Original Article

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Multidrug Resistance *Escherichia coli* in the Citarum River, Greater Bandung Area, Indonesia

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Abstract

Background: One of the main problems of the Citarum River is the contamination of *Escherichia coli* (*E. coli*) due to livestock activities, washing toilets, and industry. In addition, irrational use of antibiotics in the community and livestock can increase *E. coli* resistant strains to antibiotics. This study aimed to identify the presence of multidrug resistance (MDR) and extended-spectrum β-lactamase (ESBL) *E. coli* strains in Citarum river clusters, namely industrial, livestock, and residential clusters.

Methods: This was a descriptive study. A sample of 100 mL surface water from each Citarum cluster. Culture, antibiotic sensitivity test, and PCR to identify blaCTX-M-15 gene carriers of ESBL E. coli were carried out in the sample.

Results: There were 37 isolates of *E. coli*, with 24% of these isolates showing MDR properties, which can be found in industrial, livestock, and residential clusters at 13%, 8%, and 3% respectively. The most *E. coli* resistant antibiotics found in these samples were ampicillin (45%), followed by tetracycline (37%), and azithromycin (29%). The PCR examination did not find the blaCTX-M-15 gene carrying ESBL properties in all three Citarum river clusters.

Conclusions: The presence of *E. coli* isolates in each Citarum river cluster suggests the occurrence of river pollution due to animal, human or industrial waste. Therefore, it is necessary to make better government regulations regarding sanitation and education for the surrounding community regarding the importance of keeping the river clean.

Keywords: *Escherichia coli*, Citarum, extended-spectrum β-lactamase, multidrug-resistance

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Introduction

Citarum River is the largest and longest river in West Java Province, consisting of 19 watersheds that are the raw water source of most surrounding communities.[1] One of the problems with the Citarum River area is pollution. Analysis of raw water in several clusters of Citarum River has identified an increase in *Escherichia coli* (*E. coli*) to extreme levels that fall into the category of very heavy pollution or class IV as outlined in the Indonesian government regulation no. 82 of 2001.[2] The contamination of river water by *E. coli* is suspected because community activities such as bathing, washing, latrine, and disposals of livestock and industrial waste around the Citarum River are still occurring.[1] Pathogenic microbes in the polluted river have the potential to spread to the surrounding community through floods that occur every year in some Citarum areas, thus, increasing the risk of nearby communities contracting waterborne diseases or diseases transmitted through polluted water.

Health problems caused by *E. coli* are quite wide-ranging from gastrointestinal infections characterized by acute diarrhea accompanied by bleeding to infections of other parts of the body such as urinary tract infections and even the central nervous makeup, which impacts the increasing use of antibiotics.[3,4] More exposure to antibiotics in the body can increase antibiotic-resistant strains of *E. coli*. In

addition, using antibiotics in animal husbandry or poultry can increase the risk of multi-resistant bacterial appearance to antibiotics. Some heavy metals in industrial waste can also be mutagenic, potentially improving the adaptability of pathogenic bacteria to the environment, including changing their resistance properties.[5,6] *Enterobacteriaceae* is known as one of the bacteria that can adapt to antibiotics and has the capacity to form an expansion of the antibiotic resistance spectrum, otherwise known as extended-spectrum β-lactamases (ESBL).[7] Pathogenic bacteria with ESBL properties are one of the world's main problems today because they cause sporadic infection outbreaks.[7]

To date, the Government of West Java Province has tried to overcome the pollution of the Citarum River which is known as one of the most polluted rivers in the world through the *Citarum Harum Program* regulated by the Presidential Regulation No. 15 of 2018 on Acceleration of Pollution Control and Damage of Citarum River Flow Area.[8] However, disposal of livestock and industrial waste and activities around the Citarum River are still common, suggesting the high likelihood of contamination of antibiotic-resistant *E. Coli* bacteria in the river.[9] Therefore, it is necessary to conduct research to study the development of rivers, especially from the perspective of organic pollution. This study aimed to identify the presence of *E. coli* in the Citarum River, particularly for multidrug resistance (MDR) and ESBL strains of *E. coli* in several river clusters, such as community residential clusters (Cikapundung), livestock clusters (Cisanti), and industrial clusters (Citepus).

