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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. In the past five years, this bacteria has increased resistance to various antibiotics resulting multidrugs-resistant (MDR) phenotypic. Those antibiotics, including extended-spectrum beta-lactams, carbapenems, aminoglycosides, and fluoroquinolones. In particular, for MDR *E. cloacae*, causing longer hospitalization time, increasing medical costs, also affecting to morbidity and mortality. This study aimed to determine the minimum inhibitory concentration (MIC) of clinical *E. cloacae* towards several antibiotics. This result could be a basis to evaluate *E. cloacae* resistances for better treatment therapy in clinical settings. This *E. cloacae* was categorized as MDR bacteria since it resistant to more than three antibiotic classes including extended-spectrum beta-lactams, carbapenems, aminoglycosides, and floroquinolones.

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

1. Introduction

Enterobacter cloaceae 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). *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.

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 that are often reported to cause 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 et al research (2018) then added in its conclusion that in addition to being resistant to broad-spectral β-lactams, *E. cloaceae* is also resistant to carbapenems, aminoglycosides, and Fluoroquinolones. The phenomenon is hereinafter referred to as MDR.

The discovery of MDR *E. cloaceae* 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 the increase of research interest in *E. cloacae* resistance. Zu 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* resistance to various antibiotics, the researchers were interested in researching on *E. cloacae* 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. Results and Discussion

3.1 Results

From this time collection's period, only one carbapenem-resistant *Enterobacter cloaceae*, which is also MDR bacteria was found. This isolate was successfully amplified with PCR (Figure 1). Vitek 2 identification of this isolate was *E. cloaceae* 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 1).

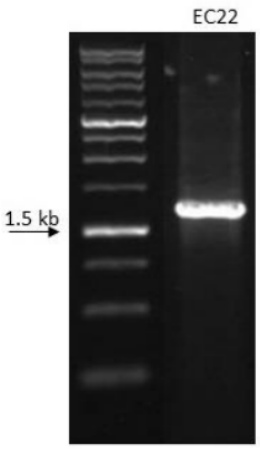

Figure 1. Visualization of PCR product amplification of EC22 16s rRNA gene. PCR product length showed 1500 bp band

Table 1. 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 cloaceae* complex	*Enterobacter cloaceae*	99.78 %	929 bp

Table 2. The minimum inhibitory concentration of EC22 against several antimicrobial categories

No	Antibiotic	Antimicrobial category	Nilai MIC (µg/ml)
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)

Bold MIC value (R) means resistant while (S) means susceptible. Antimicrobial category as follow : Ertapenem (ETP), meropenem (MEM); Extended-spectrum beta-lactam: Ceftazidim (CAZ), Ceftriaxone (CRO), Cefepime (FEP); Penicillin + inhibitor: Ampicillin-Sulbactam (SAM), Piperacillin-Tazobactam (TZP); Aminoglycoside: Gentamicin (GEN), Amikacin (AMK); Fluoroquinolone: Ciprofloxacin (CIP); Penicillin: Ampicillin (AMP); Monobactam: Aztreonam (AZT); Folate-pathway inhibitor: Trimethoprim-Sulfometoxazole (SXT); Glycylcycline: Tigecycline (TGC).

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

2.2. Discussion

DNA sequence analysis was performed using 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 cloaceae* 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. cloaceae* in high confidence.

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 are in accordance with the research was conducted by Pailhories et al (2014), which showed that the administration of ertapenem in *E. cloaceae* 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 (bioMerieux).

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

E. cloaceae resistant to various antibiotics has also been studied by Jean et al (2002) with a percentage of resistance: AMK 14%, CIP 18%, FEP 27%, TZP 51%. Khari et al (2016) reported that *E. cloaceae* resistant 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 that reported from 76 isolates of *E. cloaceae* tested as many as 36 (47.4 %) isolates were detected to contain *ampC* chromosome genes and contained plasmid-mediated AmpC β –lactamases.

Another cause of *E. cloaceae* resistance to carbapenem is 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. cloaceae* potentially resistant to those antibiotics. The existence of ESBL in *E. cloacae* was shown in Pailhories et al (2014), which proved from 50 isolates of *E. cloaceae*, 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. cloaceae*. 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. cloaceae is weak in hydrolyzing Aztreonam (AZT) and reported with a value of MIC < 1 µg/ml, meaning AZT is not an effective substrate for AmpC enzyme from *ampC* chromosome genes. This study were then strengthened by Jacoby's study (2009), which showed that the value of K_m of AmpC enzyme β-lactam from *E. cloaceae* 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. cloaceae* to AZT by MIC value 0.06 µg/ml.

Another result from Jacoby (2009) also showed that *E. cloaceae* 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. cloaceae* EC22.

Wu et al (2018) showed that *E. cloaceae* is a pathogenic bacteria resistant to ampicillin, amoxicillin clavulanate, and the first-generation cephalosporins. β-lactam-resistant *E. cloaceae* generally is caused by AmpC β-lactamase-producer. All β-lactam antibiotics used in this study resulted in resistance MIC value against *E. cloacae* in this study, including CAZ, CRO and FEP.

β-lactam resistant- *E. cloaceae* in that study was classified as AmpC-type resistance. This resistance was caused by cephalosporinase *ampC* gene mutations mediated by plasmids resulting resistance to all β-lactam antibiotics, especially third-generation cephalosporins except carbapenem and cefepime. The percentage of AmpC-type resistance in *E. cloaceae* is 50% and was followed by overexpressed of ESBL genes (Ito, 2018). According to Hanson (2003), the production of β-lactam AmpC enzymes followed by the production of enzyme ESBL β-lactamase enzyme in *E. cloaceae* caused the effects of AmpC β-lactamase decreased the effects of ESBL-type, so it was challenging to identify ESBL phenotype.

In general, *E. cloaceae* 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. cloaceae* 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. cloaceae* 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 (multi-drug resistant) *E. cloaceae* (Ferranti et al., 2018).

Wang et al (2019) started that the emergence of MDR was due to interaction between non-resistant and resistant *E. cloaceae* by conjugation or transduction process. This interaction causes the resistant plasmid genes of resistant *E. cloaceae* to move into non-resistant *E. cloaceae,* so that non-resitant E. cloaceae becomes resistant.

The emergence of MDR in *E. cloaceae* was shown in the results of a study by Huang et al (2012) which showed that thirty five *E. cloaceae* isolates from hospitals in China, harboured 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 atibiotics simultaneously to the resistance gene carried.

This study's results also prove that *E. cloaceae* can have a resistance phenotype to various antibiotics or MDR based on the discussion above. Antibiotics resistant to *E. cloaceae* are carbapenems, broad-spectrum β-lactams, Penicillins, Aminoglycosides, Fluoroquinolones, and Monobactams.

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