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ESBL-Producing *Enterobacteriaceae* Isolated from Hospital and Community Health Center Wastewater in Pekanbaru, Indonesia

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Abstract

Wastewater originating from medical facilities, particularly hospitals, serves as an important reservoir for Extended-Spectrum Beta-Lactamase (ESBL)-producing bacteria. This study assessed ESBL-producing strains of *Escherichia coli* and *Klebsiella pneumonia* in the inlet and outlet of wastewater treatment systems, as well as in downstream sewage from a community health center and a public provincial referral hospital in Pekanbaru, Indonesia, during the period of October to December 2023. The analysis included the enumeration of bacterial colonies for presumptive ESBL-producing *E. coli* and *K. pneumoniae*, phenotypic confirmation of ESBL production, and identification of ESBL-associated genes. Substantial quantities of presumptive ESBL-producing *E. coli* and *K. pneumoniae* were identified in both the inlet and outlet of hospital wastewater, as well as in downstream sewage water. In contrast, wastewater from the community health center contained only *E. coli* in the inlet samples. Phenotypic testing confirmed ESBL production in two samples (11.1%). The ESBL genes bla_{CTX}, bla_{TEM}, and bla_{SHV} were detected in seven (38.9%), three (16.7%), and one isolates (11.1%), respectively. These findings highlight wastewater as a significant reservoir of ESBL-producing bacteria and underscore the need for strengthened strategies to limit their release into the environment. Effective mitigation requires targeted measures to reduce ESBL-producing bacteria in wastewater, along with continuous monitoring and control of wastewater treatment systems to prevent further dissemination of antibiotic resistance into aquatic ecosystems.

Keywords: ESBL, *Escherichia coli*, *Klebsiella pneumonia*, wastewater

Introduction

Antimicrobial resistance (AMR)has emerged as a major global health threat with widespread implications. Approximately five million deaths annually are associated with caused by drugs-resistant pathogens.[1,2] Wastewater generated from agricultural activities, pharmaceutical manufacturing facilities, and hospital discharges has been identified as a factor that fosters AMR.[3] Despite significant advancements in technology, wastewater treatment plants (WWTPs) have substantially enhanced treatment methods, yet the full elimination of antimicrobial compounds and other emerging pollutants is still unattainable. This is primarily due to the fact that the treatment methods and systems were rarely engineered with the specific purpose of eliminating emerging pollutants like antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), and antibiotics.[4] Additionally, stressors such as the existence of antibiotics, heavy metals, pH variations, and temperature fluctuations serve as triggers that induce and spread AMR among bacteria residing in wastewater.[5]

Approcimately 70% of consumed antibiotics are estimated to be excreted into wastewater in unchanged or partially metabolized forms.[6] Hospital effluents represent significant increase antibiotic, concentrations in wastewater discharged from healthcare facilities and create favorable conditions for the dissemination

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of ARGs. Higher concentrations of ARB and antibiotic residues are therefore expected in hospital wastewater compared with other urban wastewater sources.[7] The presence of antibiotic residues, AMR, and ARG in WWTPs has been reported in multiple countries, including Indonesia. [8,9]

Wastewater systems frequently contain pathogenic bacteria that contribute to the spread of AMR, including *Klebsiella pneumoniae* and *Escherichia coli*.[9–13] Beta-lactam antibiotics are among the most commonly used antimicrobial agents and are frequently detected in wastewater environments. Exposure to these antibiotics promotes the production of beta-lactamase enzymes by Enterobacteriaceae. Among these enzymes, extended-spectrum beta-lactamases (ESBLs) represent one of the most widely disseminated resistance mechanisms worldwide. ESBLs hydrolyze penicillins and third- and fourth-generation cephalosporins. The genes encoding ESBLs commonly belong to the blaCTX-M, blaTEM, and blaSHV families. These genes are often located on plasmids, enabling horizontal transfer among bacterial species and accelerating the spread of resistanc.[14]

ESBL-producing E. coli and *K. pneumoniae* are increasingly implicated in environmental transmission pathways, particularly through wastewater from healthcare facilities. However, no comprehensive data currently exist regarding their occurrence in Indonesian hospital and community health center wastewater, creating a critical research gap. Existing studies have primarily focused on selected regions in Asia and Europe.[15]

The prevalance of ESBL-producing Enterobacteriaceae in wastewater may vary accordingto geographic factors, wastewater sources, and antimicrobial prescribing practices, underscoring the need for context-specific evidence. Therefore, this study aimed to detect ESBL-producing *E. coli* and *K. pneumoniae* in wastewater from hospitals and community health center in Pekanbaru, Indonesia. The findings are expected to provide baseline data that support strategies for mitigating the environmental dissemination of antibiotic-resistant bacteria.

