

Biodiversity of *Escherichia coli* bacterial resistance to multidrug isolated on the Dumai coast of Indonesia

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Abstract. Feliatra F, Mardalisa M, Efendi I, Adelina A, Feliatra VA. 2021. Biodiversity of *Escherichia coli* bacterial resistance to multidrug isolated on the Dumai coast of Indonesia. *Biodiversitas* 23: 10-16. Anthropogenic pollution around the Dumai coast of Indonesia disturbs marine microorganisms, including commensal bacteria such as fecal *Escherichia coli*. *Escherichia coli* have often been used as a sensitive indicator for the spread of Antibiotic Resistance Genes (ARGs) among pathogens. This study aimed to analyze the development of *E. coli* resistance, originating from the Dumai sea waters using six antibiotics against seven *E. coli* isolates (E1, E4, E6, E8, E11, E13, and E15) at the five stations. Furthermore, a sensitivity test was performed on Mueller Hinton Agar (MHA) solid media, based on the Kirby-Bauer disk diffusion method. The results showed 100% *E. coli* isolates resistant to narrow-spectrum antibiotics (Penicillin, Isoniazid, Streptomycin, and Erythromycin) at the five stations. Meanwhile, high resistance of *E. coli* against the broad-spectrum antibiotics was observed in station 4 (mangrove habitat) existed in Chloramphenicol, whilst no resistance was found in Ciprofloxacin. *Escherichia coli* isolates showed Multidrug Resistant (MDR) index value higher than 0.2 which suggests there is a high-risk of antibiotics pollution in Dumai seawater. Bioinformatics analysis of 16S rRNA gene sequences represents the identity of *E. coli* isolates at the species level (97.84% - 99.74%). This study revealed the presence of antibiotic pollution in Dumai seawater which may impact public health. Hence, the local community must be disciplined about the use of antibiotics freely.

Keywords: Antibiotic pollution, biodiversity, coastal water, *Escherichia coli*, multidrug resistant

INTRODUCTION

Sea is one of the most important ecosystems in human life, as numerous activities and inherent natural resources tend to support the welfare of communities and countries. Based on Geographic Information Systems (GIS), Indonesia features a maritime environment with a broad proportion of water (76.94%) compared to the total area of the Republic (Ramdhan and Arifin 2013). Furthermore, Dumai has been identified as a rapidly developing coastal area of the Riau Province with strategic water, and the city is characterized by dense industrial activities and human settlements, which contributes to the anthropogenic pollution of coastal areas (Zhao et al. 2014; Devarajan et al. 2016). This is evidenced by the presence of various industries, including oil and gas factories, palm oil mills, Fish Landing Centers (PPI), national and international ship ports (Yoswaty et al. 2021).

Anthropogenic pollution by communities residing in and around the coastal area disturbs marine microorganisms, including the high prevalence of antibiotic resistance (Nogales et al. 2011). Ecosystem disturbances in coastal waters may dispose of chain effects on health of living beings (Islam and Tanaka 2004; Phares et al. 2020). Like peoples of other countries, antibiotics are the drugs commonly used by peoples Indonesia. The pattern of

antibiotic consumption was increased around 65% based on research data from 2000 to 2015 in 76 countries (Klein et al. 2018). This increase is mainly due to the low levels of knowledge, income, and facilities of society (Vila and Pal 2010). High antibiotic consumption is related to self-medication behavior without medical procedures. The easy access to antibiotics without a doctor's prescription (Ain and Septian 2015) also added to their increased consumption. *Escherichia coli* multidrug-resistance was detected in Indian children that caused by demographic factors like mother's education, type of family and the access to consume antibiotics (Singh et al. 2018).

