INTERNATIONAL JOURNAL OF BIOSCIENCES AND BIOTECHNOLOGY
eISSN: 2655-9994
pISSN: 2303-3371

Identification of Amoxicillin Resistance Gene in River: A Proof Of Concept For AMR Pollution

Nurul Izzati[1*], Nurul Amri Komarudin[2,3], Sri Hasdayanti[1], Maya Aprilia[1]
[1] Department of Biotechnology, Sumbawa University of Technology, Jl. Raya Olat Maras Kecamatan Moyo Hulu, Sumbawa, Nusa Tenggara Barat, 84371, Indonesia
[2] Department of Environmental Engineering, Sumbawa University of Technology, Sumbawa University of Technology, Jl. Raya Olat Maras Kecamatan Moyo Hulu, Sumbawa, Nusa Tenggara Barat, 84371, Indonesia
[3] Department of Environmental Engineering, University of Singaperbangsa Karawang, Jl. HS. Ronggowaluyo, Teluk jambe Timur, Karawang, 41363, Indonesia

[*] Correspondence: Nurul Izzati (nurul.izzati@uts.ac.id)

Received: 06-28-2024 **Revised:** 12-10-2024 **Accepted:** 01-04-2025

Citation: Izzati, N, Komarudin, N.A., Hasdayanti, S. & Aprilia, M. (2025). Identification of Amoxicillin Resistance Gene in River: A Proof of Concept for AMR Pollution. *International Journal of Biosciences and Biotechnology*, 12 (1): 1-14. https://doi.org/10.24843/IJBB.2024.v12.i01.p01

Abstract: Antimicrobial Resistance (AMR) affects almost all nations, ranking among the top ten risks identified by the World Health Organization. The data of AMR transmission in the environment remains low in Indonesia, especially regarding rivers. AMR spread in rivers is facilitated by antibiotic-resistant genes, which can be transmitted to other microorganisms in the water. The mutated penicillin-binding protein 1A (pbp1a) is one of the genes playing a role in amoxicillin resistance. Amoxicillin is well-documented as one of the most prescribed antibiotics in primary healthcare settings, including those in Sumbawa. This study aims to identify the pbp1A gene in the Brang Biji River, Sumbawa, Indonesia, as evidence of ARG transmission in water surfaces through genetic material transfer across the microbes. The study revealed that over 300,000 CFU/mL colonies proliferated on agar medium containing 0.5 mg/mL amoxicillin at sites downstream of hospital WWTP effluent, followed by a decrease to around 50,000 CFU/mL further downstream. Two colonies, one white and one cream-colored, were susceptible to amoxicillin based on disk diffusion antimicrobial susceptibility tests. Moreover, both white and cream colonies were identified to carry the mutated pbp1A gene as determined by the PCR method. This preliminary study can provide essential insights for further analysis and more extensive research.

Keywords: Amoxicillin; AMR pollution; Mutated pbp1A; River

1. Introduction

Antimicrobial resistance (AMR) has been recognized as a global wake-up call since 2017, causing 1.27 million deaths worldwide in 2019 (Gregory, 2022). In the same year, AMR accounted for 34,500 fatalities and was associated with 133,800 fatalities in Indonesia (CDC, 2022). Furthermore, a study by Siahaan et al. (2022) revealed

that AMR poses multi-sectoral challenges in Indonesia due to prevalent antibiotic misuse practices. This has resulted in an estimation of 10 million fatalities annually by 2050 (Novianto, 2022).

The high mortality rate caused by AMR in Indonesia is attributed to recurrent antibiotic mismanagement, misuse or overuse, and inadequate monitoring of antibiotic distribution. In Indonesia, the primary users of antibiotics are humans, livestock, and the fishery sectors. Concerning human antibiotic use, patients often have access to antibiotics without a physician's prescription, consume them for shorter periods than prescribed (typically three days), and contribute to improper waste management. On the livestock side, farmers often purchase antibiotics for livestock and administer them without the veterinary oversight (Siahaan et al., 2022). Despite the marginal improvement in conditions for fishery practitioners due to government-enforced monitoring and regulation, it continues to exacerbate AMR contamination (Siahaan et al., 2022). Notwithstanding this, studies focusing on the environmental spread of AMR, particularly through water systems, remain limited.

All the aforementioned conditions contribute to the transmission of AMR in the environment, including water surfaces. Antibiotic exposure induces pathogens to adapt and develop a defensive mechanism referred to as antibiotic resistance genes (ARG) (CDC, 2024). These ARGs can subsequently infect other microbes through vertical gene transfer (VGT) during bacterial multiplication and horizontal gene transfer (HGT) mediated by mobile genetic elements (MGE), such as plasmids (Liang et al. 2024), which enable the transfer of resistance genes among bacterial species in the environment (Wang et al., 2023). As infected pathogens proliferate, the ARGs can be transmitted to their offspring (CDC, 2024). Furthermore, environmental contamination by AMR poses a threat to other organisms. Figure 1 illustrates the exposure of genetic materials in the environment, highlighting the urgent necessity to address the spread of AMR.

