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The role of *mer*A gene of mercury-resistant *Escherichia coli* from Kahayan River, Central Kalimantan, Indonesia in emerging antibiotic resistance

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Abstract. *Martani NS, Notobroto HB, Wasito EB, Jabal AR. 2022. The role of merA Gene of Mercury-resistant* Escherichia coli *from Kahayan River, Central Kalimantan, Indonesia in emerging antibiotic resistance. Biodiversitas 23: 6629-6634.* This study collected nine water samples from the upstream, middle, and downstream parts of the Kahayan river, which was heavily contaminated with mercury (Hg). The water was sampled following the collection method of surface water sample (SNI 6989.57-2008). *Escherichia coli* has been found in the four samples out of nine samples collected. The *mer*A gene of bacteria was identified at 1695 base pairs (bp), *bla* at 199 bp, *tet* at 494 bp, *cat* at 623 bp, and *gyrA* at 577 bp. The *mer*A gene from S1 from the PCR results was identified at 219 bp. However, the *mer*A gene was not amplified with a primer measuring 1695 bp. Then, sequencing was performed, and the results indicated a 48.551% homology with *E. coli* Tn5075 nucleotide sequences. On examination of the *cat* gene, all samples are positive, or target-specific bands are observed (i.e., samples 1, 5, 6, and 7). Following sequencing, 623 bp were found to have nucleotide sequences that were 100% identical to *E. coli*. In all samples, the *gyr*A gene is either positive or has a band that corresponds to the target. Meanwhile, the results of nucleotide sequence homology of 99.826% were identical to *E. coli* at 586 bp. No *bla* gene or *tet* gene was identified. Sample 1 plate 1, 3, 4, 5, and sample 5 plate 3 exhibited intermediate results for ampicillin antibiotics, while the remaining results are sensitive to the tested antibiotics. Therefore, the mercury-resistant *mer*A gene found in *E. coli* from the Kahayan river in Central Kalimantan has not been conclusively linked to antibiotic resistance.

Keywords: Antibiotic resistance, *Escherichia coli*, *mer*A, mercury resistance

INTRODUCTION

Antibiotic-resistant microorganisms were thought to be the result of the irrational application of antibiotics. *Escherichia coli* have often been used as a indicator for antibiotic resistance genes between pathogens (Feliatra et al. 2022). Bacterial isolates from natural water resources carrying heavy metal-resistance genes were frequently found to be antibiotic-resistant, as both genes were present on the same plasmid (Sultan et al. 2020; Siddiqui et al. 2020). In many bacteria, resistance to mercury (Hg) is always associated with the plasmid. In general, microorganisms detoxify mercury through a chain of metabolisms that results in one of the volatile mercury derivatives (Boyd and Barkay et al. 2012).

The mechanism of mercurial enzymatic transformation in the microorganism can be categorized into four types: (1) Hg^{2+} reduction into Hg^0; (2) organomercurial compound breakdown into Hg^0; (3) Hg^{2+} methylation; and (4) oxidation of Hg^0 into Hg^{2+}. According to Christakis et al. (2021) several bacteria exhibit a detoxification mechanism based on the intracellular reduction from Hg^{2+} into non-toxic Hg^0 by the mercury reductase enzyme encoded into *operon mer* (*merA*). Formerly produced Hg^0 will be diffused away from cells (Wagner and Dobler 2003).

Previous research de Luca Rebello et al. (2013) determined that the *merA* gene in *Escherichia coli* exposed to high levels of mercury pollution in the aquatic systems in Rio Janeiro, Brazil. According to Gaeta et al. (2022) the presence of genes conferring resistance to mercury could be connected to environmental pollution in aquatic environments. Martani (2012) observed that mercury-resistant *Escherichia coli* isolated from the Kahayan river in Central Kalimantan and previously exposed to high mercury levels was also phenotypically resistant to multiple antibiotics. A previous study hypothesized that microorganisms resistant to heavy metals did not arise naturally but rather due to particular causes, environmental heavy metal pollution is one of these factors (Zaynab et al. 2022).