Methods

This study used a cross-sectional design and was conducted from October to December 2023. A total of 18 wastewater samples were collected from the inlet and outlet of wastewater treatment systems and the downstream sewage channels of a community health center (*Puskesmas*) and a provincial referral hospital in Pekanbaru, Indonesia. Research permits were obtained from the Riau Province Investment and One-Stop Service Agency, the National Unity and Politics Agency of Pekanbaru, and the Health Offices of Riau Province and Pekanbaru.

Sterile 1-liter bottles are used for sample collection, and sample were transported to the laboratory within one hour under cooled conditions. All microbiological analyses were performed at the Microbiology Laboratory of the Faculty of Medicine, Universitas Riau. The bacterial colonies of presumptive ESBL-producing *E. coli* and *K. pneumoniae* are counted using the serial dilution method.[16] Wastewater samples are vortexed to homogenize the sediment before dilution. Each sample underwent tenfold serial dilution by mixing 1 mL of with 9 mL of sterile distilled water. This dilution process was repeated three times for each sample (10^0, 10^{-1}, 10^{-2}, and 10^{-3}). From each dilution, 100 µL was spread onto MacConkey agar supplemented with 2 µg/mL cefotaxime using a sterile glass spreader. After inoculation, the petri dishes are then incubated at 37°C for 24 hours. Colony counts were calculated by multiplying the number of colonies by the dilution factor. Presumptive *E. coli* colonies appear pink, whereas *K. pneumoniae* colonies appeared pink and mucoid. Identification was performed using biochemical including indole, triple sugar iron agar (TSIA), Simmons citrate agar (SCA), and sulfide indole motility (SIM) tests. Selected colonies from inlet, outlet, and downstream sewage samples were preserved in trypticase soy broth supplemented with 10% glycerol for ESBL confirmation and detection of ESBL genes.

Phenotypic confirmation of ESBL production was performed using the double-disk synergy test (DDST). Isolates of E. coli and K. pneumoniae were tested using antibiotic disks of amoxicillin–clavulanate, ceftriaxone, and ceftazidime placed at a distance of 20 mm. The presence of a synergistic inhibition zone between the antibiotics indicated ESBL production.[16]

Molecular detection of ESBL genes was performed at the Biomedic Laboratory, Faculty of Medicine, Universitas Riau. Preserved isolates were re-inoculation on MacConkey agar prior to DNA extraction. Genomic DNA extraction was conducted using the boiling method. Approximately 5-10 pure colonies f *E. coli* and *K. pneumoniae*, were suspended in TE buffer

Table 1 PCR Primer Sequences and Product Sizes for blaCTX-M, blaTEM, and blaSHV Genes

Primer	Sequence (5'-3')	Product Size
CTX-M-F	ATG TGC AGY ACC AGT AAR GT	593 bp
CTX-M-R	TGG GTR AAR TAR GTS ACC AGA	
SHV-F	GGT TAT GCG TTA TAT TCG CC	867 bp
SHV-R	TTA GCG TTG CCA GTG CTC	
TEM-F	ATG AGT ATT CAA CAT TTC CG	867 bp
TEM-R	CTG ACA GTT ACC AAT GCT TA	

and heated to 98°C for 10 minutes, followed by cooling toroom temperature. The suspension was centrifuged at 10,000 rpm for 10 minutes to separate the supernatant and pellet. The supernatant containing DNA was transferred to a sterile tube and stored at -20°C until further analysis. Detection of ESBL encoding genes bla_{CTX-M}, bla_{TEM}, bla_{SHV}, was performed using the PCR method. [17] The primer sequences used in this study are shown Table 1.