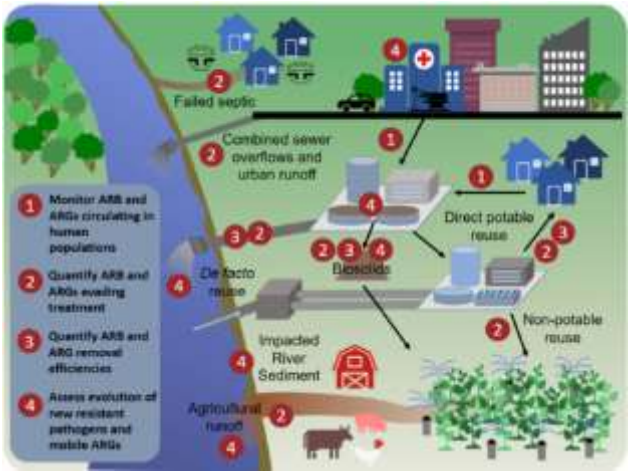

Figure 1. The transmission pathway of MGEs, antimicrobials, resistant microorganisms, and ARGs in humans and the environment (Ligouri *et al*., 2022)

In 2019, Indonesia joined the Global Antimicrobial Resistance and Use Surveillance System (GLASS), signifying the commitment to combating the AMR crisis. This was followed by the issuance of new regulations and protocols designed to mitigate the risk of antibiotic resistance cases. However, the implementation of these measures is currently lacking with few initiatives directed towards the environmental sector, despite its multisectoral accountability. Therefore, this research aims to conduct a preliminary study to identify ARGs, focusing on one of the most frequently prescribed antibiotics found in the river water in Sumbawa.

Water is considered the primary medium facilitating the entry of Mobile Genetic Elements (MGEs) into the environment (Lu et al., 2019). Moreover, the pollution caused by MGEs is alarming, as they may infiltrate marine ecological environments (CDC, 2024). On the other hand, rivers play an essential role in Sumbawa, providing key resources for agricultural and livestock hydration, as well as domestic consumption, exemplified by the Brang Biji River. This river flows through an agricultural area, livestock farms, densely populated residential areas, a public hospital, and a traditional market.

Despite an increasing amount of literature on AMR in water systems, research specifically investigating ARG contamination in Indonesian rivers remains scarce. Furthermore, while several studies have focused on the occurrence of ARGs in clinical and agricultural environments, few have examined the transmission of antibiotic resistance genes in the environmental context, particularly in regions such as Sumbawa, where rivers are integral to daily life. The Brang Biji River, which flows through agricultural land, livestock farms, and densely populated areas, supplies water for both domestic and agricultural use, therefore serving as a key vector for potential ARG transmission.

Amoxicillin is among the most prescribed antibiotics in Indonesia. A study indicated that amoxicillin is the most used antibiotic in primary healthcare and the second most used in hospitals across eight provinces in Indonesia (Siahaan et al., 2022). Data from the Indonesian Food and Drug Agency (2021) reveals that amoxicillin is the second most used antibiotic nationally. In Sumbawa, it is common practice for residents to get amoxicillin from pharmacies without a physician's prescription, particularly while suffering from toothaches or other infectious diseases. The mismanagement of antibiotics, especially amoxicillin waste, raises concerns that ARG pollutants related to amoxicillin may contaminate the Brang Biji River.

Numerous studies have shown that mutations in the penicillin-binding protein 1A (pbp1A) gene correlate with amoxicillin resistance in clinically relevant bacterial species, including Helicobacter pylori and Streptococcus pneumoniae (Gerrits et al., 2006; Attaran et al., 2021). Nonetheless, the function of this gene in environmental bacteria, particularly in river ecosystems, remains largely unexplored.

This study seeks to fill these gaps by examining the occurrence of the mutated pbp1A gene in bacterial isolates from the Brang Biji River in Sumbawa. It emphasizes the environmental spread of ARGs in an area characterized by prevalent antibiotic misuse, offering initial evidence on AMR contamination and underscoring the potential risks posed to both public health and the environment. This study is among the initial investigations in Indonesia examining the prevalence of ARGs in river systems, representing a crucial advancement in understanding the extensive impacts of AMR in the nation's ecosystems.

2. Methodology (Style: Times New Roman, 10 Points, Title Case, Bold)

2.1. Sampling site, water collection and handling

The Brang Biji River was selected as the sampling site due to its flow through the city, which encompasses diverse activities such as animal husbandry, residential areas, a public hospital, and a traditional market. Three sampling spots were designated: one directly behind the public hospital (H0) and the other two approximately two kilometers before (H1) and after (H2) the hospital. Figure 2 depicts the locations of the sampling areas. Sampling was conducted in October 2022 during the rainy season.

