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Research Article

Antimicrobial Resistance and Molecular Identification of Clinical Multi-Drug Resistant *Enterobacter cloacae*

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Abstract

Enterobacter cloacae is a Gram-negative bacteria causing nosocomial infections. This bacteria has increased resistance to various antibiotics in the past five years, resulting in a multi-drug-resistant (MDR) phenotype. In particular, MDR *E. cloacae* causes longer hospitalization time, increases medical costs, and affects morbidity and mortality. This study aimed to observe the minimum inhibitory concentration (MIC) of clinical *E. cloacae* towards several antibiotics and molecular identification of MDR *E.cloacae*. This study was conducted in a descriptive design. Secondary data was collected at the microbiology laboratory of the Teaching Hospital in Bekasi, Indonesia, from May to September 2020. Sampel was carbapenem resistant *E.cloacae*. The isolate was originated from a human clinical specimen, then was confirmed molecular identification using 16s rRNA. In this study, only one carbapenem-resistant *E. cloacae*, which is also MDR bacteria, was found. This *E. cloacae* was categorized as MDR bacteria since it was resistant to more than three antibiotic classes, including carbanemen, extended-spectrum cephalosporin, penicillins + β lactamase inhibitor, antipseudomonal penicillins + β lactamase inhibitor aminoglycoside, and penicillin. Vitek 2 identification of this isolate was *E. cloacae* complex. It showed similar results to molecular identification based on a partial sequence of 16s rRNA. BLASTn result of the trimmed sequence was *E. cloacae* with 99.78 % similarity.

Keywords: *Enterobacter cloacae, Minimum Inhibitory Concentration, Extended-spectrum beta-lactam, Multi-drug resistant*

1. Introduction

Enterobacter cloacae is a stem-shaped Gram-negative bacteria from the Enterobacteriaceae family that can not form spores (non-spores bacteria) and is a facultative anaerobe. This genus has several species that are a problem in the medical world, including the increased antibiotic resistance that causes the emergence of multidrug-resistant organisms (MDR) (Davin-Regli & Pagès, 2015). *E. cloacae* is one species of this member that brings a problem in healthcare settings and is included as a nosocomial pathogen. Recently, *E. cloacae* have increased antibiotic resistance following an increase in nosocomial diseases, including 5% cases of bacteremia in hospitals, 5% of

pneumonia, 4% of urinary tract infections, and 10% of post-operative cases of peritonitis (Wang et al., 2017).

One of the MDR *Enterobacter cloacae* studies in Indonesia was reported by (Janasuta, Sukrama, & Dwija, 2020), which stated that of 18 *Enterobacter cloacae* isolated from the urine of patients at Sanglah General Hospital in Denpasar for the period January 2015 - December 2016 resulted in resistance to 16 antibiotics with the highest proportion of resistance to ampicillin of 94, 4%.

The problem of *E. cloacae* resistance has been researched since 1990. Based on that study results, Khari et al (2016) concluded that the group of antibiotics often reported causing resistance to *E. cloacae* is the extended-spectrum beta-lactam class. Furthermore, Ferranti et al (2018) mentioned the percentage of antibiotic classes resistant to 90% extended-spectrum beta-lactams, 80% carbapenems, aminoglycoside 50%, and fluoroquinolone 30%. Jin research then added in its conclusion that in addition to being resistant to broad-spectral β-lactams, *E. cloacae* is also resistant to carbapenems, aminoglycosides, and Fluoroquinolones. The phenomenon is referred to as multi-drug-resistant (MDR) (Jin et al., 2018).

The discovery of MDR *E. cloacae* in carbapenems, β-lactam broad-spectrum antibiotics, aminoglycosides, Fluoroquinolones, and Monobactams, affects a new research interest in *E. cloacae* (Jin et al., 2018). Wu et al (2018) stated that a few data on *E. cloacae* resistance and the impact of *E. cloacae* resistance played a role in increasing research interest in *E. cloacae* resistance. Zhu et al (2020) added that the problem of *E. cloacae* resistance would impact hospitalization time, resulting in increased medical costs and the risk of morbidity and mortality.