Escherichia coli is a facultatively anaerobic, rod-shaped, Gram-negative bacteria of the family *Enterobacteriaceae* commonly found in the intestinal tract of humans and warm-blooded animals (Marflitt and Sandle 2017). *Escherichia coli* is one of the commensal bacteria used as the representative of the reservoir of antibiotic resistance genes (ARGs) in a marine community. Antibiotic resistance genes (ARGs) is generally carried by plasmid R, which can be easily transferred through conjugation (Silver et al. 1977). This unit consists of two parts, including the resistant transfer factor and the determinant of resistance, where both are responsible for the intrinsic nature. Furthermore, plasmid R is known to be very stable and evenly maintained, despite the small

amount of copy at the cell division stage of bacteria (Nordström and Austin 1989), while, the transfer process between species or genus occurs with about 100% efficiency. This molecular mechanism has generally been widely studied in *E. coli*, while, the presence of transposons and integrons in regions of bacterial chromosomes and extra chromosomes greatly influences the nature of multidrug resistance (MDR). Also, this characteristic of gram-negative bacteria is often caused by the mechanism of multidrug efflux pumps (Nikaido 2009).

Information related to bacterial resistance is very important for human life. This is due to the use of antibiotics as a therapy for the health of humans, livestock, and cultivated fish. The Indonesian government's attention to antibiotic resistance has been started in PMK No. 8 of 2015, concerning Antimicrobial Resistance Control Program (Permenkes 2015). Therefore, this study was designed to analyze the prevalence of antibiotic resistance of *E. coli* bacteria found in Dumai seawater, Riau Province, with the aim of providing an overview of the nature of multidrug resistance (MDR).

MATERIALS AND METHODS

Study area

The study was conducted in the Dumai seawater, Riau Province, Indonesia between April 2019 and May 2020.

Sampling

The process of water sampling for research materials related to water quality and microorganisms content at the sampling stations did not require any special permission because these field studies did not involve endangered or protected species. Water samples were collected (roughly 1 L) just one time by purposive sampling method at 5 different stations (three points). Each point was determined by the Geographical Positioning System (GPS) coordinate (Table 1). Station 1 was in areas surrounding Industries, station 2 was nearby Settlements, station 3 was about the Harbor, station 4 was located around the Mangrove Area, and station 5 was in an area far from anthropogenic activities. The collected water samples were transferred in sterilized bottles and processed immediately with quality assurance/quality control standards (Singh et al. 2019).

Isolation and identification of *Escherichia coli*

The MPN (Most Probable Number) method was conducted through three stages, encompassing the presumptive, confirmed, and completed tests. Production of gas, acid formation, and abundant growth were counted from coliform samples. Briefly, three sets of tubes, each containing three tubes of Lactose Broth (Merck, Germany), with Durham's tube were inoculated with water samples (10, 1, and 0.1 mL each in three sets) and incubated at 37°C for 48 h. Development of turbidity, as well as gas formation, were taken as positive results and correlated with the statistical estimate of the mean number of coliforms in the sample (Rompré et al. 2002). The *E. coli* confirmatory test was performed by streaking the pure cultures on Eosin Methylene Blue (EMB) agar medium (Cappuccino and Sherman 2005), and characterized biochemically (Srivastava et al. 2017).

Antibiotic susceptibility test

The Kirby-Bauer disk diffusion method was implemented in microbial sensitivity tests against antibiotics based on the current Clinical and Laboratory Standards Institute (CLSI) (Patel et al. 2011). The isolates were inoculated in Mueller Hinton Broth media and incubated for 18hr at 37°C. About 100µl of bacterial suspension was spread on Mueller Hinton Agar and left dried before the antibiotic disks were put on the media surface. All the samples were treated with 6 different antibiotics following (Hecht et al. 2007): Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Penicillin (10 µg), Isoniazid (2 µg), Streptomycin (10 µg), and Erythromycin (15 µg).

Determination of MDR index

The value of the MDR index in the isolates was determined by dividing the number of resistant antibiotics by the total antibiotics tested (Hecht et al. 2007).

$$\text{MDR index value} = a / b$$

Where the variable 'a' refers to the number of antibiotics that show resistance, 'b' refers to the total number of antibiotics tested. If the isolate MDR index value > 0.2, it revealed a high risk of antibiotic pollution from water tested environment (Joseph et al. 2017).