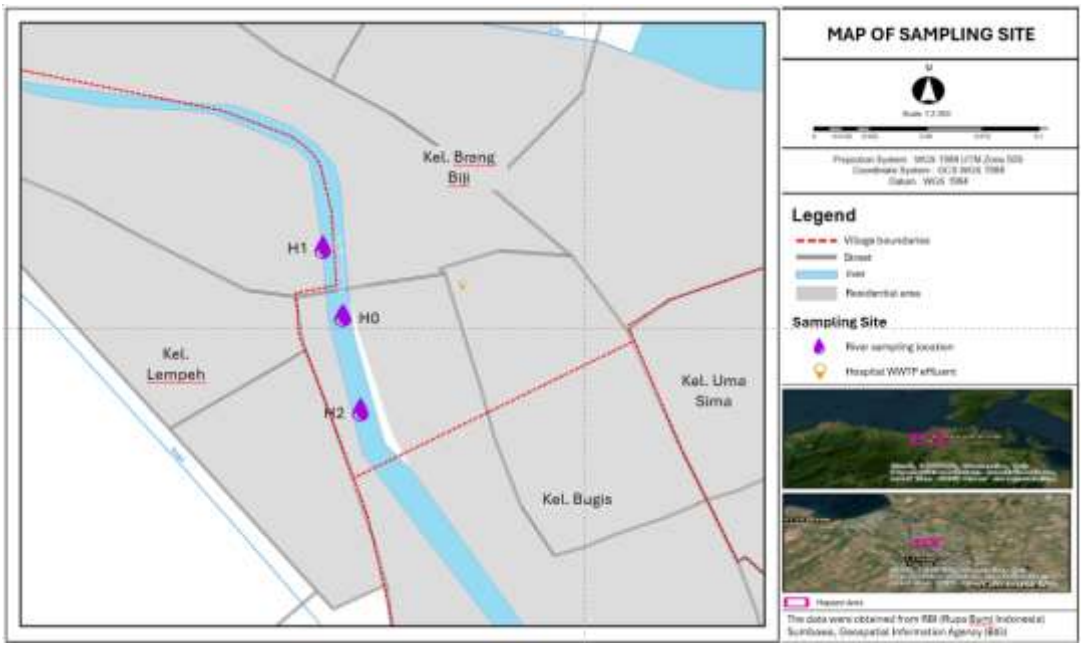

Figure 2. Sampling sites and locations in the Brang Biji River marked with purple drops. The stream flows throughout the city and ends up at the sea.

The surroundings of the sampling sites were assessed, and physical parameters including water density, pH, and temperature were measured on-site. Samples were collected using sterilized equipment and sampling personnel wore gloves and masks for hygiene. Water samples were collected in sterile 1 L bottles and maintained at low temperature in a cold box for the 20-minute transit to the laboratory. The sterile bottles were filled to full, sealed, and the water samples were processed for biological analysis on the same day as collection (Chambers, 2019).

2.2. Water sample preparation and enumeration

Enumeration was performed using Luria-Bertani (LB) agar to assess the bacterial load in the river water. LB

agar is a non-selective medium facilitating the growth of diverse microorganisms, making it ideal for the preliminary cultivation of bacteria. Upon arrival at the laboratory, the water samples were subjected to serial dilution using sterilized equipment: 10-1, 10-3, and 10-5 with sterile water. The 10-3 and 10-5 dilutions were further plated onto Luria-Bertani agar media (Madigan, 2021) supplemented with amoxicillin at a dosage of 0.5 mg/L, with each dilution plated in triplicate. Colonies were counted following a five-day incubation of the plates at 37℃. The data was analyzed using a boxplot method in Jeffreys's Amazing Statistics Program (JASP). Each colony displayed distinct characteristics, including color and contour, therefore necessitating purification by Tryptic Soy Agar (TSA) media. The purified isolates were incubated for 24 hours at 37℃. Subsequently, the isolates were preserved in a 20% glycerol stock for further analysis.

2.3. Antimicrobial Susceptibility Test (AST)

The disk diffusion method was employed to assess the susceptibility of bacterial isolates to amoxicillin. This method was selected based on its extensive use and validation by leading authorities such as the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). AST can be employed to assess whether a specific pathogen remains susceptible to an antibiotic. For this analysis, the disk diffusion or Kirby-Bauer method was conducted with minor modifications (Le Page et al., 2016). The isolates from the 20% glycerol stock were initially inoculated onto Tryptic Soy Agar (TSA) medium to obtain a pure culture. After an 18–24-hour incubation at 30°C, a suitable single colony was selected and cultured in tryptic soy broth until reaching an optical density (OD) of 0.5, in accordance with the EUCAST protocol (Khan et al., 2021). Subsequently, the suspension was swabbed onto 25 mL Mueller-Hinton agar (90 mm petri dish) in three directions and incubated for 20 hours. Each plate was then inoculated with a 6-mm-diameter disk saturated with 0.5 mg/mL amoxicillin. A 6 mm sterile filter paper disk was immersed in a 0.5 mg/mL amoxicillin for two hours to prepare it for antimicrobial susceptibility testing. All handling processes were carried out under sterile conditions, utilizing forceps for the disk movement. The saturated disk was further dried in a sterile container and correctly labeled.

2.4. Genomic Identification

Polymerase chain reaction (PCR) was selected to identify mutations in the pbp1A gene, which is recognized for imparting resistance to beta-lactam antibiotics, including amoxicillin. VeritiPro Thermal Cycler, 96 well (Applied Biosystems, A48141) was performed to identify mutated penicillin-binding protein 1A (pbp1A) using the primers Hp0597-F1 AGTTTGGGTAACTACGGATA and Hp0597-R1 CTCGTGTGAGCACCATGTTT (Gerrits et al., 2006). For the mefA gene, the PCR mixture (50 µL) consisted of 0.4 mM of each deoxy-ribonucleotide triphosphate (dNTP), 25 µL of 2x PCR Buffer KOD FX Neo 1.0 U of KOD FX NeO taq polymerase (Toyobo, KFX-201), 2.0 µL of 0.4 µM of each primer, and 50 ng of the DNA templates. The final composition involved the addition of sterile distilled water to achieve a total volume of 50 µL. The amplification reaction was performed as follows: denaturation for 2 min at 94oC, followed by 35 cycles of 30 seconds at 98oC, 30 seconds at 50/52oC, 45 seconds at 68oC, and a final extension step at 68oC for 5 min. The PCR products were electrophoresed on a 1.0% agarose gel (Toyobo, KFX-201), with a 100 bp DNA ladder (loaded 2,5 µL). The images were captured using a UV transilluminator. Figure 3 shows the research diagram.