Methods

This study was a cross-sectional descriptive study. The research materials comprised surface water samples from 3 Citarum River flow clusters taken in September 2020; the residential cluster (Cikapundung), the industrial cluster (Citepus), and the livestock cluster (Cisanti). A 100 mL sample was taken using a sterile bottle and inserted into a coolbox. The water sample was then immediately taken to the microbiology laboratory of the Faculty of Medicine Universitas Padjadjaran Bandung for further *E. coli* culture.

A dilution procedure of samples was performed in the microbiology laboratory, and 1 mL of each series of dilution was taken via pipettes. The sample was inserted into a petri dish labelled, added 15 mL to eosin methylene blue and incubated at 37 ^{0}C for 24–48 hours. The *E. coli* colony showed dark-colored, circular characteristics flattened with or without metallic sparkle. The total presumptive *E. coli* observed from three clusters was 54 isolates. After subculture, biochemical tests of glucose fermentation, lactose, indol, citrate, H2S, and gas production were conducted and confirmed as 37 isolates of *E. coli*.

The Kirby-Bauer method was used for antibiotic sensitivity tests based on the Clinical & Laboratory Standards Institute (CLSI).[10] Firstly, isolate bacteria were suspend in a 0.9% NaCl solution until 0.5 MacFarland turbidity is achieved (equivalent to 1.5 x 108 cells/mL). Furthermore, the suspension was inoculated into Mueller Hilton agar. Some antibiotic discs used for sensitivity tests were Tetracycline (TE), trimethoprim/sulfamethoxazole (SXT), nalidixic acid (NA), gentamicin (CN), ampicillin (AMP), azithromycin (AZM), and ciprofloxacin (CIP). The antibiotic disc was placed and incubated in an inverted position at 37 ^{0}C for 18–24 hours. Furthermore, with or without clavulanic acid, ESBL was determined using ceftazidime (CAZ) and cefotaxime (CTX) discs. Next, calculate the inhibition zone and adjust the inhibition zone's interpretation to observe the antibiotic's sensitivity.

The blaCTX-M-15 gene, which carried ESBL properties in *E. coli* isolates, was also determined for each *E. coli* isolate from all 3 clusters using the following procedure. Phenol-chloroform DNA extraction was a procedure that utilized the principle of liquid-liquid extraction. A total of 200 µL of TRIsure was added and then homogenized. The sample was then incubated for 5 minutes at room temperature. After adding 40 µL of chloroform, it went through a vortex for 15 seconds, followed by incubation for 3 minutes. Next, the samples were centrifuged at 12,000 gram for 15 minutes at 4 ^{o}C, which resulted in the organic phase (DNA), interphase (Protein), and water (RNA). Sixty µL of ethanol (100%) was added, homogenized inversely, and subsequently incubated for 3 minutes at room temperature. A total of 2,000 gram were centrifuged for 5 minutes at 4 ^{o}C. Meanwhile, a 200 µL of sodium citrate 0.1 M with ethanol (10%) was added to DNA pellets that appeared after this process. This solution was kept homogenous for 30 minutes and then incubated for 3 minutes, centrifuged in another 2,000 gram for 5 minutes at 4 ^{o}C and kept dry for 15 minutes, followed by adding 25 µL of sodium hydroxide. The samples were further centrifuged in 12,000 gram for 10 minutes at 4