Table 1. The geographic coordinates of sampling stations

Sites	Point 1	Point 2	Point 3
Station 1	01° 41' 27.38" N; 101° 26' 29.96" E	01° 41' 34.33" N; 101° 26' 31.12" E	01° 41' 40.17" N; 101° 26' 33.59" E
Station 2	01° 41' 21.77" N; 101° 25' 55.89" E	01° 41' 26.61" N; 101° 25' 57.03" E	01° 41' 32.44" N; 101° 25' 59.72" E
Station 3	01° 41' 52.32" N; 101° 25' 3.80" E	01° 41' 54.24" N; 101° 25' 16.90" E	01° 41' 52.22" N; 101° 25' 33.11" E
Station 4	01° 42' 18.87" N; 101° 24' 13.19" E	01° 42' 20.49" N; 101° 24' 16.53" E	01° 42' 31.04" N; 101° 24' 20.48" E
Station 5	01° 42' 40.44" N; 101° 24' 21.70" E	01° 42' 46.94" N; 101° 24' 25.79" E	01° 42' 54.60" N; 101° 24' 29.04" E

Note: N: North; E: East

DNA extraction and characteristic of 16S rRNA bacteria

The pure culture of *E. coli* was used as samples subjected to amplification process by PCR techniques (Senbadejo 2017). We used a Qiagen PCR kit and universal 16S rRNA primers like 24F (5'-AGAGTTTGATCCTGGCTCAG-3') (Lane 1991) and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3') (Massol-Deya et al. 1997). The visualization of PCR products was done on agarose gel (1%) for the confirmation test. The PCR product was sequenced to identify homology levels of samples with GenBank data sequences (<http://www.ncbi.nlm.nih.gov/>) using the BLASTN software.

Phylogenetic tree construction

The *E. coli* samples were aligned with *E. coli* sequences downloaded from the GenBank data using ClustalW software. The phylogenetic tree was constructed using the UPGMA model in the MEGA X software.

RESULTS AND DISCUSSION

The central industrial area of Indonesia is well known in Dumai city, Riau Province due to high anthropogenic activities (Yoswaty et al. 2021). It is located on the east coast of the island of Sumatra and is situated in Malaysia's outer waters bordering.

The Indonesian Ministry of the Environment stated on the quality standards about the total of *coliform* bacteria and *E. coliform* in the marine environment must not exceed 1000 CFU/100 mL and 200 CFU/100 mL, respectively (Hidup 2004). The density of *E. coli* at the 5 stations in Dumai seawater has exceeded the Ministry of the Environment standards (Figure 1), with the lowest value at station 5 (2.37×10^2 CFU/100 mL), which was identified as an area far from anthropogenic pollution. However, the density of *E. coli* at stations 2 and 3 (1.1×10^3 CFU/100 mL), known as an area prone to anthropogenic pollution (Feliatra et al. 2020; Feliatra et al. 2021), showed a higher coliform count because of the location near the harbor and densely settlements. Conversely, station 4 (7.45×10^2 CFU/100 mL) features a mangrove habitat, while station 1 (4.23×10^2 CFU/100 mL) is an industrial area (Lukistyowati et al. 2019). Indonesia's Ministry of Environment is in line with the international regulations issued by WHO, which stipulated a maximum limit of *E. coliform* (≤ 200 CFU/100 mL) for activities in the field of aquaculture (Santé et al. 2004). A similar study was revealed in river Krishna, India, where is the total coliform and fecal coliform counts were found in a range of 16×10^2 to 24×10^6 CFU/100 mL (Dhakayanaika and Kumara 2010).