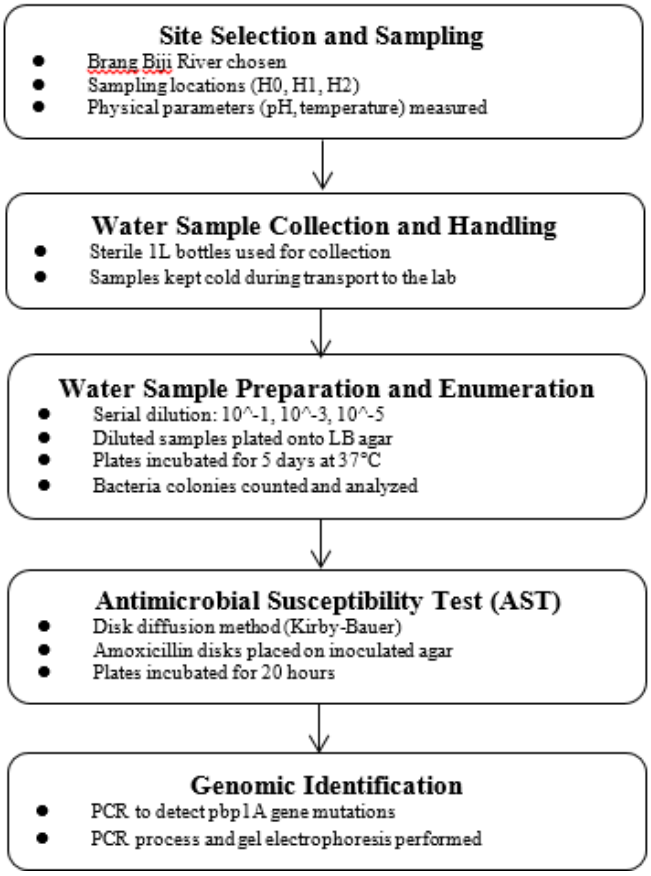

Figure 3. The Research Diagram

3. Results and Discussions

The environmental dimension of AMR spread needs to be considered, especially in Indonesia, wherein data remains scarce. The water surface, including rivers, is known to be a suitable medium for ARG transmission. A major challenge is that ARGs can be transferred across bacteria through mobile genetic elements (MGEs), necessitating an understanding of their movement in the environment alongside Antibiotic-Resistant Bacteria (ARB) and ARGs. This study serves as a preliminary investigation of ARGs associated with amoxicillin resistance, acting as a proof of concept.

Amoxicillin, a β-lactam antibiotic, is effective against several gram-positive pathogens such as Streptococcus. Similar to penicillin, it includes most Streptococcus species and has improved efficacy against Listeria monocytogenes and Enterococcus spp. (Akhavan et al., 2024). Additionally, it provides protection against Haemophilus influenzae, certain Escherichia coli, Actinomyces spp., Clostridium species, Salmonella spp., Shigella spp., and Corynebacteria species (Shulman et al., 2012). To combat antibiotic resistance, an extra amino group was incorporated into amoxicillin, forming an amino-penicillin structure (Bernatová, 2013). Amoxicillin selectively blocks penicillin-binding protein (PBP) transporters, inhibiting the formation of peptidoglycans. The proteins to which the penicillin binds are accountable for facilitating glycosyltransferase and transpeptidase reactions, leading to the formation of cross-links between D-alanine and D-aspartic acid within bacterial cell walls (Sauvage et al., 2016).

Amoxicillin is the most often administered antibiotic in the primary healthcare facilities in Indonesia. Amoxicillin ranked second in hospitals in 16 districts of 8 provinces between 2012 and 2014. During the same time period, amoxicillin was the most frequently prescribed medication in 15 districts of 8 provinces (Siahaan et al., 2022). Meanwhile, amoxicillin was the second most administered antibiotic at a major hospital in Sumbawa between 2019 and 2021 (Prasaja et al., 2024). Considering the accessibility of amoxicillin in Sumbawa, it is imperative to investigate its associated ARGs polluting the water surface.

The Brang Biji River traverses Sumbawa city, with a specific location collecting liquid from the hospital's WWTP effluent. The water sample was collected a day following heavy rainfall, with two piles of rubbish observed near the sampling area. In situ assessments were conducted to evaluate the physical characteristics at the sampling area, focusing on four indicators: pH, temperature, total suspended solids (TSS), and total dissolved solids (TDS). The consolidated findings are presented in Table 1.

Table 1. Physical characteristics of three sampling areas at Brang Biji River

No.	Sampling Spot	Coordinate	pH	T (ºC)	TSS (mL/g)	TDS (ppm)
1	H0	8°29'25.7"S 117°25'02.1"E	5.7	28.1	86.0	14.0
2	H1	8°29'33.5"S 117°25'06.8"E	5.7	26.6	86.0	14.0
3	H2	8°29'20.3"S 117°25'00.0"E	5.7	29.2	85.7	14.0

The river's pH was determined to be slightly acidic, recorded at 5.7 across the entire tested area, although the temperature remained within standard limits. The solid abundance and density in the river were reported to be rather high, categorized as grade three or four (The Fifth National Report of Indonesia to the Convention on Biological Diversity, 2024). Total suspended solids (TSS) can indicate water salinity (Rusydi et al., 2018), which affects the osmotic pressure of bacterial cells. Conversely, turbidity, represented by the total dissolved solids (TDS) number, signifies suspended particles and offers a habitat for pathogens or other microorganisms (Ilmu et al., 2024).

3.1. The presence and abundance of microbial communities

The presence and abundance of microbial communities were evaluated by observing bacteria potentially resistant to amoxicillin on LB agar medium supplemented with 0.5 mg/mL amoxicillin. LB medium is a versatile growth medium used for cultivating a wide variety of non-fastidious bacteria (Peterson et al., 2021). Given that this medium is often a complex growth medium, it can support the cultivation of gram-positive pathogens. Furthermore, gram-positive bacteria can be isolated from mixed cultures by employing selective agents or differential media that inhibit the growth of gram-negative bacteria while allowing the growth of gram-positive bacteria using LB medium (Madigan, 2021). Colony growth was quantified, as depicted in Figure 4, serving as presumptive isolates for further analysis.

