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Abstract. *Inggraini M, Nurfajriah S, Priyanto JA, Ilsan NA. 2021. Antimicrobial susceptibility and molecular species identification of clinical carbapenem-resistant bacteria. Biodiversitas 22: 557-564.* Antibiotic is the first option treatment for infectious diseases both in human and animal. However, the excessive usage and misuse of antibiotics have driven antibacterial resistances worldwide and the increasing case of antibiotic resistance leads to limited options for treatment. This study aimed to observe antimicrobial susceptibility and molecular identification of carbapenem-resistant human clinical bacteria. A total of nine isolates in this study were collected in 2020 from a teaching hospital in Indonesia. All isolates were originated from various human clinical specimens, including urine, blood, pus, and sputum. Identification using 16s rRNA-based showed that these isolates were closely related to *Klebsiella pneumoniae* (1/9), *A. baumannii* (5/9), *Escherichia coli* (2/9), and *Lysinibacillus fusiformis* (1/9). According to minimum inhibitory concentration using Vitek Automated Machine, four isolates of multi-drug resistant (MDR) bacteria were found. In contrast, five of them were categorized as extensively-drug resistant (XDR). Interestingly, all of the XDR isolates belonged to *A. baumannii*. These isolates were resistant to at least seven different antimicrobial classes. A comparison of partial 16s rRNA showed two *E. coli* had similar variance. While in *A. baumannii* isolates, we found one of five isolates had a different variance sequence, which suggests different clonality among this species. This study gives an insight into the prevalence of carbapenem-resistant bacteria with XDR criteria in Indonesia.

Keywords: 16s rRNA, antibiotic, antimicrobial resistance, clinical bacteria, carbapenem-resistant bacteria

INTRODUCTION

Carbapenem is a last-line agent antibiotic that can be used to treat infectious diseases in clinical settings. This antibiotic has high efficacy for the treatment of severe infections (Hawkey and Livermore 2012). Carbapenem is a beta-lactam antibiotic class with a similar mechanism with penicillins and cephalosporins through penicillin-binding sites, then inhibiting cell wall synthesis (Zhanel et al. 2007). However, carbapenem is characterized as having a more broad-spectrum than penicillins and cephalosporins.

The misuse and excessive usage of carbapenem have driven to antibacterial resistance worldwide, making carbapenem less effective against emerging bacterial. The term "ESKAPE" has arisen for bacteria that cause a problem in the hospital and can escape from several antimicrobial treatments, such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species (Rice 2008). Therefore, World Health Organization (WHO) has announced that carbapenem-resistant bacteria becomes a priority for exploring a new antimicrobial to fight against since it leaves limited options for treatment (WHO 2017).

A study in the US revealed that *A. baumannii* was the most common carbapenem-resistant pathogen with 44.8%, followed by *P. aeruginosa* with 14.2%, and Enterobacteriaceae with only 1% from the total of carbapenem-resistant clinical bacteria (Nordmann and Poirel 2019). These bacteria can be found in various sources, including blood, sputum, urine, and others. The current study in Indonesia found that in 2015-2016, from total of 1082 clinical bacteria in the hospital, 10.7% were carbapenem-resistant (Kuntaman et al. 2018). A study in Aceh showed that from 212 methicillin-resistant *Staphylococcus haemolyticus*, 99.1% of them were resistant to carbapenem (Suhartono et al. 2019). In a particular study for the most common pathogen *A. baumannii*, from a total of 24 isolates, 23 (95.8%) of them were multidrug-resistant bacteria (MDR). These isolates were resistant to several antibiotic classes, including aminoglycoside, carbapenem, quinolone, cephalosporin, penicillin, and tigecycline. Most of the patients were dead, up to 75% of the total patient

(Gustawan et al. 2014). These data depicted the high prevalence and emergence of carbapenem-resistant bacteria, particularly in Indonesia.