PCR amplification was performed using a BIO-RAD T100 Thermal Cycler in a total reaction volume of 20 µL, consisting of 10 µL GoTaq Green Master Mix (2×) (Promega), 1 µL of each forward and reverse primer, 3 µL nuclease-free water, and 5 µL template DNA. The PCR cycling conditions were adjusted according to the specific genes under investigation. Cycling conditions followed standard protocols for ESBL gene detection, consisting of an initial denaturation (6 min at 95°C for the bla_{CTX-M} gene, 5 min at 96°C for the bla_{SHV} gene and bla_{TEM} gene), followed by denaturation (1 min at 95°C for the bla_{CTX-M} gene, 1 min at 96°C for the bla_{SHV} gene and bla_{TEM} gene), annealing (45 seconds at 51°C for the bla_{CTX-M} gene, 1 min at 60°C for the bla_{SHV} gene and 1 min at 58°C bla_{TEM} gene), and elongation at 72°C for 1 min to all gene (cycles 1-4 repeated 35 times). Then a final extension 5 min at 72°C for the bla_{CTX-M} gene and 5 min at 72°C for the bla_{SHV} gene and bla_{TEM} gene. A final extension step was performed at 72 °C for 5 minutes. PCR products were analyzed using electrophoresis on 1.5% agarose gel and visualized using a gel documentation system.

Results

Presumptive ESBL production *E. coli* and *K. pneumoniae* were detected in wastewater samples collected from the hospital and community health center, including inlet, outlet, and downstream sewage locations. The bacterial colony counts are presented in Table 2.

In the hospital wastewater system,*K. pneumoniae* was detected at all sampling points.The highest bacterial load was observed downstream sewage ($1.3×10^6$ CFU/100 mL), followed by the outlet ($1×10^6$ CFU/100 mL) and the inlet ($7×10^5$ CFU/100 mL). Similarly, *E. coli* was detected at all three points, with the highest concentration also found downstream ($8×10^5$ CFU/100 mL). In contrast, samples collected from the community health center showed no detectable levels of *K. pneumoniae* and only a minimal presence of *E. coli*, with $1×10^6$ CFU/100 mL observed at the inlet and no detectable colonies at the outlet or downstream.

Table 2 Colony Counts of Presumptive ESBL-Producing *E. coli* and *K. pneumoniae*

Healthcare Facility	Location	Colony Count of Presumptive ESBL Production Bacteria (CFU/100 mL)*	
		K. pneumoniae	*E. coli*
Hospital	Inlet	$7x10^5$	$3x10^5$
	Outlet	$1x10^6$	$7x10^5$
	Downstream	$1.3x10^6$	$8x10^5$
Community Health Center	Inlet	0	$1x10^6$
	Outlet	0	0
	Downstream	0	0

Table 3 Phenotypic Confirmation of ESBL Production and Detection of ESBL-Encoding Genes

Isolate Number	Species	Location	DDST Result *	ESBL-Encoding Genes		
				bla_{CTX}	bla_{TEM}	bla_{SHV}
1	*E. coli*	Inlet puskesmas	Negative	Positive	Positive	-
1B	*E. coli*	Inlet hospital	Positive	Positive	Positive	-
1C	*E. coli*	Inlet hospital	Negative	-	-	-
2A	*E. coli*	Inlet hospital	Negative	Positive	-	-
2B	*E. coli*	Inlet hospital	Negative	Positive	-	-
2C	*K. pneumoniae*	Inlet hospital	Negative	-	-	-
3A	*E. coli*	Outlet hospital	Negative	Positive	-	-
3B	*E. coli*	Outlet hospital	Positive	-	-	-
3C	*E. coli*	Outlet hospital	Negative	-	-	-
4A	*K. pneumoniae*	Outlet hospital	Negative	-	-	-
4B	*K. pneumoniae*	Outlet hospital	Negative	-	-	-
4C	*K. pneumoniae*	Outlet hospital	Negative	Positive	-	-
5A	*E. coli*	Downstream hospital	Negative	-	-	-
5B	*E. coli*	Downstream hospital	Negative	-	-	-
5C	*E. coli*	Downstream hospital	Negative	-	-	-
6A	*K. pneumoniae*	Downstream hospital	Negative	-	-	-
6B	*K. pneumoniae*	Downstream hospital	Negative	Positive	Positive	Positive
6C	*E. coli*	Downstream hospital	Negative	-	-	-