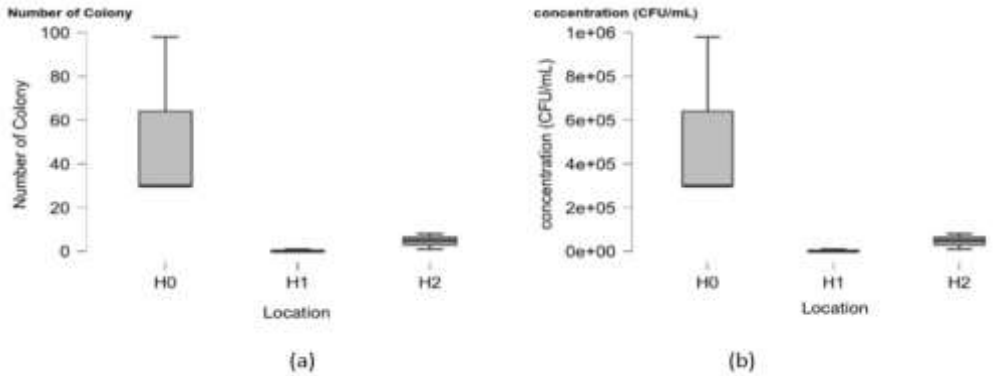

Figure 4. The number (a) and the concentration (b) of colonies isolated from three sampling areas at the Brang Biji River. H0 represents the stream that has direct link to the wastewater treatment plant (WWTP) effluent from hospital, H1 is the area before the hospital WWTP effluent and H2 after hospital WWTP effluent.

Figure 4 illustrates the distribution of presumptive bacteria resistant to Amoxicillin. The highest colony concentration was observed at H0, indicating that the WWTP effluent may influence resistance. The stream downstream of the WWTP effluent had a higher colony concentration compared to upstream, suggesting a probable contribution from the effluent. The difference in concentration between H0 and H2 may be attributed to the transmission of resistant bacteria via the river stream.

Although WWTPs are not explicitly designed to reduce the transmission of AMR to water environments, their potential in this regard is significant (Liguori et al., 2022). The research results suggest that ARG contamination in the Brang Biji River may be affected by the effluent. Moreover, concerns have been raised regarding the regeneration of resistant microbes in distribution systems, particularly in cases involving nonpotable recycled water (Zhu et al., 2021). Consequently, key monitoring sites for AMR in the environment, including wastewater, recycled water, and affected receiving waters, have been identified (Pruden et al., 2018).

The elevated concentration of bacterial isolates in the H0 area suggests a correlation between hospital activities and AMR contamination. Furthermore, the improper disposal of antibiotic waste from the hospital and local

residences may contribute to this issue. The densely populated settlement between the hospital and the river necessitates attention to local residents' actions about antibiotics.

A study reported that 10% of Indonesian citizens store antibiotics in their homes, and 86.1% obtain antibiotics without a legal prescription. Furthermore, the government must intensify efforts to supervise the implementation of AMR policies more effectively to prevent easy access enabled by lax oversight (Siahaan et al. 2022). The lack of proper waste management in Sumbawa is evidenced by the discovery of two piles of trash near the sampling sites, a circumstance that warrants attention.

3.2. Antimicrobial Susceptibility Test

The colonies obtained from the isolation procedures were further characterized based on their morphology, resulting in just two colonies displaying distinct colors and shapes, as seen in Figure 5. Subsequently, these two colonies were purified on Tryptic Soy Agar (TSA) medium in preparation for Antimicrobial Susceptibility Testing (AST) analysis. Following purification on TSA medium, the AST analysis was conducted using Muller Hinton Agar medium (Eucast, 2024).

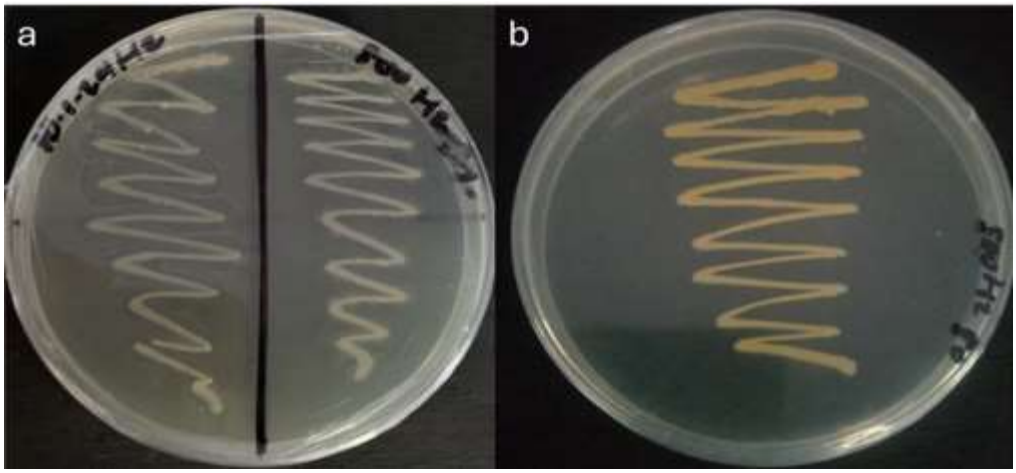

Figure 5. The purified colony on TSA medium. The white color (a) was the most prevalent colony in all three sampling areas, whilst the creme (b) was exclusively observed in the H2 area, which is downstream from hospital WWTP effluent.