MPN testing has been widely used for monitoring the water quality from coliform bacteria contamination. The pH range of Dumai seawater at the 5 stations (6.7 - 7.3) strongly supports the growth of *E. coli* bacteria (Tururaja and Moge 2010), with the presence of dissolved oxygen at about 8.0-8.6 ppm, which was categorized in good condition. Furthermore, the tidal currents range from 0.06-

0.2 m/s, and temperatures of 30.1-30.7°C were identified as supporting factors for the high density of *E. coli* which is currently at an unhealthy level. This is the cause of low awareness on the part of the community and government towards the management and sanitation of waste streams, which subsequently threatens public health. *Escherichia coli* which pollutes the waters can cause diarrhea, in the form of endotoxins produced by virulence factors (Prejit and Latha 2007).

Based on the identification of morphological, biochemical, and molecular processes, there were only 7 out of 17 isolates confirmed as *E. coli*. Other isolates only show homology under 95% after the sequencing analysis (genus level). E1 and E4 were isolated from station 1, E6 and E8 were represented from stations 2 and 3 respectively, E11 and E13 were collected from station 4, and last station 5 was represented by E15. All isolates were tested to antibiotic sensitivity based on the provisions of the current Clinical and Laboratory Standards Institute (CLSI), and the three criteria include resistance (R) with a 0-10 mm resistance zone, intermediates (I) at 11-19 mm, and sensitive (S) at above 20 mm (Wayne 2010). Furthermore, this study uses two groups of antibiotics, including broad-spectrum (Ciprofloxacin and Chloramphenicol) and narrow-spectrum (Penicillin, Isoniazid, Streptomycin, and Erythromycin) in Table 2. The selection of antibiotics was based on the common types that farmers used in pond feeds (Jayaprakash and Bright 2005). The results obtained from antibiotic power on *E. coli* isolates are shown in Figure 2.

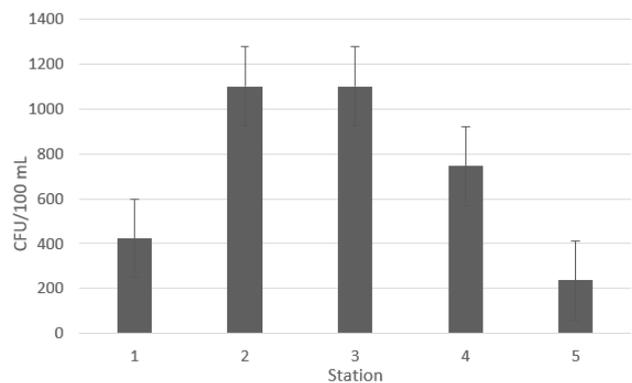


Figure 1. *Escherichia coli* density from 5 stations in Dumai seawater, Riau Province, Indonesia

Table 2. Percentage of *Escherichia coli* isolates resistance against antibiotics

Antibiotics	S	I	R	Percentage (%)			
				S	I	R	
Broad-spectrum	Ciprofloxacin	1	6	0	14	86	0
	Chloramphenicol	0	5	2	0	71	29
Narrow-spectrum	Penicillin	0	0	7	0	0	100
	Isoniazid	0	0	7	0	0	100
	Streptomycin	0	0	7	0	0	100
	Erythromycin	0	0	7	0	0	100