The identification of carbapenem-resistant bacteria is important to enrich the existing knowledge in epidemiology and future treatment. Yet, such identification of clinical bacteria is challenging, particularly for species from the genus of *Acinetobacter*, because not only their relevance of causing the disease, but also *A. baumannii, A. nosocomialis,* and *A. pittii* are closely related. It has been known as *Acinetobacter calcoaceticus-A. baumannii* complex (ACB complex). This group is difficult to differentiate using the phenotypic approach. Identification tools either manual, such as API20NE, or semi-automated, including Vitek 2, Pheonix, and Microscan WalkAway, causing misidentification up to 25% (Higgins et al. 2010). The genomic approach is promising due to its high accuracy than the phenotypic approach. One of the alternatives is using 16s rRNA sequence identification to identify clinical bacteria with good resolution. It has a 96% concordance rate for the genus level and 87.5% concordance rate for species level (Srinivasan et al. 2015). The aim of this study is to observe the antimicrobial susceptibility patterns of carbapenem-resistant human clinical bacteria using semi-automated Vitek 2 and then to identify those bacteria using a 16s rRNA gene sequence for better accuracy. The rationale for using Vitek 2 because this machine is considerably the common tool in Indonesian hospitals. This approach will give information for future treatment and the diversity of carbapenem-resistant bacteria from clinical sources.

RESULTS AND DISCUSSION

Antimicrobial susceptibility and identification based on Vitek 2

Starting from May to September 2020, nine isolates that were non-susceptible to at least one representative of carbapenem antibiotic, including ertapenem and meropenem were collected. According to Vitek 2 identification, these nine isolates belonged to *Klebsiella pneumoniae* (2/9), *Acinetobacter baumannii* (5/9), and *Escherichia coli* (2/9). All isolates were recovered from various human specimens, such as urine (2/9), blood (1/9), pus (3/9), and sputum (3/9). According to Magiorakos et al. (2012), *K. pneumoniae* and *E. coli* are in the same Enterobacteriaceae group for the emergence status of antibiotic resistance.

The criteria of multi-drug resistance (MDR) of this group is resistant to more than three antibiotic classes. In this group, resistant to more than eight classes will be categorized as extensively-drug resistant (XDR). While for *A. baumannii*, isolate will be concluded as XDR if resistant to more than seven antibiotic classes. *K. pneumoniae* and *E. coli* in this study were categorized as MDR pathogen since these isolates were resistant to at least 6 classes, such as carbapenem class (ertapenem and meropenem), extended-spectrum beta-lactamase (ceftazidime, ceftriaxone, cefepime), penicillin + inhibitor (ampicillin-sulbactam, piperacillin-tazobactam), aminoglycoside (gentamicin), penicillin (ampicillin), and monobactam (aztreonam). As many as one *K. pneumoniae* and two *E. coli* were resistant to fluoroquinolone class ciprofloxacin, two *K. pneumoniae* but none *E. coli* were resistant to aminoglycoside class gentamicin, one *K. pneumoniae* and one *E. coli* were resistant to folat-pathway inhibitor class trimethoprim-sulfamethoxazole. All of *A. baumannii* were concluded as XDR pathogen since they were resistant to seven antibiotic classes, such as carbapenem, extended-spectrum beta-lactam, and penicillin inhibitor, aminoglycoside, fluoroquinolone, monobactam, and folate-pathway inhibitor (Table 1). As a note, all of these MDR and XDR isolates in this study were susceptible to glycylcycline class tigecycline.

The minimum inhibitory concentration of all carbapenem-resistant isolates is in Table 1. Value in bold means non-susceptible (intermediate or resistant) with criteria according to CLSI 2018. Antibiotic classes that were

tested including Carbapenem: Ertapenem (ETP), meropenem (MEM); Extended-spectrum beta-lactam: Ceftazidime (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)

Molecular identification and sequence analysis of 16s rRNA gene

All isolates were successfully amplified with similar PCR product length in ±1300 bp (Figure 1). Molecular identification based on a partial sequence of 16s rRNA showed 8 of 9 were similar to those identified by Vitek 2. PCR products of these *16s rRNA* genes were sent for Sanger sequencing. Sequences then were analyzed with BLASTn in NCBI website.

