a reference from CLSI M-100 to determine the status of resistance, susceptible, or intermediate for each isolate.

RESULT

Isolate Identification

The identification of the bacterial isolate was conducted through a combination of TSI biochemical assays and conventional polymerase chain reaction (PCR) analysis. Biochemical assays provided preliminary insights into the metabolic characteristics and phenotypic traits of the isolate, allowing for initial classification based on enzymatic activity, substrate utilization, and other metabolic markers. To confirm the identification, conventional PCR assays targeting species-specific gene regions were employed. Out of a total of 50 samples, the two most promising isolates were selected, as presented in (Table 1),

Table 1. Identification results for each isolate)

Isolate Code	TSI Result	PCR Result	Suspected Result
S30	K/A (G)	-	*Shigella* sp.
S45	K/A (G)	-	*Shigella* sp.

NOTE:
- TSI = *Triple Sugar Iron Agar*, composed of two regions, which are the top (slanted) part and bottom (butt) part.
- TSI results = Alkaline showed as red (K), acid showed as yellow (A); reading results from (slant/butt)
- (G) = Presence of gas

The TSI results showed similarity to *Salmonella* sp. due to its fermentative profile (alkaline slant, acidic butt in the TSI test). However, the *invA* was absent in these isolates. These results suggest that both isolates S30 and S45 are most likely suspected as members of the *Shigella* genus.

Antibiotic Susceptibility Testing

The study evaluated the resistance of two selected isolates (S30 and S45) against antibiotics that are known to be effective towards *Shigella* sp.: CIP, LFX, SXT, and AMC. Antibiotics of appropriate concentration is acquired from diluting antibiotic solid stock, according to the manual of CLSI M100 31st edition. The average diameter of the inhibition zone of tested antibiotics on each isolate is shown in Table 2. Results were then compared with the standard reference of CLSI for *Shigella* sp. (Table 3).

Table 2. Average diameter of inhibition zone of each isolate (mm)

Isolate Code	CIP	LFX	AMC	SXT
S30	29.57	25.3	19.87	0*
S45	0*	10.02	33.79	13.86

(*) Shows a clear zone diameter of less than 10mm, with a small gap that is almost to the point of negligible

Abbreviation:
CIP = Ciprofloxacin
LFX = Levofloxacin
AMC = Amoxicillin-Clavulanate
SXT = Sulfamethoxazole-Trimethoprim

Table 3. Reference of clear zone diameter for *Shigella* sp. (mm)

Interpretation	CIP	LFX	AMC	SXT
Susceptible (S)	≥ 26	≥ 21	≥ 18	≥ 16
Intermediate (I)	22–25	17–20	14–17	11–15
Resistance (R)	≤ 21	≤ 16	≤ 13	≤ 10

Source: Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing, M100, 31st Edition (see ref. 5)

Abbreviation:
CIP = Ciprofloxacin
LFX = Levofloxacin
AMC = Amoxicillin-Clavulanate
SXT = Sulfamethoxazole-Trimethoprim

Comparison with the standard reference (Table 3) revealed varying degrees of resistance in each isolate (Table 4). Starting from CIP, other samples showed significant susceptibility with inhibition zones 29,57 mm, though S45 showed no response, indicating resistance against this antibiotic. Isolates tested towards LFX showed that resistance was observed in the main sample S45, but another isolate, S30, shows a susceptibility with inhibition zones range of 25,33 mm. No isolates showed resistance to AMC, but only S30 strains were resistant against SXT.

Table 4. Summary of antibiotics susceptibility assay

Isolate Code	CIP	LFX	AMC	SXT
S30	S	S	S	R*
S45	R*	R	S	I

(*) It shows a clear zone diameter of less than 10mm so it might be subject to retrial at different concentrations.

Abbreviation:
S = Susceptible
I = Intermediate
R = Resistant
CIP = Ciprofloxacin
LFX = Levofloxacin
AMC = Amoxicillin-Clavulanate
SXT = Sulfamethoxazole-Trimethoprim

DISCUSSION

The findings showed resistance in isolate samples, especially to the SXT mechanism of action, which is used to block the biosynthesis of nucleic acids and protein in bacteria. It also indicates a possible resistance towards similar antibiotics such as those from the Sulphonamides class. This pattern hints at possible overuse or wrong use of these antibiotics, which might be connected to how available they are or how they are prescribed in public health and the fishing industry in Indonesia.

The high resistance in some samples to Ciprofloxacin and Levofloxacin, which are important antibiotics, raises worries about treatment success and public health safety (6). The significant resistance in SXT matches earlier research, which shows that environmental pollution with low amounts of antibiotics can lead to resistant bacteria over time. Some samples, such as S45, showed clear resistance to several antibiotics (CIP&LFX), indicating these strains could have multi-drug resistance, likely gained through gene

change/adaptation, caused by overtime exposure in sewage sludge.

The broad resistance found in samples from biofilm-rich areas supports the idea that riverbeds with sludge can be a source of resistance genes. These environments probably allow for good bacterial growth and sharing of resistance traits. If this sewage sludge is used as fertilizer, resistant bacteria could end up in agricultural soils, which presents a serious health risk to consumers and makes managing resistance harder (7).

To enhance future studies, incorporating quantitative antibiotic analysis through minimum inhibitory concentration (MIC) testing would provide more detailed and nuanced information (8). MIC testing offers several advantages over qualitative methods, including precise measurement of the lowest antibiotic concentration that inhibits bacterial growth, improved sensitivity for detecting subtle differences in susceptibility, and the ability to reveal emerging resistance patterns earlier. This quantitative approach allows for more accurate comparisons between samples and antibiotics, aids clinicians in making informed decisions about dosing and treatment selection and facilitates standardized comparisons across studies and laboratories.

Furthermore, analysing the distribution of MIC values in bacterial populations can offer insights into resistance mechanisms. By implementing MIC testing, researchers can gain a more comprehensive understanding of antibiotic resistance patterns in environmental samples, potentially leading to more effective strategies for managing and mitigating the spread of resistance in aquatic ecosystems and related industries.

CONCLUSION

This study highlights a critical issue of antibiotic resistance in bacterial isolates from river environments, emphasizing the urgent need for improved antibiotic usage protocols and waste management in Indonesia. The high resistance to commonly used antibiotics underscores the role of environmental contamination in spreading resistance. These findings recommend strict regulations on antibiotic disposal and better practices in the public health and aquaculture sectors to reduce environmental impact.

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