The deterioration in the quality of the Kahayan River has been mainly attributable to illegal gold mining (PETI) activities carried out by the people for succeeding generations (Environment Agency of Central Kalimantan 2017). The 2007 data from 2,264 gold drums operating in Central Kalimantan revealed 1,563 engine units inside the Kahayan Watershed. In the past three months, 1.5 tons of

Hg^{2+} had been dumped on the Kahayan River, resulting in Hg^{2+} concentrations of 0.014 mg/L. In addition, seven other rivers in Central Kalimantan were contaminated with Hg^{2+} concentrations ranging from 0.002 to 0.007 mg/L, exceeding the permitted threshold of PP No. 82 of 2001 about Management of Water Quality and Water Pollution Control, which is equal to 0.001 mg/L (Widodo et al. 2019; Wikurendra et al. 2022).

Prior studies indicated that mercury-resistant *Escherichia coli* was also resistant to antibiotics. Water quality monitoring conducted by the Environmental Agency of Central Kalimantan Province in 2017 showed that four locations were found to contain high levels of mercury. Consequently, it was necessary to determine whether *Escherichia coli* found in Kahayan river, Central Kalimantan, had already developed mercurial resistance genotypically. This study aimed to analyze *mer*A Gene of Mercury-resistant *Escherichia coli* in Emerging Antibiotic Resistance from Kahayan River, Central Kalimantan.

MATERIALS AND METHODS

Study area

Samples were collected from the Kayahan River of Central Kalimantan, Indonesia in 2018. There were nine sampling locations on considering easy access to location, and near a mining company in the area.

Measuring mercury content in water samples

Initially, 125 mL of water was taken from previously collected water samples, replicated twice, and filtered using a porous membrane filter before being filled into sterile bottles. Then, 100 mL of water was filled into tubes and added with 5 mL concentrated sulphuric acid, 2.5 mL concentrated nitric acid, and 5 mL $SnCl_2$ solution before the tubes were covered. The solution was mixed for 90 sec using a magnetic stirrer, and then the solution was diluted up to the threshold mark using mineral-free water. Next, in the atomic absorption spectrophotometer (AAS), a hollow cathode lamp and air rate were set, and the instrument was connected to a PC to record the results. Samples were then analyzed using AAS and recorded their absorbance from each solution. Eventually, the mercury absorbance curve was drawn by applying extrapolation, and mercury concentration was converted.

Isolation of *Escherichia coli* from water samples

This method used three tube groups. A single group consisted of five tubes in which each was filled with 10, 5, and 5 mL lactose broth medium and sterilized Durham flasks, respectively. After that, each tube of groups 1, 2,and 3 was added by 10, 1, and 0.1 mL of an examined water sample using a measuring pipette aseptically. All test tube was incubated at a temperature of 35°C for 24 hours. If inadequate gas (<10 %) was detected within Durham tubes, incubation was extended by 24 hours (a total of 48 hours incubation). If, after 24 hrs of incubation, gas was determined to be adequate (≥ 10 %), the presumptive test was declared positive. Positive tubes from each group were recorded, and an MPN table was used to determine coliform MPN from each 100 mL water sample examined.

Positive cultures from each group were subcultured in test tubes filled with liquid BGLB Brilliant Green Lactose Bile Broth medium completed with Durham tubes. Medium volume and culture were added based on its original presumptive group. Test tubes were then incubated for 24 hours at a temperature of 35°C. If air bubbles were formed, the test result was declared positive and contained *E. coli*. Positive results from each group were recorded, and an MPN table was used to determine the MPN of *E. coli* from the respective 100 mL water sample examined.

The positive culture from the determination test was streaked on an EMB medium and incubated for 24 hours. Subculture colonies were then stained using gram staining. Positive results were indicated by metallic green colonies, gram staining revealing rod-shaped, gram-negative, non-spore-forming bacteria observed using a microscope, and the IMVIC biochemical reaction test results. If the complementary result was positive, the isolated bacteria were confirmed to be *E. coli*.