KP1 isolate, which is identified as *K. pneumoniae* in Vitek 2, was closed related to *Lysinibacillus fusiformis* with 97.6% identity of 370 bp (23.8%) aligned. As many as 8 of 9 sequence isolates showed strong confidence with ID > 99% and 0.0 E-value (Table 2). Phylogenetic tree based on nucleotide sequences displayed 4 of 5 *A. baumannii* isolates were closely related to *A. baumannii* ATCC 17978 reference strain. However, *A. baumannii* AB32 was separated into a different branch (Figure 2). Graph of nucleotide sequence alignment showed that *A. baumannii* AB32 was different in one nucleotide than *A. baumannii* remaining isolates AB27, AB46, AB53, and AB91. Those four remaining isolates had a similar sequence. *E. coli* EC133 and EC143 had 4 nucleotide variances than *E. coli* ATCC 25922 reference strain. Nucleotide analysis showed EC143 and EC133 were identical although had 4 nucleotide differences than reference strain (Figure 3).

Discussion

Antimicrobial resistance is now becoming major threats worldwide. Government starts to pay more attention to carbapenem-resistant since WHO and CDC announced this emergence (WHO 2017). This study found multi-drug resistant *K. pneumoniae* (MDR-KP) and extensively-drug resistant *A. baumannii* (XDR-AB) from human clinical specimens. MDR-KP infections have been known correlated to high mortality rate of 40-50%, particularly in critically severe patients (Xu et al. 2017). Some European countries such as Italy, Turkey, and Greece are recently considered infections due to carbapenemase-producing *Enterobacteriaceae* as endemic cases (Bassetti et al. 2018). This infection has been known challenging to treat since combination therapies resulting in unsatisfactory outcomes (Bassetti et al. 2015). On the other hand, *A. baumannii* infections cause several diseases, including pneumonia, bacteremia, meningitis, and surgical site infections (Manchanda et al. 2010). *A. baumannii* has high flexibility in genetical drive through plasmid or transposon. Moreover, *A. baumannii* possesses intrinsic carbapenemase gene such as Oxacillinase-51 (OXA-51) that causes carbapenem-resistant phenotype if it is over-expressed (Peleg et al. 2008). XDR-AB infections are becoming an emerging threat in clinical settings due to resistance to last-line antibiotics. This situation leads to limited options for treatment (Katsiari et al. 2018). The outbreak caused by XDR-AB has been reported in several countries (Gray et al. 2016; Vilacoba et al. 2013).

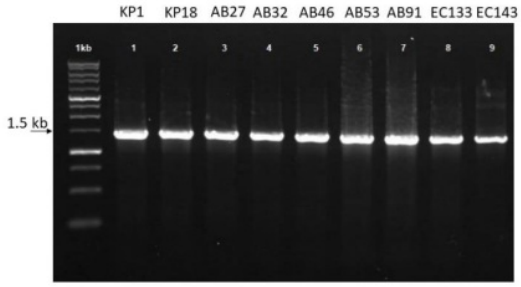

Figure 1. PCR product band of 16s rRNA amplification of isolates. Product size was approximately 1500 bp. 1-9 represented lane order in agarose gel for each isolate

Table 1. Antimicrobial susceptibility (minimum inhibitory concentration) and identification of carbapenem-resistant clinical bacteria according to Vitek 2. Minimum inhibitory concentration (MIC) unit in this table is μg/ml. NT means antimicrobial MIC measurement was not tested for this species