*DDST= Double Disk Synergy Test; Phenotypic screening using the Double Disk

Figure 1 Positive Double Disk Synergy Test (DDST)

The results of phenotypic ESBL confirmation and the detection of ESBL-encoding genes can be summarized in Table 3. Only two isolates (11.1%) were confirmed as ESBL producers by phenotypic testing. The ESBL genes $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV} were detected in 7 isolates (38.9%), 3 isolates (16.7%), and 1 isolate (11.1%) respectively. In one *K. pneumoniae* isolate obtained from the downstream sewage of the hospital, all three genes were found simultaneously. Despite higher bacterial colony counts observed at the wastewater outlet and downstream location, the frequency of ESBL genes detection was comparatively lower.

Synergy Test (Figure 1) identified several isolates showing clear synergistic expansion of the inhibition zone toward amoxicillin–clavulanate, confirming ESBL production. Molecular analysis

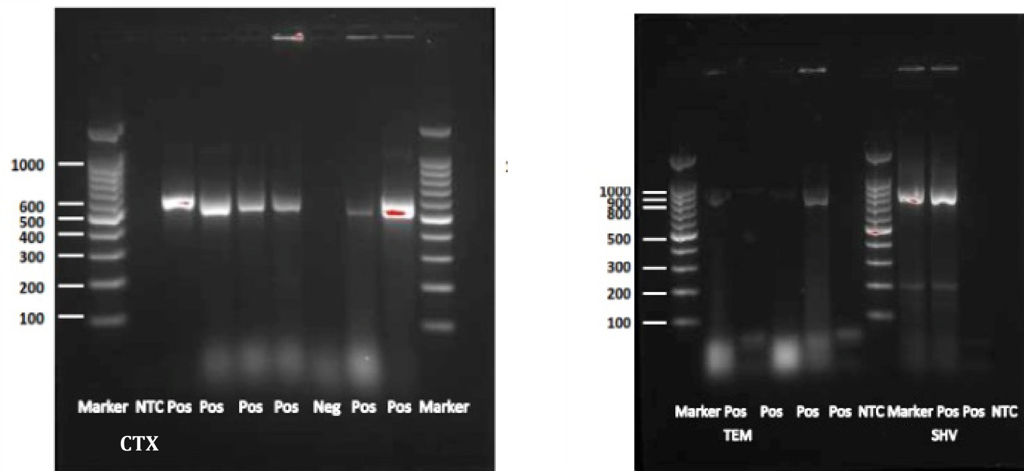

Figure 2 PCR Results for bla_{CTX-M}, bla_{TEM}, bla_{SHV} Genes

using PCR (Figure 2) confirmed the presence of ESBL-encoding genes, with amplification products corresponding to blaCTX-M, blaTEM, and blaSHV detected in several isolates. The combined phenotypic and genotypic findings demonstrate that a subset of the wastewater-derived *E. coli* and *K. pneumoniae* isolates carries ESBL-associated resistance determinants.

Discussion

Wastewater originating from hospitals represents an important environmental reservoir for *extended-spectrum beta-lactamas*e (ESBL).[15] This present study evaluated the occurrence of ESBL-producing *E. coli* and *K. pneumoniae* in wastewater from healthcare facilities in Pekanbaru. Notably, considerable amounts of presumptive ESBL-producing *E. coli* and *K. pneumoniae* were identified in both the inflow and outflow of hospital wastewater, as well as in downstream sewage water. Conversely, puskesmas wastewater exhibited the presence of only *E. coli* in the incoming wastewater.

These findings were derived from descriptive analysis, as no statistical testing was applied due to the descriptive nature of the study. Colony-forming units (CFU) of presumptive ESBL-producing bacteria were quantified at multiple sampling points (inlet, outlet, and downstream) in both healthcare facilities. Both *K. pneumoniae* and *E. coli* were detected in substantial quantities in hospital wastewater, with higher bacterial loads observed at the outlet and downstream sampling points. In contrast,, the community health center wastewater showed limited contamination, with *E. coli* detected only at the inlet and no detectable bacteria at the outlet or downstream locations. Lower contamination levels in community health centers wastewater are expected because these facilities generallyhave lower patient volumes, reduced antibiotic onsumption, fewer invasive procedures, and smaller wastewater discharge compared with hospitals.