Antibiotic resistance test

The cotton swab was dipped into bacteria culture and then dispersed evenly on the surface of the Mueller-Hinton agar dish using the streak method. Agar was allowed to solidify for five minutes before paper discs were saturated with antibiotics (ampicillin, tetracycline, chloramphenicol, and nalidixic acid) at a certain level. The culture was then incubated at 35°C for 24 to 48 hrs. The resulting inhibition zone was measured (in mm) and then compared to the Clinical Laboratory Standard Institute (CLSI) reference table for the inhibition zone (Widowati et al. 2008).

Table 1. Primers used for amplification of resistant genes in *Escherichia coli*

Primer name	Sequence (5-3)	Size	Source
cat-F, *cat*-R	5`-CCTGCCACTCATCGCAGT-3` 5`-CCACCGTTGATATATCCC-3`	623 bp	Zhang et al. (2022)
tet-F, *tet*-R	5`-TTGGCATTCTGCATTCACTC-3` 5`-GTATAGCTTGCCGGAAGTCG-3`	494 bp	Nohr-Meldgaard et al. (2021)
gyrA-F, *gyrA* R	5`-CTGAAGCCGGTACACCGT-3` 5`-GGATATACACCTTGCCGC-3`	577 bp	Rigouts et al. (2015)
bla-F, *bla*-R	5`-ATACCGCGCCACATAGCAGAA-3` 5`-AGTATTCAACATTTCCGTGTCG-3`	199 bp	Effendi et al. (2018)
*mer*A-F, *mer*A-R	5`-CGGGATCCATGAGCACTCTCAAAATCACC-3` 5`-TCCCCCGGGATCGCACACCTCCTTGTCCTC-3`	1695 bp	Boy and Barkay (2012)

Identification of mercury- and antibiotic-resistance *Escherichia coli* genes

Extraction with QIAamp DNA Mini Kit (QIAGEN) into a 1.5 mL microtube containing samples, added 180 µL of Tissue Lysis Buffer (TLB) solution and 20 µL proteinase-K. The microtube is then shaken using a mini mixer and incubated at 56°C for 1-3 hrs per night until lysis, then rotated for a few seconds. Next, 200 µL of LB solution (Lysis Buffer) was put into a micro tube, then shaken using a mini mixer for 15 sec and incubated at 70°C for 10 min. Next, the tube is rotated for a few seconds, then 200 µL of ethanol 100% is added, stirred, and shaken using the mini mixer for 15 sec. Then, the tube is rotated again for a few seconds.

After adding ethanol, a clear and white liquid will be formed. Then, all fluids are carefully transferred into the two mL mini spin-column and collection tubes were available, then properly closed and centrifuged at 6,000 g (8,000 rpm) for one minute. Collection tubes containing liquid are removed, then the mini spin-column is placed in a new collection tube, then 500 µL of AW-1 solution is added (column wash buffer-1) and centrifuged at 8,000 rpm for one min. Collection tube filled with discarded liquid. The mini spin column is then placed in a new collection tube, then 500 µL of AW-2 solution is added (column wash buffer-2) and centrifuged at 14,000 rpm for three minutes. The collection tube containing liquid is thrown back. The mini spin column is moved to 1.5 mL of clean or new microtube, then 200 µL of AE Buffer (Elution Buffer) or distilled water is carefully added.

After standing for 1-3 min, the samples are centrifuged at 8,000 rpm for 1 min. The DNA is then stored at -20°C until it is used in the amplification process. Extraction with QIAamp DNA Mini Kit (QIAGEN) into a 1.5 mL microtube containing tissue samples (25 mg), added 180 µL of TLB solution and 20 µL proteinase-K. The microtube is then shaken using a mini mixer and incubated at 56°C for 1-3 hours per night until lysis, then rotated for a few seconds. Then, 200 µL of AL solution was put into a micro tube, shaken using a mini mixer for 15 sec, and incubated at 70°C for 10 min. The tube is rotated for a few seconds, then 200 µL of ethanol 100% is added, stirred, and shaken using the mini mixer for 15 sec. The tube is rotated for a few seconds. The PCR amplification process was carried out in the reaction mixture with a volume of 20 µL per sample as follows: GoTaq®Green master mix (Promega, Madison WI USA) as much as 12.5 µL, nuclease-free water as much as 0.5 µL, primer (reverse and forward) each with 1 µL and a DNA template of 5 µL. The MJ thermal cycler is utilized to perform amplification. Table 2 shows the PCR cycle conditions for all tested genes.