The two colonies were subjected to AST analysis. Initially, a single colony from TSA medium was transferred into Tryptic Soy Broth (TSB) and incubated for 18 hours or until it reached an optical density (OD) of 0.5. Subsequently, the suspension was swabbed onto Muller Hinton Agar (MHA) medium following the European Committee on Antimicrobial Susceptibility Test (EUCAST) performance standards. The agar disk-diffusion method was employed to assess the inhibitory zone towards amoxicillin. A clear zone around a disk indicated that the antibiotic had inhibited the growth of bacteria in that area. The AST results are presented in Figure 6. The breakpoints for the efficacy of the amoxicillin antibiotic may be referenced from CLSI and EUCAST, categorizing it into non-meningitis Streptococcus pneumonia and Enterobacteriaceae, as detailed in Table 2.

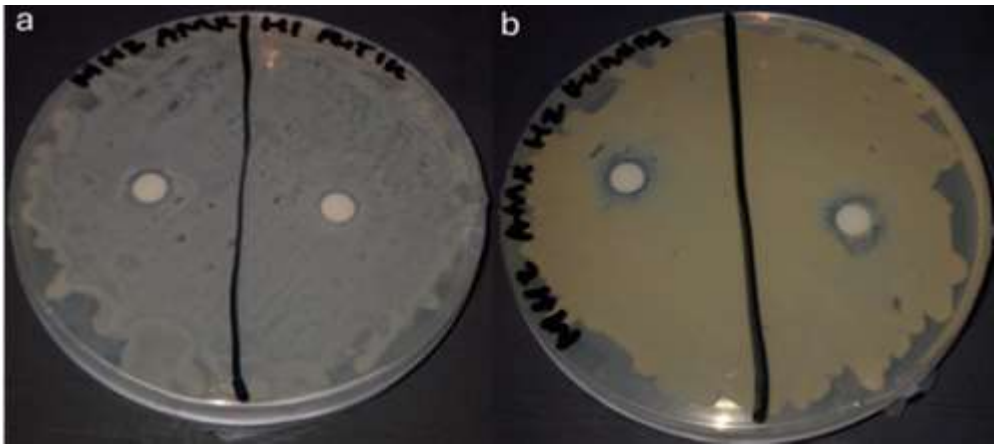

Figure 6. Agar disk diffusion methods for antimicrobial susceptibility test: (a) The inhibitory zone by white colony and (b) creme colony. The disks were infused with amoxicillin 0.5 mg/mL and placed two on each plate.

Table 2. The breakpoint standard for the inhibition zone of amoxicillin antibiotic, as per CLSI and EUCAST

Standard	non-meningitis *Streptococcus pneumonia*			*Enterobacteriaceae*		
	susceptible	**intermediate**	**resistant**	**susceptible**	**intermediate**	**resistant**
CLSI	≥20 mm	15-19 mm	≤14 mm	≥18 mm	15-17 mm	≤14 mm
EUCAST	≥20 mm		≤19 mm	≥22 mm	15-17 mm	≤21 mm

Source: EUCAST, (2024) and Clinical and Laboratory Standards Institute, (2019)

Figure 5 illustrates that neither isolate exhibited a zone of inhibition with amoxicillin at a concentration of 0.5 mg/mL. In comparison to EUCAST and CLSI standards, the isolates were categorized as the resistance bacteria since both breakpoint standard were set at ≤ 14 mm and ≤ 19 mm for non-meningitis S. pneumonia, ≤ 14 mm and ≤ 21 mm for Enterobacteriaceae, respectively. Therefore, both isolates indicated resistance to amoxicillin antibiotic. AST relies on the identification of resistance determinants in bacterial isolates or directly in clinical specimens (Jenkins et al., 2012). This finding suggests that further examination of both strains is essential to gain a comprehensive understanding of AMR contamination.

The observation of antibiotic resistance in bacterial isolates, as evidenced by the lack of inhibition zones in the antimicrobial susceptibility testing (AST), holds significant clinical implications. Resistance to widely used antibiotics poses a substantial challenge in clinical settings due to its impact on treatment options for bacterial infections. The emergence of resistance to amoxicillin, a widely prescribed antibiotic, can restrict the efficacy of empirical therapy for various bacterial infections.

Recent studies indicate a global increase in antibiotic resistance, leading to a rise in treatment failures and complications associated with bacterial infections (Laxminarayan et al., 2020). Infections due to multidrug-resistant bacteria are particularly alarming, as they frequently necessitate the administration of alternative, more potent antibiotics that entail higher costs, increased toxicity, and limited availability (WHO, 2014).

Moreover, the development of antibiotic resistance can undermine the success of infection control measures and increase the risk of healthcare-associated infections, leading to prolonged hospitalizations, higher morbidity and mortality rates, and increased healthcare expenditures (Bhavnani et al., 2021). The spread of resistant bacteria in healthcare facilities and communities might undermine public health initiatives to contain infectious diseases and prevent outbreaks (European Centre for Disease Prevention and Control, 2019).

Overall, the prevalence of antibiotic resistance in bacterial isolates highlights the urgent need for antimicrobial stewardship initiatives, improved infection control practices, and the development of novel therapeutic strategies to combat resistant infections and preserve the efficacy of existing antibiotics.

Although our AST data demonstrated the bacterial isolate's resistance to amoxicillin, further investigation is necessary to elucidate the specific resistance mechanisms at play. Understanding these mechanisms is crucial for guiding treatment decisions and implementing targeted strategies to address antibiotic resistance, particularly in water environments.