Note collections of isolates					Antibiotics													
Isolate	**Vitek ID**	**Partial 16s rRNA ID**	**Source**	**Collection date**	**ETP**	**MEM**	**CAZ**	**CRO**	**FEP**	**SAM**	**TZP**	**GEN**	**AMK**	**CIP**	**AMP**	**KZ**	**AZT**	**SXT**	**TGC**
KP1	*K. pneumoniae*	*Lysinibacillus fusiformis*	Urine	1 June 2020	**≥8**	**≥16**	**≥64**	**≥64**	**≥64**	**≥32**	**≥128**	**≥16**	**≥64**	≤0.25	**≥32**	**≥64**	**≥64**	≤20	1
KP18	*K. pneumoniae*	*K. pneumoniae*	Blood	6 May 2020	**≥8**	**≥16**	**≥64**	**≥64**	**≥64**	**≥32**	**≥128**	**≥16**	**≥64**	**≥4**	**≥32**	**≥64**	**≥64**	**≥320**	2
AB27	*A. baumannii*	*A. baumannii*	Pus	5 Jun 2020	NT	**≥16**	**≥64**	**≥64**	**≥64**	**8**	**≥128**	**≥16**	4	**≥4**	NT	**≥64**	NT	**≥320**	2
AB32	*A. baumannii*	*A. baumannii*	Sputum	5 Sep 2020	NT	**≥16**	**≥64**	**≥64**	**≥64**	**≥32**	**≥128**	**≥16**	4	**≥4**	NT	**≥64**	NT	**≥320**	2
AB46	*A. baumannii*	*A. baumannii*	Sputum	6 Sep 2020	NT	**≥16**	**≥64**	**≥64**	**≥64**	**≥32**	**≥128**	**≥16**	8	**≥4**	NT	**≥64**	NT	≤20	1
AB53	*A. baumannii*	*A. baumannii*	Pus	9 Sep 2020	NT	**≥16**	**≥64**	**≥64**	**≥64**	**16**	**≥128**	**≥16**	4	**≥4**	NT	**≥64**	NT	**≥320**	2
AB91	*A. baumannii*	*A. baumannii*	Sputum	3 May 2020	NT	**≥16**	**≥64**	**≥64**	**≥64**	**≥32**	**≥128**	**≥16**	4	**≥4**	NT	**≥64**	NT	**≥320**	2
EC133	*E. coli*	*E. coli*	Pus	17 Mar 2020	**≥8**	**≥16**	**≥64**	**≥64**	**≥64**	**≥32**	**≥128**	**≥16**	≤2	**≥4**	**≥32**	**≥64**	**≥64**	≤20	2
EC143	*E. coli*	*E. coli*	Urine	19 Sep 2020	**≥8**	**≥16**	**≥64**	**≥64**	**≥64**	**≥32**	**≥128**	1	≤2	**≥4**	**≥32**	**≥64**	**≥64**	**≥320**	2

Note: The minimum inhibitory concentration of all carbapenem-resistant isolates. Value in bold means non-susceptible with criteria according to CLSI 2018. Antibiotic classes that were tested including Carbapenem: Ertapenem (ETP), meropenem (MEM); Extended-spectrum beta-lactam: Ceftazidime (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)

Table 2. BLASTN result against NCBI database of partial 16s rRNA trimmed-nucleotide sequence

Isolate	Description	Max score	Query cover (%)	E-value	Identity (%)	Sequence length (% coverage of full length)	Accession no.
KP1	*Lysinibacillus fusiformis* strain SKTU8	634	100	1e-177	97.57	370 bp (23.8%)	MK652860.1
KP18	*Klebsiella pneumoniae* strain IGM6-9	1711	100	0.0	99.79	933 bp (60%)	MT197279.1
AB27	*Acinetobacter baumannii* ATCC 17978	1814	100	0.0	100	982 bp (61.9%)	CP053098.1
AB32	*Acinetobacter baumannii* strain rY22	1808	100	0.0	99.9	982 bp (61.9%)	MN173901.1
AB46	*Acinetobacter baumannii* ATCC 17978	1814	100	0.0	100	982 bp (61.9%)	CP053098.1
AB53	*Acinetobacter baumannii* ATCC 17978	1814	100	0.0	100	982 bp (61.9%)	CP053098.1
AB91	*Acinetobacter baumannii* ATCC 17978	1814	100	0.0	100	982 bp (61.9%)	CP053098.1
EC133	*Escherichia coli* strain 192	1631	99	0.0	99.89	886 bp (57%)	MH671478.1
EC143	*Escherichia coli* strain 192	1631	99	0.0	99.89	886 bp (57%)	MH671478.1