The gene amplification results were detected by electrophoresis of each amplicon by ten µL on 1.5% agarose gel in the Tris Acetate EDTA buffer for 25 min. In addition, DNA staining was carried out by soaking the gel in ethidium bromide solution (0.5 µg per 100 mL TAE buffer) for 15 minutes. The results were documented with a digital camera.

The QIA quick gel extraction kit was used to purify the DNA extracted from the PCR results. Next, DNA purification was carried out by pro-sequencing PCR using one of the primers used in the previous PCR. Labeling with the BigDye ® XTerminator™ Purification kit is completed at this stage. Next, the purification of DNA from PCR labeling pro-sequencing was done with the BigDye XTerminator™ purification kit from ABI, and then dry DNA was stored until the time was applied to the sequencer machine after being mixed with reagent sequencing from the Applied Biosystems. Finally, in the PCR results of purified labeling pro-sequencing, containing nucleotide fragments amplified from the desired genome region, nucleotide sequence analysis was carried out by the direct sequencing method with application to the ABI DNA 310 sequencer from Applied Biosystems, Inc. This procedure requires capillary buffers containing POP 6 polymer and a tube for 0.5 mL sequencing from Applied Biosystems, Inc.

RESULTS AND DISCUSSION

Results

Bacteria isolation was then performed from water samples. Four of the nine samples tested positive for the presence of *E. coli*, i.e., S1, S5, S6, and S7. In contrast, no *E. coli* was identified in samples S2, S3, S4, S8, or S9. The result of inhibition zone evaluation from antibiotic resistance test using paper disc diffusion (Kirby Bauer) method was presented in Table 3, and then it was compared to CLSI table 2014. On examination of paint genes, all samples are positive or have bands corresponding to the target (samples 1, 5, 6, and 7). After sequencing, the results of nucleotide sequences homology of 100% identical to *E. coli* were 623 bp. The *gyr*A gene is positive or is found to be the band corresponding to the target in all samples (samples 1, 5, 6, and 7). The analysis of the *mer*A gene in each sample of bacteria (S1, S5, S6, and S7) yielded negative results.

Table 2. PCR conditions

Primer	Cycling conditions					
	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
cat	94°C (2 min)	94°C (1 min)	49-58°C (1 min)	72°C (1 min)	72°C (5 min)	30
tet	95°C (5 min)	94°C (30 sec)	53-60°C (30 sec)	72°C (30 sec)	72°C (10 min)	35
*gyr*A	95°C (5 min)	94°C (45 sec)	53-60°C (45 sec)	72°C (45 sec)	72°C (10 min)	35
bla	94°C (5 min)	94°C (30 sec)	55°C (30 sec)	72°C (1 min)	72°C (10 min)	30
*mer*A	95°C (5 min)	95°C (1 min)	63°C (2 min)	72°C (3 min)	72°C (5 min)	30

Table 3. Measurement result of inhibition zone diameter in the Institute of Tropical Disease (ITD), University of Airlangga, Surabaya, in 2014