One approach to further analyze the resistance phenotype is to conduct additional susceptibility testing using a panel of different antibiotics with diverse mechanisms of action. This may assist in identifying alternative antibiotics that might remain effective against the resistant isolate. Testing for susceptibility to beta-lactamase inhibitors, such as clavulanic acid in combination with amoxicillin, can elucidate the presence of beta-lactamase enzymes that confer resistance to amoxicillin.

In addition to phenotypic testing, molecular techniques offer valuable tools for detecting resistance genes and characterizing the genetic basis of antibiotic resistance. Polymerase chain reaction (PCR) assays targeting recognized resistance genes, including those that encode beta-lactamases or efflux pumps, can be employed to identify specific genetic determinants of resistance (Liu et al., 2019). Sequencing of resistance-associated genes and genomic analysis of the bacterial isolate can further elucidate the genetic mechanisms underlying antibiotic resistance, e.g. metagenomic studies for the AMR surveillance in the environment.

Furthermore, whole-genome sequencing (WGS) offers comprehensive insights into the genomic architecture of the resistant isolate, facilitating the identification of novel resistance determinants, mutations in antibiotic target genes, and mobile genetic elements associated with horizontal gene transfer (Didelot et al., 2016). Comparative genomic analysis with reference strains and databases can facilitate the identification of genetic signatures associated with antibiotic resistance and inform the development of tailored therapies.

By employing a combination of susceptibility testing, molecular techniques, and genomic analysis, we can achieve a thorough comprehension of the resistance mechanisms in the bacterial isolate and guide strategies for antimicrobial stewardship, infection control, and antibiotic development.

3.3. Mutated Penicillin Binding Protein 1A as the ARG marker to amoxicillin

Numerous studies suggest that the primary cause of amoxicillin resistance is several point mutations in the

pbp1A gene, leading to a decrease in the affinity between amoxicillin and PBP-transpeptidase (Francesco, 2011). Enzymes known as PBPs are involved in the creation and upkeep of the bacterial cell wall's peptidoglycan layer (Gerrits et al., 2006). Tran et al. (2022) [40] identified seven amino acids from Helicobacter pylori that play a role in amoxicillin resistance. Among these, site-directed mutagenesis has demonstrated that the Ser414 to Arg substitution is the primary cause of the Hardenberg strain's amoxicillin resistance, while spontaneous transformation has indicated its prevalence in clinical AmxR strains (Pruden et al. 2018). Using the primers designed by Gerrits et al. (2006), The size of pbp1A is ranges from 1500 to 1800 bp, contingent upon the location of mutated gene within H. pylori. The PCR result from this study is presented in Figure 7.

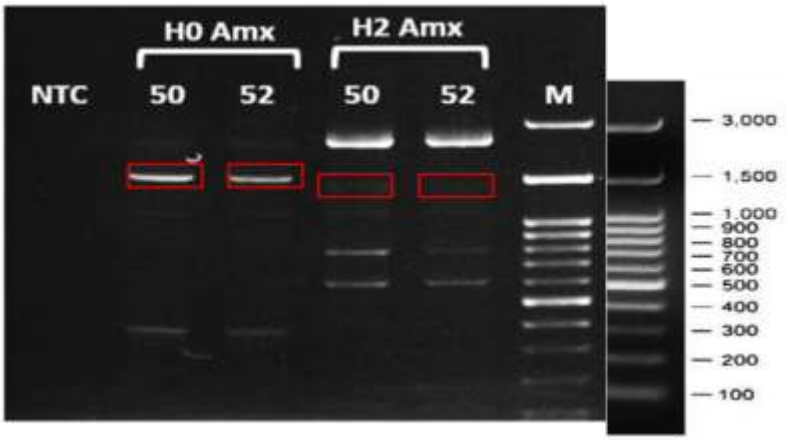

Figure 6. PCR results of white (H0 Amx) and creme (H2 Amx) isolates. The annealing temperature were 50 and 52. Both isolates showed a band in the range of 1500-1700 bp.

Our investigation into the genetic basis of antibiotic resistance in the bacterial isolates involved the use of polymerase chain reaction (PCR) targeting the mutated penicillin-binding protein 1A (pbp1A) gene, a known determinant of resistance to beta-lactam antibiotics such as amoxicillin (Smith et al., 2018). Figure 6 demonstrates the positive PCR result, indicating the presence of the mutated pbp1A gene in the isolates that furnish molecular evidence of the genetic mechanism responsible for amoxicillin resistance.

A study from Bangladesh has indicated that mutations in the pbp1A gene of H. pylori highly correlate with amoxicillin resistance in clinical isolates (Fauzia et al., 2023). Additionally, mutations in pbp1A have been identified in Streptococcus pneumoniae strains resistant to amoxicillin (Contrears-Martel et al., 2006). The detection of the mutated pbp1A gene through PCR validates the role of altered penicillin-binding proteins (PBPs) in conferring resistance to amoxicillin in the bacterial isolates. Mutations in pbp1A can lead to reduced affinity of PBPs for beta-lactam antibiotics, thereby rendering the bacteria less susceptible to inhibition by these antibiotics (Jacoby et al. 1996). The presence of the mutated pbp1A gene provides a molecular explanation for the observed resistance phenotype in our AST results.

Furthermore, the identification of the mutated pbp1A gene highlights the importance of genetic surveillance in monitoring the spread of antibiotic resistance determinants within bacterial populations. Mutations in pbp1A have been implicated in the development of resistance not only to amoxicillin but also to other beta-lactam antibiotics, including penicillins and cephalosporins (Bernal-Bayard et al., 2019). Thus, the presence of the mutated pbp1A gene in our isolates underscores the potential for cross-resistance to multiple beta-lactam antibiotics, posing challenges for treatment selection and patient management.