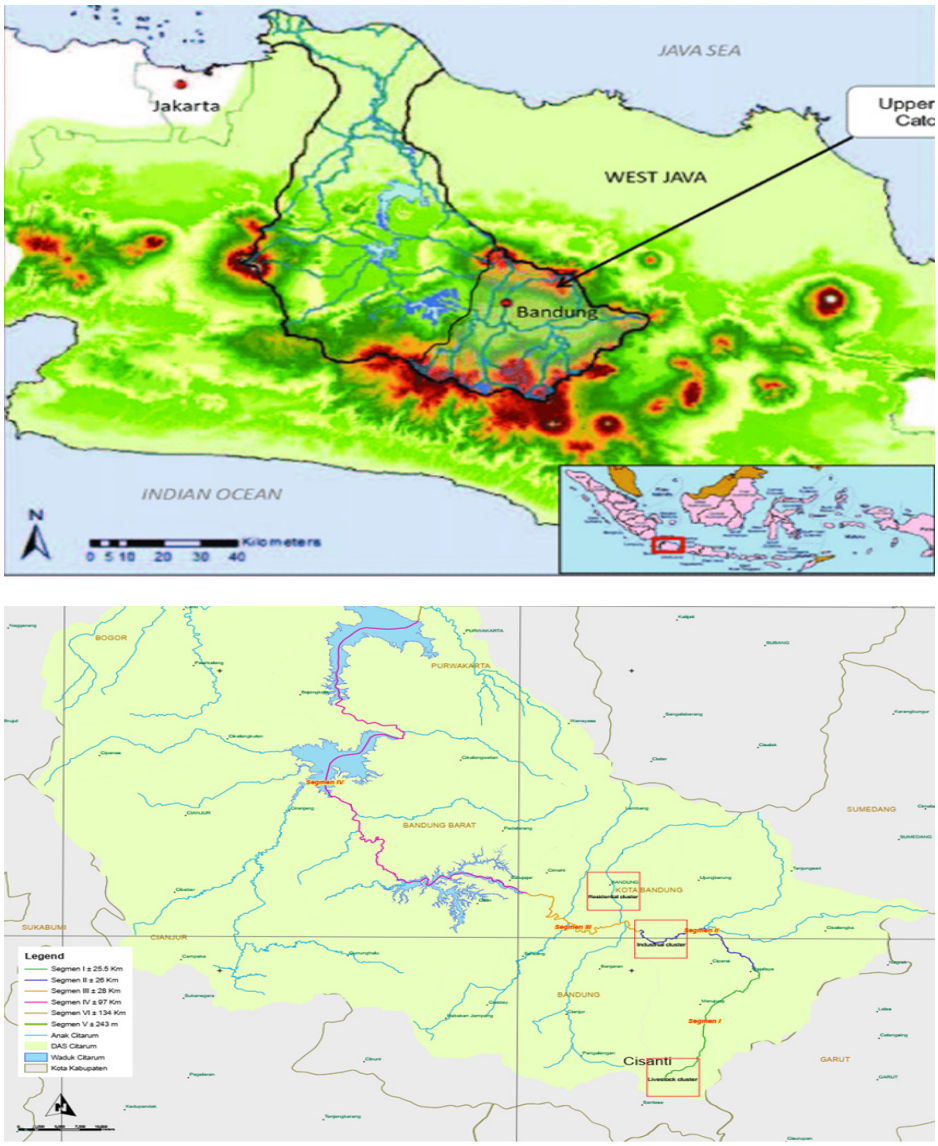

Figure 1 Map of Citarum River

°C. After forming the supernatant, the pH was set to 7.5 using EDTA 1 mM and quantifying the DNA A260/A280. The supernatant was then stored in the refrigerator at -20 °C.

Amplification was performed with a mixture containing 160 µmol/L dNTP, 1X GoTaq buffer (Promega Corporation, Madison, WI), 2 mM $MgCl_2$, 1.25 U GoTaq DNA Polymerase (Promega), a final volume of the primary reaction of 25 µL, and a 1 µL DNA template. Amplification was used hot start techniques to avoid non-specific/non-target amplification. GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) was used as an amplification tool. Amplification was carried out according to the first stage of denaturation with a temperature of 94 °C for 5 minutes, followed by 35 cycles of denaturation with a temperature of 94 °C for 30 seconds, annealing with a temperature of 55 °C for 30 seconds, extension 72 °C for 1 minute, and the last extension stage with a temperature of 72 °C for 5 minutes. The results were observed by means of gel electrophoresis in agarose (1.5%) with ethidium bromide. The research had been approved by the Research Ethics Committee of the Universitas Padjadjaran (774/UN6.KEP/EC/2020).

Results

In total, 37 isolates of *E. coli* isolates were obtained from the residential cluster

Table 1 Overview of *E. coli* Isolates' Overall Sensitivity to Various Antibiotics

Antibiotics	Antimicrobial Susceptibility Test		
	Resistance(%)	Intermediate (%)	Sensitive(%)
Ampicillin	45	8	47
Azithromycin	29	-	71
Cefotaxime	3	5	92
Ceftazidime	5	3	92
Ciprofloxacin	13	30	57
Gentamicin	3	-	97
Nalidixic acid	29	5	66
Tetracycline	37	-	63
Trimethoprim/Sulfamethoxazole	10	3	87

(Cikapundung; n=11 isolates), industrial clusters (Citepus; n=12 isolates), and livestock clusters (Cisanti; n=14 isolates) river water. The upstream of the Citarum River (Cisanti kilometres 0), which was a nature reserve with water quality protected from pollution, no *E. coli* isolates were found.