Lack of data on *E. cloacae's* resistance to various antibiotics, the researchers were interested in researching on *E. cloacae's* susceptibility to carbapenems, β-lactams, aminoglycosides, fluoroquinolones, and monobacterial antibiotics. This study aimed to determine the minimum inhibitory concentration (MIC) value of several antibiotic groups inhibiting the *E. cloacae's* growth. The results of this study can then be used to monitor and control treatment therapies so that an effective and efficient system can be obtained in the treatment of *E. cloacae*.

2. Materials and Methods

2.1. Secondary data collection of *Enterobacter cloacae* MIC

The study design was descriptive research with MIC value of *E. cloacae* as secondary data. MIC value and biochemical identification were using automated bacterial identification and antimicrobial susceptibility Vitek 2 (bioMérieux, Marcy l'Etoile, France) from May to September 2020 in the microbiology laboratory of the Teaching Hospital in Bekasi, Indonesia. *E. cloacae* was recovered from pus specimen. For information, besides *E. cloacae*, other pathogenic bacteria have been successfully isolated, including *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Escherichia coli*. Bacterial identification based on the 16s RNA gene was performed as a confirmation.

According to CLSI (2018), non-susceptible carbapenem criteria are bacteria that have a Minimum Inhibitory Concentration (MIC) ≥ of 4 mg/L for at least one of imipenem, ertapenem, and meropenem. Antimicrobial susceptibility testing was performed using Vitek 2 (bioMérieux), including Ertapenem (ETP), Meropenem (MEM), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ampicillin-Sulbactam (SAM), Piperacillin-Tazobactam (TZP), Gentamycin (GEN), Amikacin (AMK), Ciprofloxacin (CIP), Ampicillin (AMP), Cefazolin (KZ), Aztreonam (AZT), Trimethoprim-Sulphamethoxazole (SXT) and Tigecycline (TGC).

2.2. DNA extraction

DNA was extracted using Promega Wizard Genomic DNA Purification Kit (USA). Bacteria were cultured in 3 ml Tryptic Soy Broth (TSB) with a 100 rpm shaker for 24 hours. Culture as much as 1 ml was centrifuged at 13,000 g for 2 minutes. The supernatant was disposed of while the pellet was resuspended with 480 µl 50 nM EDTA. The suspension was added with 60 µl lysozyme 10 mg/ml then was incubated at a temperature of 37 oC for 60 minutes. Pellet was added with 600 µl of nuclei lysis solution. The mixture was incubated at a temperature of 80 oC then was cooled. The mixture was then added with 3 µl of Rnase solution, then was incubated at a temperature of 37 oC for 60 minutes. The mixture was added with 200 µl protein removal solution, then was centrifuged at 13,000 g for 2 minutes. The supernatant was discarded, the pellet was washed with 60 µl ethanol 70%. Pellet was dried with the lid open for 15 minutes. Pellet was resuspended with 100 µl of DNA rehydration solution, then was incubated at 65 C for 1 hour. The quantity and purity of gDNA were measured using the TM 1000 nanodrop spectrophotometer.