	Plate (mm)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
S1										
AMP	13.06	15.85	12.03	12.98	12.01	15.19	15.20	17.64	18.18	16.32
C	25.06	25.69	23.20	27.64	29.61	24.22	29.52	27.48	31.91	27.46
NA	25.80	25.19	25.08	29.86	26.14	26.82	27.48	24.78	26.88	25.48
TE	23.36	24.10	23.40	28.74	24.78	23.39	24.36	25.39	24.52	24.69
S5										
AMP	15.80	15.31	13.45	16.34	15.77	16.49	15.52	17.46	16.02	18.42
C	26.86	26.68	30.49	29.11	24.22	29.73	27.59	29.40	27.91	29.42
NA	24.20	25.59	26.12	26.79	26.58	27.21	26.22	27.92	28.61	28.63
TE	26.38	25.06	25.51	24.86	24.51	28.91	25.12	27.28	24.52	26.85
S6										
AMP	17.61	15.75	15.19	16.38	15.29	17.92	15.18	15.28	16.20	16.51
C	27.60	27.43	29.29	29.52	27.82	26.59	27.41	27.94	25.89	23.86
NA	26.81	24.52	26.34	26.32	25.42	25.28	23.94	24.55	24.69	23.92
TE	25.42	24.86	25.43	27.09	24.14	23.58	23.62	22.78	26.22	22.08
S7										
AMP	16.01	17.62	16.48	17.83	16.08	15.68	16.82	19.92	17.62	17.28
C	28.89	27.82	23.22	29.01	24.75	25.01	26.83	27.15	28.02	27.93
NA	26.42	25.38	27.86	26.61	29.22	25.43	25.82	29.23	26.68	26.08
TE	25.56	23.19	25.69	25.74	25.01	24.25	24.63	28.61	25.43	29.78

In this study, nine water samples were obtained from the Kahayan river water, which spread in Kuala Kurun Sub-district Gunung Mas Regency, Bukit Rawi and Pangkoh Sub-district, Pulang Pisau Regency. The nine samples contained mercury exceeding the threshold. Among the nine water samples, there were four water samples containing *E. coli*. One sample was detected for *E. coli* containing MerA from Tumbang Miri Village, Kuala Kurun Sub-district, Gunung Mas Regency.

The measurement of mercury (Hg) content in Kahayan river water using the AAS method revealed that mercury levels were above the 0.001 mg/L standards set by the regulation of PP No. 82 of 2001 on water quality management and water pollution control (Javendra, 1995). It was caused by contamination from unauthorized gold mining. Of the nine samples, S5 was the highest mercury content at 1.22 mg/L, while S4 was the lowest at 0.26 mg/L. Afterward, bacteria were isolated from water samples. Four of the nine samples tested positive for the presence of *E. coli*, i.e., S1, S5, S6, and S7. In contrast, no *E. coli* was detected in samples S2, S3, S4, S8, and S9.

The result of inhibition zone evaluation from antibiotic resistance test using paper disc diffusion (Kirby Bauer) method was presented in Table 3; then it was compared to CLSI table 2014. On examination of paint genes, all samples are positive or have bands corresponding to the target (samples 1, 5, 6, and 7). Then after sequencing, the results of nucleotide sequences homology of 100% identical to *E. coli* were 623 bp. Furthermore, the *gyr*A gene is positive in all samples (samples 1, 5, 6, and 7).On the other hand, analysis of the *mer*A gene in all bacteria samples (S1, S5, S6, and S7) produced negative results.

Discussion

The mercury concentration in the Kahayan river in Central Kalimantan exceeded the WHO-approved threshold of 0.001 mg/L (Widowati et al. 2008). It resulted from illegal gold mining that contaminated river water with mercury when extracting gold particles. The mercury levels in the water samples collected from this river ranged from 0.26 mg/L in sample S4 to 1.22 mg/L in sample S5. The variance in mercury concentration was influenced by the number of gold miners in each area. Consequently, more gold miners would increase mercury levels in the water and vice versa.

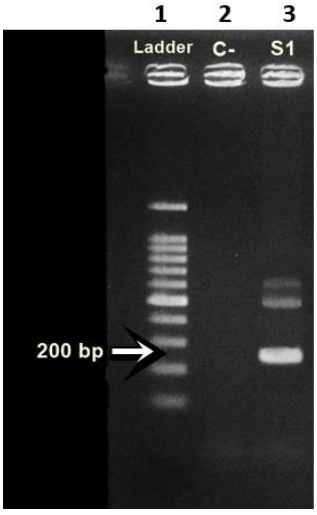

Figure 2. optimization results *mer*A gene from S1. Lane 1: DNA Ladder, Lane 2: Nuclease Free Water, Lane 3: sample 1