All 37 isolates collected were tested on their sensitivity to several antibiotics such as ampicillin, tetracycline, azithromycin, nalidixic acid, ciprofloxacin, trimethoprim/sulfamethoxazole, ceftazidime, cefotaxime, and gentamicin, as shown in Table 1.

In general, the results showed that the highest resistance to *E. coli* was ampicillin (45%), tetracycline (37%), azithromycin (29%), and quinolones such as nalidixic acid (29%). Some isolates of *E. coli* also showed intermediate sensitivity to some antibiotics, with the highest for ciprofloxacin (30%).

The proportion of *E. coli* isolate sensitivity to several types of antibiotics from each Citarum River cluster was shown in Figure 2.

The characteristics of *E. coli* isolate resistance in each cluster to the number of antibiotics tested were presented in Table 2.

Overall, *E. coli* isolates were most commonly found resistant to only 1 class of antibiotics, recorded at 30%. The number of *E. coli* isolates resistant to ≥3 antibiotics, or MDR, is 24%. Among the 64% of antibiotic-resistant *E. coli* isolates in livestock clusters, as many as 21% are MDR. In comparison, industrial clusters are mostly antibiotics-resistant at 92%, with a proportion of MDR isolates being found at 41%. There are 73% antibiotic-resistant isolates in residential clusters, but most (45%) are only resistant to 1 class of antibiotic; MDR isolates, on the other hand, have only one isolate (9%). The identification of the blaCTX-M-15 gene, which carries ESBL properties in *E. coli* isolates, was found to be negative in each *E. coli* isolate from all 3 clusters.

Discussion

There is no data on the antibiotic sensitivity pattern of *E. coli* isolates from the Citarum

Table 2 *Escheria coli* Isolate Sensitivity Pattern based on the Number of Antibiotics

Clusters	Number of Antibiotic-Resistant n (%)								
	R0*	R1*	R2*	R3*	R4*	R5*	R6*	Total	MDR
Livestock** (n=14)	5 (36)	3 (21)	3 (21)	2 (14)	-	1 (7)	-	14 (100)	3 (21)
Industrial** (n=12)	1 (8)	3 (25)	3 (25)	3 (25)	1 (8)	1 (8)	-	12 (100)	5 (41)
Residential** (n=11)	3 (27)	5 (45)	2 (18)	-	-	1 (9)	-	11 (100)	1 (9)
Total	9 (24)	11 (30)	8 (22)	5 (13)	1 (3)	3 (8)	-	37 (100)	9 (24)

Note: R0*= no antibiotic resistance, R1*= resistant to 1 class of antibiotics, R2*= resistant to 2 classes of antibiotics, R3*= resistant to 3 classes of antibiotics, R4*= resistant to 4 classes of antibiotics, R5*= resistant to 5 antibiotics, R6*= resistant to 6 classes of antibiotics. MDR= Multidrug resistance, if resistance ≥3 classes of antibiotics (CDC). ** Livestock clusters (Cisanti), industrial clusters (Citepus), and residential clusters (Cikapundung)