2.3 PCR amplification, interpretation, and analysis of 16s rRNA gene

16s rRNA gene was amplified using primer 1387r (5' GGGCGGWGTGTACAAGGC 3') and primer 63f (5' CAGGCCTAACACATGCACATC 3') with a product amplicon length of 1300 bp (Marchesi et al., 1998). A total of 50 µl of the total reaction was used with a composition of 25 µl GoTaq Green Master Mix (Promega), 5 µl primer 1387r (10 pmol), 5 µl primer 63f (10 pmol), 4 µl gDNA as a template (100 ng/µl), and 11 µl nuclease-free water. The amplification process was performed in 30 cycles with pre denaturation conditions of 94 oC for 30 seconds, annealing 55 oC for 4 seconds, elongation 72 oC in 1 minute 45 seconds, and post-elongation of 72 oC for 10 minutes. Amplicon visualization was performed in agarose gel and under UV transilluminator. PCR product was sequenced by PT Genetika Science First Base. On sequencing analysis, raw sequences were trimmed according to the quality of the chromatogram. The trimmed sequence was aligned with BLASTN to the National Center for Biotechnology Information (NCBI) database to see the highest similarities. Phylogenetic trees were created using Molecular Evolutionary Genetics Analysis (MEGA) version X by the neighbor-joining method. The nucleotide

variation was described manually according to the alignment of each isolate for the species *E. cloacae.*

3. Results and Discussion

3.1. Results

Starting from May – September 2020, one isolate of *E. cloacae* code EC22 that was resistant to several antibiotics was isolated (Tabel 1).

Table 1. The minimum inhibitory concentration of EC22 against several antimicrobial categories

No	Antibiotic	Antimicrobial category	Nilai MIC (µg/ml)	Category
1	ETP	Carbapenem	**≥8**	**R**
2	MEM	Carbapenem	**≥16**	**R**
3	CAZ	Extended-spectrum cephalosporin	**≥64**	**R**
4	CRO	Extended-spectrum cephalosporin	**≥64**	**R**
5	FEP	Extended-spectrum cephalosporin	**16**	**R**
6	SAM	Penicillins + β lactamase inhibitor	**≥32**	**R**
7	TZP	Antipseudomonal penicillins + β lactamase inhibitor	**≥128**	**R**
8	GEN	Aminoglycoside	**≥16**	**R**
9	AMK	Aminoglycoside	**≥64**	**R**
10	CIP	Fluoroquinolone	≤0.25	S
11	AMP	Penicillin	**≥32**	**R**
12	KZ	First-generation cephalosporin	**≥64**	**R**
13	AZT	Monobactam	<1	S
14	SXT	Folate-pathway inhibitor	≤20	S
15	TGC	Glycylcycline	2	S

Note: Bold MIC value (R) means resistant while (S) means susceptible

E. cloacae that are grouped as *Enterobacteriaceae*, could be categorized as multi-drug resistance (MDR) pathogens if they are resistant to at least three classes of the antimicrobial category listed (Magiorakos et al., 2012). Based on its Vitek 2 antimicrobial susceptibility test, *E. cloacae* EC22 was categorized as an MDR pathogen due to resistance to carbanemen, extended-spectrum cephalosporin, penicillins + β lactamase inhibitor, antipseudomonal penicillins + β lactamase inhibitor aminoglycoside, and penicillin.

This isolate was successfully amplified with PCR (Figure 1). Vitek 2 identification of this isolate was *E. cloacae* complex. It showed similar results to molecular identification based on a partial sequence of 16s rRNA. BLASTn result of trimmed-sequence was *E. cloacae* with 99.78 % similarity (Table 2).

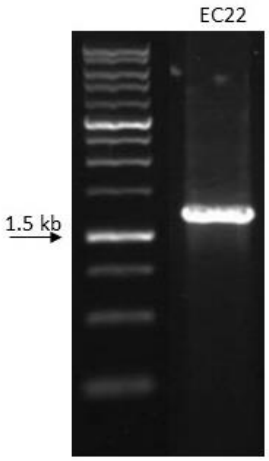

Figure 1. Visualization of PCR product amplification of EC22 16s rRNA gene. PCR product length showed 1500 bp band

Table 2. BLASTn results of 16s rRNA sequence of EC22. BLASTn was performed using the NCBI platform

Isolate	Vitek 2 ID	Molecular 16s rRNA sequence ID	Maximum identity	Sequence length
EC22	*Enterobacter cloacae* complex	*Enterobacter cloacae*	99.78 %	929