Phenotypic ESBL confirmation and molecular detection of ESBL-encoding genes were performed to characterize the antimicrobal resistance profiles of the isolates. A summary of these results is presented in Table 3. Despite the high number of presumptive colonies, only 11.1% of isolates were phenotypically confirmed as ESBL producers. This discrepancy likely reflects the fact that growth on cefotaxime-containing media indicates reduced susceptibility but does not necessarily confirm functional ESBL enzyme expression.

These results suggest that hospital effluents may serve as an important source of antibiotic-resistant bacteria due to intensive antibiotic usage and selective pressure in clinical settings. Previous studies have also reported higher ESBL prevalence in hospital wastewater compared to community settings. Previous research has indicated that hospital wastewater exhibits the highest prevalence of ESBL-*E. coli* (33.98%), surpassing urban areas and animal waste.[15] This difference is to the higher consumption of antibiotics in hospitals compared with community settings. Hospital wastew`ater has also been reported to contain approximately 25% higher concentration of antibiotics and antibiotic

resistance genes than urban wastewater.[18] The extensive use of antibiotics in hospitals is also implicated in the genesis of ESBL,[19] transforming hospitals into environments highly conducive to ESBL-producing bacteria.[11,15]

of antibiotics and antibiotic resistance genes than urban wastewater.[20,21] In addition, antibiotic resistance genes (ARGs) can be transferred among bacterial populations in wastewater environments.[22,23] Previous studies have demonstated a direct relationship between clinical isolates of *E. coli* a nd the abundance of ESBL-producing *E. coli* detected in hospital wastewater.[24]

Hospital wastewater may ultimately enter environmental water bodies used foragricultural, domestic, or recreational purposes. Subsequently, antibiotic-resistant bacteria and/or ARGs may be transmitted to humans. Proper wastewater treatment methodologies can mitigate bacterial numbers and resistance gene prevalence. In many developing nations, wastewater from agriculture, communities, and hospitals often undergoes insufficient treatment, with limited functional treatment facilities available.[25] According to the World Health Organization (WHO), insufficient wastewater management can contribute to environmental contamination with resistant bacteria such as ESBL-producing *E. coli,* thereby threatening aquatic ecosystems and public health.[16]

PCR amplification targeting ESBL-encoding genes successfully identified the presence of three major ESBL genes—blaCTX-M, blaTEM, and blaSHV—in several bacterial isolate. DNA bands of approximately 593 bp were observed in multiple positive samples, corresponding to the expected amplicon size of the $bla_{CTX\text{-}M}$ genes. DNA bands of approximately 867 bp were visible in several positive isolates for both bla_{TEM}, bla_{SHV} genes. It is important to note that the presence of bla genes does not always correlate with ESBL phenotypic expression. Some genes may be silent or expressed at low levels, which may explain why more isolates were PCR-positive than phenotypically ESBL-positive.

The detection of these three ESBL genes indicaes the presence of genetic genetic determinants encoding ESBLs, which enzymatically hydrolyze and inactivate a broad spectrum of β-lactam antibiotics, including third-generation cephalosporins. This finding highlights the potential threat posed by multidrug-resistant Gram-negative pathogens such as *E. coli* and *K. pneumoniae* and emphasizes the importance of continuous molecular surveillance and effective infection control strategies to limit the spread of ESBL-producing organisms in both clinical and environmental settings.

Among the detected genes, bla_{CTX} was the most frequently identified. This observation is consistent with previous meta-analyses reporting blaCTX-M as the predominant ESBL gene worldwide, with a prevalence of 66.56% (95% CI: 53.98–78.15), followed by blaTEM (49.88%, 95% CI: 35.01–64.76) and blaSHV (21.58%, 95% CI: 13.71–30.49)).

Several limitations should be acknowledged. Sampling was limited to one hospital and one community health center in Pekanbaru, which may not fully represent the regional situation. Nevertheless, the findings support the study's objective by demonstrating that healthcare-associated wastewater can serves as an important reservoir of ESBL-producing *E. coli* and *K. pneumoniae*. Future research should include additional healthcare facilities and environmental sampling site, such as markets, rivers, and agricultural runoff areas, to provide a more comprehensive understanding of ESBL dissemination in the region.

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