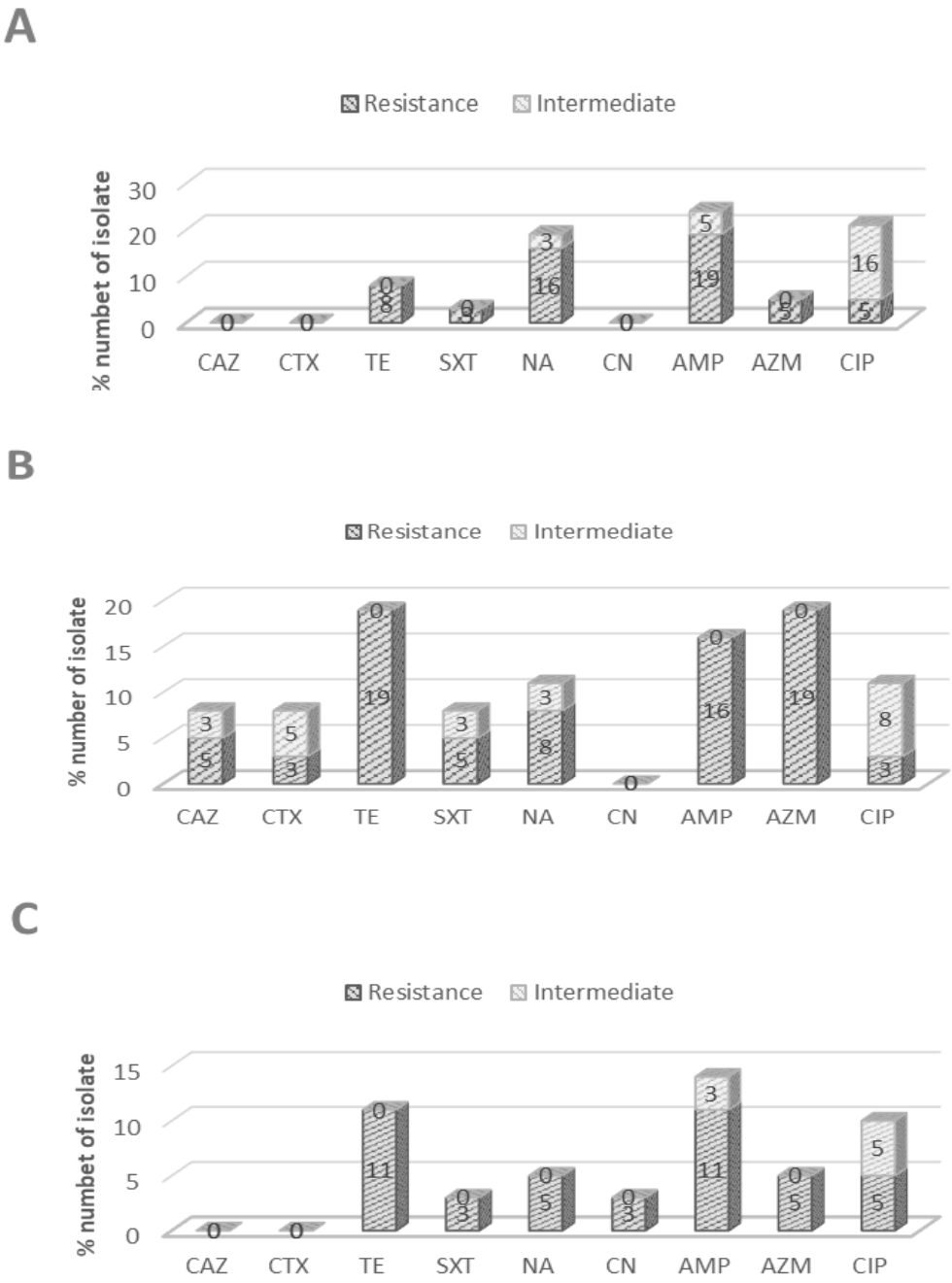

Figure 2 Sensitivity Pattern of *E. coli* Isolates in 3 Clusters of Citarum River. (A) livestock clusters (Cisanti); (B) industrial clusters (Citepus); and (C) residential clusters (Cikapundung).

Note: AMP= ampicillin; CTX= cefotaxime; CAZ= ceftazidime; TE= tetracycline; STX= trimethoprim/ sulfamethoxazole; NA=nalidixic Acid; CN = gentamicin; AZM = azithromycin; CIP = ciprofloxacin

river, including the identification of ESBL *E. coli*. Research on the sensitivity patterns of *E. coli* isolates from various other rivers in Indonesia mainly were assessed for resistance to commonly used antibiotics such as amoxicillin, chloramphenicol, sulfamethoxazole, and streptomycin, but nothing specific to ESBL *E. coli*.[11,12] As for some other countries, the presence of ESBL *E. coli* in rivers has begun to be identified, such as in the Netherlands and

South Africa, which have found ESBL isolates at 17% and 28%, respectively, with samples originating from river water and wastewater treatment plants.[13,14]