3.2. Discussion

According to its MIC value, EC22 MIC was resistant to ETP, MEM, CAZ, CRO, FEP, SAM, TSP, GEN, AMK, AMP, while EC22 was non-susceptible to CIP, AZT, SXT and TGC. The study results follow the research was conducted by Pailhoriès et al (2014), which showed that the administration of ertapenem in *E. cloacae* produces a value of MIC >8 µg/ml or belongs to the category of resistance. MIC test in the study was performed by semi-automated method Vitek 2 (bioMérieux).

The susceptible CIP antibiotic isolate results were also reported by Linde et al (2002) that the CIP administration in *E. cloacae* taken from 2 patients resulted in a MIC value of 0.25 or susceptible category. Other studies showed *E. cloacae* resistance to carbapenem and CAZ antibiotics. This study reported that *E. cloacae* were resistant to Imipenem and CAZ with MIC values >256 µg/ml (Jiang et al., 2005). Susceptibility tests used the E-test method.

E. cloacae resistance to various antibiotics has also been studied with a percentage of resistance: AMK 14%, CIP 18%, FEP 27%, TZP 51% (Jean et al., 2002). Khari et al (2016) reported that *E. cloacae* resistance to class β-lactam antibiotics was caused by the presence of *ampC* chromosome genes obtained from gene transfer between bacterial populations through plasmids called plasmid-mediated AmpC β -lactamases. The presence of *ampC* chromosome genes was proven by PCR reported from 76 isolates of E.

cloacae tested as many as 36 (47.4 %) isolates were detected to contain ampC chromosome genes and plasmid-mediated AmpC β –lactamases.

Another cause of *E. cloacae* resistance to carbapenem in the presence of Extended-spectrum beta-lactamase (ESBL). ESBL is an enzyme that can hydrolyze antibiotics penicillin, first, second, and third-generation cephalosporins and monobactam group resulting in *E. cloacae* potentially resistant to those antibiotics. The existence of ESBL in *E. cloacae* was shown in Pailhoriès et al (2014), which proved from 50 isolates of *E. cloacae*, 25 isolates (50 %) were ESBL-positive isolates. The *ampC* chromosome gene is a gene that encodes the β-lactamase group of AmpC enzyme. The location β-lactamase AmpC enzymes are found in the periplasm *E. cloacae*. This enzyme is active in penicillin breakdown but is more active in cephalosporins and can hydrolyze cephamicyn such as cefoxitin, cefotetan, ceftazidime, cefotaxime, and ceftriaxone; and monobactams such as aztreonam, although the β-lactamase enzymes of the AmpC group in hydrolyzing aztreonam is very weak. The efficacy of this enzyme will then affect the MIC value (Jacoby, 2009).

E. cloacae is weak in hydrolyzing Aztreonam (AZT) and reported a value of MIC < 1 µg/ml, meaning AZT is not an effective substrate for AmpC enzyme from *ampC* chromosome genes. This study was then strengthened by Jacoby's study (2009), which showed that the value of K_m of AmpC enzyme β-lactam from *E. cloacae* was weak when hydrolyzing AZT. This means that β-lactamase AmpC enzymes are not efficient enough to hydrolyze AZT, thus affecting the susceptibility of *E. cloacae* to AZT by MIC value 0.06 µg/ml. Jacoby (2009) also showed that *E. cloacae* had a high value of K_m enzyme β-lactamase AmpC against CAZ with MIC 215 µg/ml. The evidence was followed the results of this study, which showed the CAZ MIC was ≥64 µg/ml (resistant) against *E. cloacae* EC22.