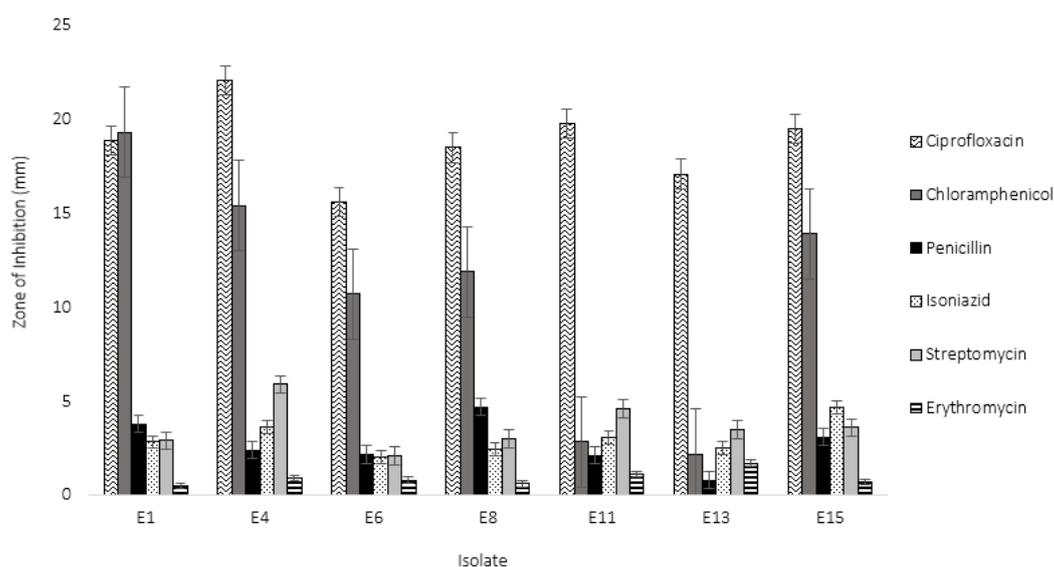


Figure 2. Results of the average resistance test of *Escherichia coli* isolates against antibiotics

The result from Table 2 and Figure 2 show that most *E. coli* tested have high resistance against all the narrow-spectrum antibiotics (Penicillin, Isoniazid, Streptomycin, and Erythromycin). The *E. coli* resistance to Chloramphenicol was found on isolates E11 and E13, whilst, no resistance of *E. coli* to Ciprofloxacin was found. The condition was also reported in Malaysia coastal waters that showed a high percentage of bacteria resistance to narrow-spectrum than broad-spectrum such as Chloramphenicol (Kian et al. 2012). The sensitivity test of Ciprofloxacin is quite good, because of its penetration properties in bacteria cells or tissues, and its inherent action of inhibiting Topoisomerase II and IV (Chohan et al. 2005). Antibiotics pollution not only occurred in developing countries but also happened in the universal, it is a global problem regards anthropogenic effects (Na et al. 2018).

Both isolates (E11 and E13) were isolated from the mangrove habitat (station 4), which is location near with aquaculture pond. The fact of biogeochemical cycles in mangrove ecosystems was contributed by anthropogenic waste and there has been a correlation to ARG potency in microbiota (Jiang et al. 2021). For example, by using Chloramphenicol in fish feed as an effort of farmers to treat fish infections. Consequently, it may affect heterotrophic bacteria from the water where it becomes resistant to Chloramphenicol as same as narrow-spectrum (Manivasagan et al. 2011; Javid et al. 2020). This reflected high antibiotic pollution in the water. It also conferred that indigenous bacteria could be considered as bio-indicators of antibiotic pollution (Al-Bahry et al. 2009).

The multidrug resistance (MDR) index value of each sample was calculated to determine the *E. coli* resistance to multidrug (Ab Rahman et al. 2015; Mardalisa et al. 2021). The Multidrug Resistance (MDR) index based on the data in Table 2 was calculated to be 0.714 (> 0.2). Even though, this study has small data, its patterns were succeeded to

support the recent study about antibiotic pollution in Indonesia (Reverter et al. 2020). It is assumed that usage of antibiotics more freely may lead to the development of *E. coli* resistance, so its bad effect contributed to infection problems in humans and animals (Krisnaningsih et al. 2005). This is supported by drug providers who sell antibiotics without a doctor's prescription (Ain and Septian 2015). The Indonesian government already set the rules on PMK RI No. 8 of 2015 to avoid the use of inappropriate and high antibiotic doses, due to the inherent potential of MDR (Permenkes 2015).