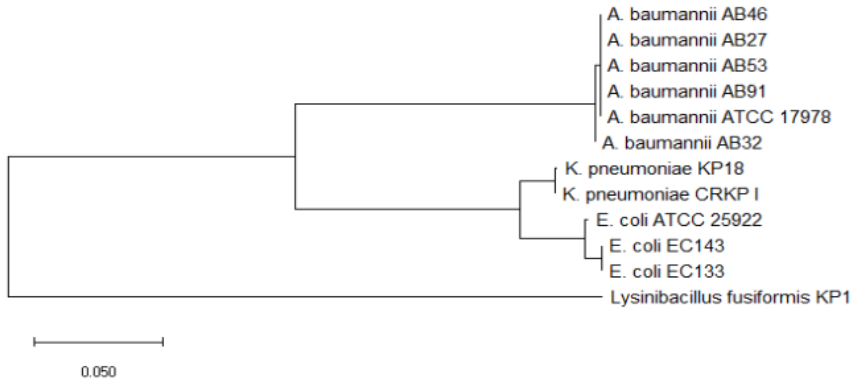

Figure 2. Phylogenetic tree of partial 16s rRNA sequence of isolates. The tree was generated using the Neighbor-joining method in MEGAX software

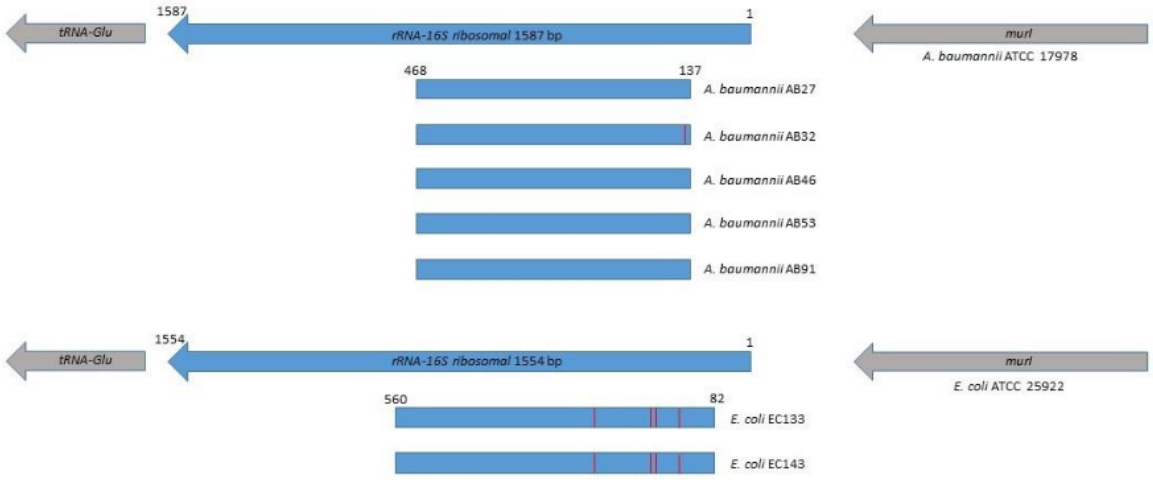

Figure 3. BLASTN graph sequence of 16s rRNA of *A. baumannii* and *E. coli*. Sequences were aligned and compared to their reference strain from the NCBI database. The red mark inside sequence bar means nucleotide variance position.

