

Detection of ESBL and MBL in *Acinetobacter* spp. and Their Plasmid Profile Analysis

Md Fazlul K. Khan¹, Shah S. Rashid^{1,*}, Aizi Nor M. Ramli¹, Ukaegbu C. Ishmael¹ and Mohammad Nazmul H. Maziz²

¹Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang, Gambang, 26300 Pahang, ²Graduate School of Medicine, Perdana University, Jalan MAEPS Perdana, Serdang, 43400 Selangor, Malaysia

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Abstract

The prevalence of extended spectrum β -lactamases (ESBLs), metallo β -lactamases (MBL), and plasmid-mediated resistance is a severe threat to current lactam therapy leading to treatment failure. Therefore, in this research, an investigation has been conducted to study the presence of ESBL and MBL genes in *Acinetobacter* spp. and their relationship to antimicrobial resistance. Fifty *Acinetobacter* spp. isolates were collected from both pediatric and adult patients at Sungai Buloh Hospital, Kuala Lumpur, Malaysia. The Double Disk Synergy Test (DDST) and the E-test have been used to detect ESBL and MBL production. The plasmid was extracted using the Close and Rodriguez (1982) method with modification in addition to the QIAprep Spin Kit. Among the fifty *Acinetobacter* isolates, eleven (22%) were ESBL-positive, while none of the strains produced MBLs. Fourteen isolates were positive for a plasmid with an occurrence rate (POR) of 28 %. These fourteen isolates appeared to harbor one or more plasmids with a maximum of four plasmids. Four isolates had single plasmids, and five carried four plasmids, while four and one isolates had two and one plasmids respectively. The sizes of the plasmid DNA were found to range between 1.8 kb and 14 kb. This study emphasizes the problematic raising frequency of ESBL, MBL, and plasmid-mediated resistance. This awareness highlights an urgent need to prevent and control such bacteria.

Keywords: *Acinetobacter* spp., ESBL; MBL, Malaysia, Plasmid profile.

1. Introduction

Acinetobacter spp. has appeared as a significant widespread nosocomial pathogen implicated with a fatality. They are aerobic, non-fermentative, and gram-negative bacilli (Howard *et al.*, 2012). *Acinetobacter* spp. causes many diseases like pneumonia, septicemia, wound sepsis, urinary tract infections, endocarditis, meningitis, and some clinical diseases including blood stream infections (BSI) (Zurawski *et al.*, 2012). Over the last decades, multidrug-resistant (MDR) clinical isolates of *Acinetobacter* spp. have been reported as a consequence of the extensive use of potent broad-spectrum antimicrobial agents in hospitals throughout the world (Towner, 2009). Furthermore, *Acinetobacter* spp. contaminates different types of commercial food such as meat, fruits, vegetables and various types of livestock. *Acinetobacter* spp. associated with this contaminated food can multiply in the digestive tract of humans through environmental routes of transmission (Wong *et al.*, 2017; Zhang *et al.*, 2013). *A. baumannii* is the most virulent of all the species based on clinical data by statistical analysis and studies of animal models (Chusri *et al.*, 2014). The identification of ESBL producing *Acinetobacter* spp. is essential due to its multidrug resistance property (Bush, 2010). Possible risk factors related to the development of colonization or

infection of hospitalized patients with *Acinetobacter* spp. extended the length of hospital stay, the severity of the disease, offensive procedures, and treatment (Ibrahimagic *et al.*, 2017). Studies showed that the environmental strains of *Acinetobacter* spp. Possess antibiotic resistance mechanisms (Al Atrouni *et al.*, 2016). The emerging antibiotic resistance is a serious global concern, resulting in treatment failures and increasing healthcare costs. The production of β -lactamase is the most common cause of bacterial resistance to beta-lactam antibiotics. Metallo- β -lactamases is a set of enzymes that catalyze the hydrolysis of carbapenems and a broad range of β -lactam drugs (Palzkill, 2013). The antibiotic resistance by ESBLs and MBLs gene production is increasing significantly in the clinical isolates of *Acinetobacter* spp. around the world. Studies have shown that bacteria producing ESBLs or MBLs are associated with higher mortality and morbidity (Delgado-Valverde *et al.*, 2013; Oberoi *et al.*, 2013). Extended-spectrum beta-lactamases (ESBLs) confer resistance to various groups of antibiotics (penicillins, cephalosporins, and aztreonam) (Aqel *et al.*, 2014). ESBLs are mostly encoded by plasmid- encoding resistance genes for a variety of antimicrobial agents (Cheaito and Matar, 2014). MBLs gene encoded by transposons transfers large transferable plasmids among different bacterial species and genera (Al-Marjani *et al.*, 2013). Plasmids are extra-chromosomal, circular, double-stranded DNA molecules,

* Corresponding author e-mail: samiur@ump.edu.my; tsamiur@yahoo.com.