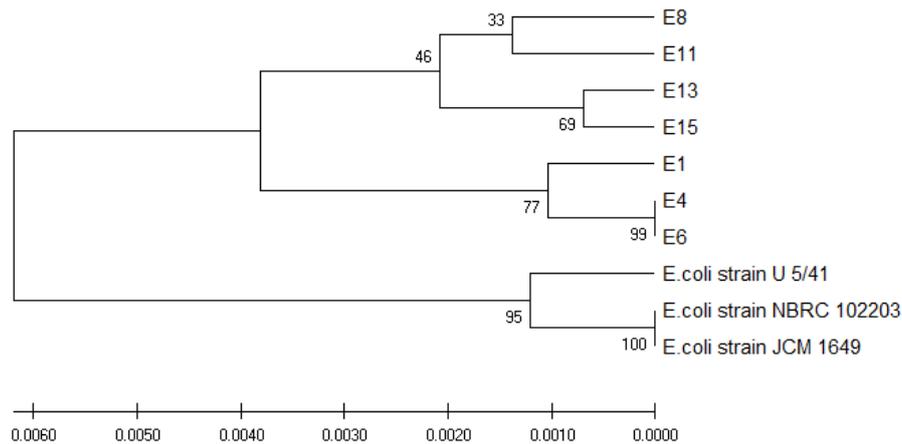
Most of the Gram-negative bacteria including *E. coli* are pathogenic to humans. A previous study reported that antibiotic resistance genes (ARGs) in plasmid R were transferred between the pathogenic and nonpathogenic Gram-negative bacteria in the aquatic environment with high antibiotic pollution (Zhang et al. 2011). *Escherichia coli* could be a host for the transmission of antibiotic resistance genes (ARGs) in the food chain via fish and other infected marine biotas. The possibility of cross-resistance to produce proteins encoded by the affiliated resistance genes (ARGs) in plasmid R (Al-Bahry et al. 2006).



Figure 3. Gel electrophoresis of 16S rRNA from *Escherichia coli* isolates. L: 1kb DNA ladder

Table 3. BLASTN analysis result from *Escherichia coli* isolates

Isolate	Species	Strain	Access code	Query coverage	Homology	Station
E1	<i>E. coli</i>	NBRC 102203	NR 114042.1	98%	98.68%	1
E4	<i>E. coli</i>	JCM 1649	NR 112558.1	76%	99.74%	1
E6	<i>E. coli</i>	JCM 1649	NR 112558.1	76%	98.74%	2
E8	<i>E. coli</i>	NBRC 102203	NR 114042.1	98%	98.12%	3
E11	<i>E. coli</i>	U 5/41	NR 024570.1	99%	98.62%	4
E13	<i>E. coli</i>	NBRC 102203	NR 114042.1	98%	97.84%	4
E15	<i>E. coli</i>	U 5/41	NR 114042.1	99%	98.41%	5

**Figure 4.** Phylogenetic tree for *Escherichia coli* isolates using UPGMA method

Identification of *E. coli* isolates was done by molecular test that used DNA barcode 16S rRNA. All isolates were successfully amplified by PCR technique using 24F and 1541R primers (Mardalisa et al. 2021). The results showed a single band for all samples at a size of around 1500 bp in 1% agarose gel (Figure 3). The 16S rRNA gene is the highly conserved region to identify the diversities in prokaryotic organisms and evolutionary relationships between prokaryotic strains (Mardalisa et al. 2020).

16S rRNA gene analysis is based on the detection of sequence differences (polymorphisms) in the hypervariable regions of the 16S rRNA gene which 97% of sequence similarity is recognized in the same species. Bioinformatic analysis showed that all samples show the homology level range from 97.84% to 99.74% to *E. coli* data in GenBank (Table 3) (Kim et al. 2014). Homology percentage in BLASTN analysis result showed the statistically significant similarity that reflects common ancestry based on sequence DNA (Pearson 2013).

The identification of the kinship for *E. coli* isolates was conducted using the UPGMA method, characterized by Multiple Alignments (MA) that aligns sequences of similar length (Figure 4). Based on the principle of Wunsch Needleman Algorithm, the branch length from the parent to the two-child nodes was the same (Malendes and Bunyamin 2017).

The study revealed that *E. coli* isolates obtained from Dumai seawater showed multidrug resistance. Therefore, it

is necessary for all the local communities to be disciplined in using antibiotics.

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