Four isolated samples tested positive for the presence of *E. coli*, i.e., S1, S5, S6, and S7. It was affected by the variation in physical and chemical properties of the upstream or downstream sample locations, including mercury content, pH, temperature, and turbidity. Following isolation, the bacteria's antibiotic resistance was assessed. The diffusion disc method (Kirby-Bauer) was used to evaluate the four samples for antibiotic resistance to ampicillin, tetracycline, chloramphenicol, and nalidixic acid. Antibiotic test results for sample 1 plate 1, 3, 4, 5, and sample 5 plate 3 indicated intermediate results when testing the ampicillin. The test revealed that the findings were sensitive to the tested antibiotics.

This study discovered that bacteria isolated from a natural water source with heavy metal resistance genes also had antibiotic resistance genes, and both genes were identified in the same plasmid (Yamina et al. 2012). Eighty percent of all mercury-resistant *E. coli* Hydrargyrum Resistant Escherichia coli (Hgr *E. coli*) were resistant to ampicillin, 58 percent to nalidixic acid, 30 percent to tetracyclin, and 18 percent to chloramphenicol (Tiwari et al. 2022). The mercury resistance gene (*merA*) has been hypothesized to be present in bacteria capable of surviving at a concentration of 0.01 mg/L $HgCl_2$ (Chandan et al. 2017). However, in the current investigation, *mer*A was found positive at a 1.22 mg/L mercury level. *E. coli* isolated from the mercury-contaminated Yamuna river at a concentration of 3.76 mg/L had the 1695-bp *mer*A gene. In the most recent investigation, PCR analysis of the *mer*A gene revealed the presence of a band; further sequencing analysis revealed a nucleotide sequence homology of 48.551%. It means that the *mer*A gene is 219 bp instead of *E. coli*. On examination of paint genes, positive or target-specific bands were detected in all samples (samples 1, 5, 6, and 7). After sequencing, the identical nucleotide sequences of *E. coli* were determined to be 623 base pairs. The *gyr*A gene is positive in all samples (samples 1, 5, 6, and 7). After sequencing, the results of nucleotide sequences homology of 99.826% were identical to *E. coli* at 586 bp. In all samples, the *bla* and *tet* genes were negative.

Mechanisms of cross-resistance, co-resistance, and co-regulation/co-expression of metal and antibiotic resistance were studied by Chandan et al. (2017). When one resistance system confers resistance to both an antibiotic and metal, this is known as cross-resistance. Co-resistance is when an organism has antibiotic and metal resistance that is physically co-located on the same genetic element, such as a plasmid. Meanwhile, co-regulation/co-expression occurs when the expression of the metal and antibiotic resistance system is controlled by a common regulator. It has not been conclusively demonstrated that the *mer*A gene causes the establishment of antibiotic resistance. Sample 1 plate 1, 3, 4, 5, and sample 5 plate 3 exhibited intermediate results for ampicillin, while the remaining samples demonstrated sensitivity to the antibiotics tested. The *mer*A gene contributes to the development of antibiotic resistance. Both are located on the same plasmid, and the *mer*A gene affects the expression of antibiotic-resistance genes. It is consistent with the findings of Tiwari et al. (2022) and Sultan et al. (2020), who observed that bacterial isolations from natural water bodies that carry genes for heavy metal resistance genes are also typically resistant to antibiotics, which are located on the same plasmid. Overall, the mercury-resistant *mer*A gene found in *E. coli* from the Kahayan river in Central Kalimantan has not been conclusively linked to antibiotic resistance.

In conclusion, the current research discovered *merA*-positive *E. coli*. The *mer*A gene in S1 was measured to be 219 base pairs by PCR. Sample 1 plate 1, 3, 4, 5 and sample 5 plate 3 on ampicillin antibiotics demonstrated intermediate results, while the remaining showed antibiotic sensitivity. Therefore, the mercury-resistant *mer*A gene in *E. coli* from the Kahayan river in Central Kalimantan has not been conclusively proven to produce antibiotic resistance.

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