Antimicrobial resistance in microorganisms is a natural phenomenon. It has been accumulated from excessive usage of antimicrobial agents both in humans, animals, and plants (Sugden et al. 2016). Antimicrobial agent is still becoming a primary option for infectious disease treatment, however, about 50% of antimicrobial prescription are unnecessary (Elshamy and Aboshanab 2020). It drives antimicrobial resistance changing dramatically. Several groups of bacteria naturally possess resistance factors it called intrinsic resistance factor. *A. baumannii* naturally has $bla_{\text{OXA-51}}$ gene that encodes oxacillinase enzyme. This enzyme contributes to oxacillin antibiotic resistance. Besides of intrinsic resistance factor, acquired resistance is a big problem worldwide. Bacteria have high genomic flexibility due to plasmid, insertion sequence, and transposable element that able to spread genetic material to either intraspecies or interspecies. This leads to antimicrobial resistance that can be spread over the world rapidly (Elshamy and Aboshanab 2020).

Carbapenem is the latest developed β-lactam antibiotic possessing a β-lactam ring and a five-group ring differ from penicillin. This different structure confers high stability against β-lactamase, including extended-spectrum β-lactamase (ESBL) such as ceftazidime, ceftriaxone, and cefepime (Meletis 2016). Carbapenems have a broad spectrum activity for treatment against infectious disease caused by multi-drug resistant (MDR) pathogens (Lee and Bradley 2019). Since 21st century, bacteria that confer ESBL genes have arisen including *Enterobacteriaceae* group and *A. baumannii*, except for carbapenemase gene. As a result, the usage of carbapenems in clinical setting has increased. It drives resistance due to β-lactamase or enzyme that is able to hydrolyze carbapenems antimicrobial, known as carbapenemase (Durante-Mangoni et al. 2019). Generally, it has been reported three main mechanisms of carbapenem

resistance including enzyme-mediated gene (carbapenemase), porin-mediated resistance, and efflux pump overproduction (Elshamy and Aboshanab 2020).

The most common mechanism is enzyme-mediated resistance. This mechanism is causing global threat since β-lactamase genes are frequently attributed by transposons, inside the plasmids, or another mobile transposable element, which can be horizontally transferred both intraspecies and interspecies (Meletis 2016). According to functional structure, carbapenems are divided into three classes including β-lactamase class A, B, and D. Class A and D β-lactamases possess a serine residue at the active site, thus called serine β-lactamases. Class B β-lactamase contains metal (zinc ions) at the active site (Jacoby and Munoz-Price 2005). KPC enzyme (*K. pneumoniae* carbapenemase) is one of the most important class A carbapenemase. Bacteria that contain *bla*$_{KPC}$ gene often found resistant to other antimicrobials such as aminoglycosides class, fluoroquinolones, extended-spectrum-β-lactamases, making them MDR. *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ are the most frequently reported from total of thirteen types of *bla*$_{KPC}$ gene (Djahmi et al. 2014). *bla*$_{KPC}$ is conferred by plasmid drives to spread horizontally to another species (Cuzon et al. 2010). New Delhi metallo β-lactamase (*bla*$_{NDM}$) and Verona Integron encoded MBL (*bla*$_{VIM}$) were the important class B carbapenemase so far. These carbapenemase groups bring resistance to almost β-lactams including carbapenems but not aztreonam (Doi and Paterson 2015). In particular *bla*$_{NDM}$, it confers resistance to enteric pathogens such as *K. pneumoniae* and *E. coli*. Class D carbapenemases comprise oxacillinase (OXA) enzymes that able to hydrolyze oxacillin. *bla*$_{OXA}$ genes are often found in *Acinetobacter* species, however, it is relatively weak carbapenemase activity but lacks of inhibitors for them (Tzouvelekis et al. 2012).