Wu et al (2018) showed that *E. cloacae* are a pathogenic bacteria resistant to ampicillin, amoxicillin-clavulanate, and the first-generation cephalosporins. β-lactam-resistant *E. cloacae* generally is caused by AmpC β-lactamase-producer. All β-lactam antibiotics used in this study resulted in resistance MIC value against *E. cloacae*, including CAZ, CRO, and FEP. β-lactam resistant- *E. cloacae* in that study were classified as AmpC-type resistance. This resistance was caused by cephalosporinase *ampC* gene mutations mediated by plasmids resulting in resistance to all β-lactam antibiotics, especially third-generation cephalosporins except carbapenem and cefepime. The percentage of AmpC-type resistance in *E. cloacae* is 50% and was followed by overexpression of ESBL genes (Ito et al., 2018). The production of β-lactam AmpC enzymes followed by the production of enzyme ESBL β-lactamase enzyme in *E. cloacae* caused AmpC β-lactamase decreased the effects of ESBL-type, so it was challenging to identify ESBL phenotype (Hanson, 2003).

In general, *E. cloacae* is one of the *Enterobacteriaceae* members that is resistant to third-generation cephalosporins. However, Jin et al (2018) reported that out of 55 strains of *E. cloacae* were isolated from 12 hospitals in 11 cities in China, 50 strains were detected, resulting in 8 types of carbapenemase. Ertapenem (ETP) and meropenem (MEM) were

carbapenem class that has been resistant to these isolates. The recent study in accordance with this study showed by Tian et al (2020), who reported that 85 *E. cloacae* revealed a percentage of 100 % resistant to ertapenem, 51.8 % to imipenem, and 42.4 % to meropenem. Carbapenemase is a β-lactamase enzyme capable of hydrolyzing and inactivating carbapenem-type of antibiotics. One of the genes that encode the enzyme carbapenemase is *bla*NDM, which causes strong resistance because the plasmids that carry this gene often carry other antibiotic resistance genes such as ESBL and AmpC MDR *E. cloacae* (Ferranti et al., 2018).

Wang et al (2019) started that the emergence of MDR was due to interaction between non-resistant and resistant *E. cloacae* by conjugation or transduction process. This interaction causes the resistant plasmid genes of resistant *E. cloacae* to move into non-resistant *E. cloacae,* so that non-resitant E. cloacae becomes resistant. The emergence of MDR in *E. cloacae* was shown in the results of a study by Huang et al (2012), which showed that thirty-five *E. cloacae* isolated from hospitals in China harbored 25.7% carbapenemase resistance genes, 65.7% ESBL genes, 77.1% aminoglycoside resistance genes, and 68.6% quinolone resistance gene. These isolates were categorized as MDR pathogen because it was resistant to antibiotics simultaneously to the resistance gene carried.

Based on the discussion above, this study's results also prove that E. cloacae can have a resistance phenotype to various antibiotics or MDR. Antibiotics resistant to *E. cloacae* are carbapenems, broad-spectrum β-lactams, penicillins, aminoglycosides, fluoroquinolones, and monobactams. DNA sequence analysis was performed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information, National Institute for Health, USA (www.blast.ncbi.nlm.nih.gov). DNA sequence encodes 16S rRNA on *Enterobacter cloacae* EC22 showed the total score of 1705 with 929 bp aligned with a subject. The percentage of overall analysis (query coverage) was 100%, while the similarity identification percentage was 99.78 %. Thus the EC22 isolate was *E. cloacae* in high confidence.

Conclusion

DNA sequence encodes 16S rRNA on *Enterobacter cloacae* EC22 showed the total score of 1705 with 929 bp aligned with a subject. The percentage of overall analysis (query coverage) was 100%, while the similarity identification percentage was 99.78 %. Thus the EC22 isolate was *E. cloacae* in high confidence. This *E. cloacae* was categorized as MDR bacteria since it was resistant to more than three antibiotic classes, including carbanemen, extended-spectrum cephalosporin, penicillins + β lactamase inhibitor, antipseudomonal penicillins + β lactamase inhibitor aminoglycoside, and penicillin.

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