The results of this study align with findings from other research conducted in Southeast Asia, which identifies waterways as significant reservoirs of AMR contamination. Lu et al. (2019) found similar evidence of ARGs in river systems in China, highlighting the contribution of human and agricultural waste to the proliferation of resistant bacteria in aquatic ecosystems. Siahaan et al. (2022) reported the widespread misuse of antibiotics in agriculture and aquaculture in Indonesia, corroborating the findings of this study that associate antibiotic misuse with environmental contamination.

This study provides novel insights into the presence of the mutated pbp1A gene in environmental isolates, particularly in river samples, which has not been extensively documented in previous research. Although studies such as Gerrits et al. (2006) have reported pbp1A mutations in clinical isolates of Helicobacter pylori, few have explored this gene's presence in environmental bacterial populations. The detection of pbp1A in this study expands our understanding of how environmental bacteria may act as reservoirs for clinically relevant resistance genes, highlighting the need for further research into the environmental persistence and spread of ARGs.

One possible explanation for the relatively high abundance of resistant isolates in this study compared to other similar studies (e.g., Liang et al. 2024) could be the unregulated access to antibiotics in the region, which promotes widespread misuse and improper disposal into local water systems. The specific role of agricultural runoff and

waste from healthcare facilities may also be a key factor in the elevated levels of contamination observed.

The presence of antimicrobial-resistant bacteria and ARGs, as evidenced by both phenotypic resistance to amoxicillin and the detection of the mutated pbp1A gene, highlights significant environmental and public health risks. Rivers like the Brang Biji are vital resources for communities, yet their contamination with AMR poses a potential pathway for the spread of resistant pathogens through human use, agricultural practices, and wildlife interaction. These findings highlight the urgent need for improved antibiotic stewardship practices in the region.

The integration of environmental surveillance for AMR into current public health frameworks could greatly improve early detection of AMR hotspots and enable more targeted mitigation efforts. This may include stricter regulation of antibiotic sales, preventing the unregulated disposal of antibiotics, and educating the public on the risks associated with improper antibiotic use. Additionally, industries such as agriculture and aquaculture should be incentivized to adopt practices that curtail the use of antibiotics, hence diminishing environmental contamination.

In conclusion, the positive PCR result for the mutated pbp1A gene offers molecular evidence of the genetic foundation of antibiotic resistance in the bacterial isolates, emphasizing the need for comprehensive molecular surveillance and antimicrobial stewardship to curb the spread of resistance determinants and preserve the efficacy of beta-lactam antibiotics

4. Conclusions

This study sought to examine the presence of antimicrobial resistance (AMR) contamination in a river environment, highlighting the identification of antibiotic-resistant genes and their corresponding phenotypes as indicators of environmental pollution. Our research presents compelling evidence of AMR contamination in the Brang Biji River ecosystem through a multi-facet methodology, including enumeration, AST, and PCR analysis targeting the mutated pbp1A gene.

The enumeration analysis revealed a substantial presence of bacterial colonies in the river water sample, suggesting microbial proliferation and potential environmental contamination. Notably, among the numerous colonies observed, two distinct colonies exhibiting different color and shape were identified, prompting further investigation. Subsequent AST testing of these selected colonies revealed positive results, indicating resistance to the antibiotic amoxicillin. Further molecular analysis through PCR targeting the mutated pbp1A gene, a known determinant of resistance to beta-lactam antibiotics such as amoxicillin, yielded positive results in the selected colonies. The detection of this resistance gene provides molecular evidence of antibiotic resistance and supports the hypothesis of AMR contamination in the river.

These findings indicate that the river is a potential reservoir for AMR contamination, likely due to human and agricultural activities. Given the vital role of the river in local agriculture, livestock, and domestic water needs, this contamination poses substantial threat to environmental and public health.

This study offers a preliminary assessment of AMR in the Brang Biji River; nevertheless, further research is required to comprehensively understand the extent of AMR contamination. Future studies should explore additional resistance mechanisms, including the presence of other ARGs that may contribute to multi-drug resistance. Expanding the geographic scope of sampling to other regions and examining seasonal variations in AMR levels could offer deeper insights into the dynamics of resistance spread. Moreover, studies evaluating the direct health hazards to humans and animals dependent on polluted water supplies is crucial for guiding public health interventions.

Author Contributions

Conceptualization, N.I and N.A.K; methodology, N.I.; validation, N.I., N.A.K and S.H.; formal analysis, S.H. N.I, and N.A.K; investigation, S.H. and M.A; resources, N.I.; data curation, S.H. and M.A; writing—original draft preparation, N.I.; writing—review and editing, N.A.K.; visualization, N.A.K.; supervision, N.I.; project administration, M.A.; funding acquisition, N.I. All authors have read and agreed to the published version of the manuscript.” The relevant terms are explained at the CRediT taxonomy.

Funding

This research was sponsored by Universitas Teknologi Sumbawa (HITS), a seed grant for research..

Informed Consent Statement

Not applicable

Data Availability

Not applicable

Acknowledgements

Anyone who contributed to the research or manuscript, but not a listed author, should be acknowledged. This may include technical support, material donation, etc. Do not repeat the support already covered by Author Contributions or Funding. Do not include thanks to anonymous reviewers and editors, inessential words, or effusive comments.

Conflicts of Interest

The authors declare no conflict of interest

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Nomenclature (Style: Times New Roman, 10 Points, Title Case, Bold)

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Appendix (Style: Times New Roman, 10 Points, Title Case, Bold)

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