The first KPC enzyme (*bla*$_{KPC-2}$) was reported in 1996 from *K. pneumoniae* clinical isolates in North Carolina, USA (Yigit et al. 2008). Since then, *K. pneumoniae* that produces KPC was massively spread in US (Kitchel et al. 2009). A hospital in Greece reported an outbreak caused by KPC-2 producer *K. pneumoniae* in 2007. In 2008 and 2009, KPC-3 producer *K. pneumoniae* was reported in Columbia and Italy, respectively (Agodi et al. 2011). In 2008, NDM-producer *K. pneumoniae* isolate was recovered in a Swedish patient with Indian descent who had been traveled to New Delhi, India. It was a great concern since United Arab Emirates found isolates harbored *bla*$_{NDM-4}$, *bla*$_{NDM-1}$, *bla*$_{NDM-7}$, *bla*$_{NDM-5}$, *bla*$_{OXA-181}$, *bla*$_{KPC-2}$ carbapenemase genes in IncX3 plasmid, in 2009 (Moutfah et al. 2019). Besides, OXA-48 producer *K. pneumoniae* was firstly found in 2001 from Istanbul, Turkey (Poirel et al. 2004). Since then, some countries had an outbreak caused by OXA-48 producer *K. pneumoniae* in France (Cuzon et al. 2011), Belgium (Glupczynski et al. 2012), Netherland and Russia (Poirel et al. 2012), also Egypt (Elshamy et al. 2018).

Based on the 16S rRNA sequence, 8 out of 9 isolates in this study, were belonged to the Enterobacteriaceae family, a producer of extended-spectrum β-lactamase (ESBL), while another isolate coded as KP1 was related to Bacillaceae. Interestingly, of 9 isolates, five isolates were highly similar to *Acinetobacter baumanii* (identity 99-100%). This species was commonly known as a leading cause of bacterial infections worldwide, nearly 1.000.000 cases yearly, of which 50% are resistant to various antibiotics, including carbapenems (Spellberg and Rex 2013). Carbapenem-resistant *Acinetobacter baumannii* (CRAB) has been attributed as an important nosocomial pathogen (Piperaki et al. 2019). Several mechanisms likely include carbapenem resistance in this bacterium, including naturally produce

carbapenemase (OXA-51-group carbapenemase), active efflux, and reduced permeability of the outer membrane (Viehman et al. 2014). Besides, two isolates were identified as *Escherichia coli*. Similar results with this study, this species has also been reported resistant to carbapenem antibiotics. For example, eighty-one isolates (29.03%) collected from calves in India were resistant to at least one of three carbapenem antibiotics through active efflux pump mechanism and production of Metallo β-lactamase (Murugan et al. 2019). Isolate coded as KP18 was closely related to *Klebsiella pneumoniae*, a gram-negative bacterium belonging to the Enterobacteriaceae family. Carbapenem-resistant *K. pneumoniae* (CRKP) is characterized with the ability to produce various types of carbapenemase including class A (*K. pneumoniae* carbapenemase, KPC), class B or metallo-β-lactamases (MBL), and class D (OXA-48-like carbapenemases) (Reyes et al. 2019). Remarkably, this present study was firstly reported that a rod-shape Gram-positive bacterium, *Lysinibacillus fusiformis*-related isolate (KP1) was resistant to at least one of the carbapenem antibiotics used in this study. This species could find in a patient with a history of intravenous drug abuse and splenectomy (Wenzler et al. 2015). Consistently, as shown in Figure 2, KP1 isolate (*L. fusiformis*) was more distantly grouped than other isolates.

Clonality determination is an alternative way of observing strain dissemination in a certain ward. Our approach of using a partial 16s rRNA sequence is to predict clonality among isolates. This study found at least 2 different clonalities among 5 carbapenem-resistant *A. baumannii*, suggesting these 2 different clonality strains might spread in that hospital. In conclusion, this study gives an insight into the presence of carbapenem-resistant *A. baumannii, K. pneumoniae, E. coli* and *L. fusiformis*. This study also provides more identification for better understanding in terms of treatment management.

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