The presence of *E. coli* in rivers can be one indicator of human and animal excreta pollution.[15] This study identified 37 isolates of *E. coli* from the Citarum River, most likely due to pollution of livestock waste, bathing activities, washing, latrine, and the impact of industrial waste around the river flow considering the origin of samples being taken from clusters of livestock, industrial, and residential areas.[1] This reasoning is reinforced by the absence of bacterial isolates in cultures from the kilometer-0 (Km-0) upstream sample of the Citarum River, which is a nature reserve with ecosystems that are still preserved. *E. coli* isolates were also identified from samples of drinking water and raw water in residential clusters in the Cikapundung River area in a study conducted in 2018.[16]

The consumption of polluted river water as raw material for communities surrounding the area increases the risk of waterborne disease transmissions, where *E. coli* is the most common bacteria that can cause gastrointestinal infections. Some pathogenic *E. coli* strains are also known to have the ability to infect parts of the body outside the digestive tract, such as the urinary tract and even the central nervous system; therefore, the range of *E. coli*-related infections is becoming wider, which would significantly impact the increasing use of antibiotics to treat these various infections. The antibiotic groups commonly used as the first and second lines of *E. coli* infection therapy are β-lactams, trimethoprim-sulfamethoxazole, fluoroquinolones, and aminoglycosides.[17] In Indonesia, the most common antibiotic agents used in treating urinary tract and gastrointestinal infections caused by *E. coli* in both communities and health facilities are ampicillin, tetracycline, trimethoprim, fluoroquinolone, cephalosporin generation III, gentamicin and aminoglycosides.[18] Furthermore, antibiotics without detection in the community are common place until they reach 40–80% of the population.[19] This study shows that the antibiotics with the highest resistance level is ampicillin, which is 45% in line with the high use of ampicillin in the community.[18,19] In addition to ampicillin, quinolone, and tetracycline groups showed high resistance levels of 42% and 37%, respectively, followed by aminoglycosides trimethoprim-sulfamethoxazole. Moreover, many farms in Indonesia also use antibiotics to accelerate the growth of farm animals, thereby increasing the potential appearance of resistant *E. coli* in the river.[20] Antibiotics that are often used in farms include penicillin and tetracycline.[20] This research shows that *E. coli* isolates from livestock clusters (Cisanti) have high resistance to ampicillin and tetracycline.

Bacteria in the environment, such as in rivers, can have resistant properties due to various factors, primarily due to the excessive use of antibiotics in humans and animals. Other causes are mutations in specific genes that occur in bacteria and the horizontal gene transfer process.[21] Wastewater treatment plants around the Citarum River can also play a role in spreading antibiotic-resistant *E. coli* bacteria because it is a waste collection from various sources that eventually become a medium for the spread of these forms of bacteria.[22] In this study, industrial clusters became the most prevalent cluster of *E. coli* isolates that are MDR at 41%. Based on its geographical location along the river, the industrial cluster acts as a meeting point of streams that consolidate waste from textile factories, human, and farm waste; therefore it is possible that *E. coli* with MDR properties is most commonly found in industrial clusters. Additionally, findings from several studies conducted at the Citarum River water indicate increased heavy metal content.[23] Heavy metal content in rivers could trigger genetic mutations in bacteria, making them more resistant to extreme environmental conditions, including giving the appearance of bacteria resistant to antibiotics.[6] The influence of river water pollution on the appearance of *E. coli* MDR isolates also occurs in various rivers around the world.[24,25] Although this study found no ESBL *E. coli* colonies in all three Citarum River clusters, *E. coli* isolates with resistant properties, including MDR, were found in residential clusters, livestock, and industrial, indicating river pollution has occurred due to livestock activities, bathing, washing, latrine of residents around the river, and industrial waste.

A few limitations of the study include the small amount of *E. coli* isolates collected and the absence of molecular examination on the samples to identify possible mutations or genes that carry resistance properties. Thus, further research is needed to carry out molecular examinations with larger samples.

In conclusion, identification of *E. coli* isolates in each cluster of the Citarum River suggests that river pollution by industrial waste and animal or human excrement has

occurred through the presence of *E. coli* isolates with MDR, but none were found with ESBL. Therefore, it is necessary to make better government regulations regarding sanitation and education for the surrounding community regarding the importance of keeping the river clean.

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