and play an essential role in bacterial multi-drug resistance. Plasmid-mediated antibiotic resistance is common in *Acinetobacter* spp. and plasmid profile analysis examines the total bacterial plasmid content (Nazmul MHM *et al.*, 2016). In Asia, one of the three most common causes of bacteremia and nosocomial pneumonia is due to *Acinetobacter* spp. (Chung *et al.*, 2011; Kuo *et al.*, 2012). There are approximately one million (range, 600,000 to 1,400,000) cases globally per year (Spellberg and Rex, 2013). Therefore, the outcome of this study might highly be effective for an early recognition of infections, the choice of antibiotics, limiting clinical use of antibiotics, the failure treatment of broad-spectrum β -lactams, and for the control of the prevalence of antibiotic-resistance mechanism, which is necessary to prevent the spread of multi-drug resistance throughout the world.

2. Materials and Methods

For this study, fifty *Acinetobacter* spp. isolates were obtained from the blood, skin, pus, respiratory secretions, urine, and sputum of both pediatric as well as adult patients of Sungai Buloh Hospital, Kuala Lumpur, Malaysia. Furthermore, the presence of both ESBL and MBL genes were confirmed by Double Disk Synergy Test (DDST) and E-test. In DDST, discs of third-generation cephalosporins and augmentin were kept 30mm separately from center to center on inoculated Muller-Hinton Agar (MHA). A perfect extension of the edge of the inhibition zone of cephalosporin to the augmentin disc was interpreted as positive for the ESBL production.

The phenotypic confirmatory test was evaluated for ESBL-producing *Acinetobacter* spp. Briefly, a 0.5 MacFarland's suspension of each strain was spread on a Muller – Hinton agar (MHA) plate. Ceftazidime (30 μ g) and ceftazidime / clavulanic acid (30 μ g/ 10 μ g) discs were placed aseptically on the agar plate. The distance was 15mm between the two discs (edge to edge), and the culture was incubated at 37°C overnight. Based on The Clinical and Laboratory Standards Institute (CLSI), ESBL production was confirmed when the inhibition zone increased to more than or equal to 5mm surrounding the combined antimicrobial agent compared to the single antimicrobial agent (CLSI, 2017). The increase in the zone diameter is due to the inhibition of the β -lactamase by the clavulanic acid (Thomson *et al.*, 2018). The procedures for

Table 1. Antibiotype of isolated *Acinetobacter* spp.

SL	Total isolates (50)	Antibiotyping										ESBL (+)	MBL (+)
		TPZ	CAZ	CFP	CIP	AK	MEM	IPM	PRL	AMP	CN		
1	Number of resistance	34	37	32	32	28	41	37	27	46	28	11	0
2	Percentage of resistance	68	74	64	64	56	82	74	54	92	56	22	0

Tazobactam 10/ Piperacillin 75-TZP, Ceftazidime-CAZ, Cefoperazone-CFP, Ciprofloxacin-CIP, Amikacin-AK, Meropenem-MEM, Imipenem-IPM, Piperacillin-PRL, Ampicillin-AMP, Gentamicin-CN.

Acinetobacter spp. was tested to detect the ESBL production and the occurrence rate of ESBL-producing strains. Among the fifty isolates of *Acinetobacter* spp., 22 % were found to produce ESBL in the phenotypic test and the E-test. The percentage of ESBL-negative isolates (78 %) were observed in both the phenotypic analysis and the E-test are shown in table 2. None of the strains was found to produce MBLs.

Table 2. Detection of ESBL-producing strains using the phenotypic test and the E-test.

Organism	Total Number of the organism	Phenotypic test for ESBL		E-test for ESBL	
		Positive	Negative	Positive	Negative
<i>Acinetobacter</i> spp.	50	11(22%)	39(78%)	11 (22%)	39(78%)

the E-test were followed according to the manufacturer's instructions.

Close and Rodriguez's (1982) method was used with modification to detect plasmids. The manufacturer's instructions were followed to extract plasmid DNA using extraction kit (QIAprep spin kit, USA). The Supercoil DNA marker was used to estimate the plasmid size. It was used in the electrophoresis gel each time along with the plasmids as a molecular weight (range, 2.0kb to 10.0kb) marker, bought from Promega, USA.

3. Results

About fifty isolates of *Acinetobacter* spp. were identified by conventional bacteriological tests. Ten different types of commonly prescribed antibiotics have been used in this study to evaluate and compare the antibiotic resistance rate between the isolates.

All the clinical isolates (fifty) of *Acinetobacter* spp. showed a high frequency of resistance to ampicillin, meropenem, imipenem, and ceftazidime with a resistance rate of 92 %, 82 %, 74 %, and 74 % respectively (Table 1). Some of the isolates showed moderate resistance to the antibiotics tazobactam-piperacillin, ciprofloxacin, piperacillin, gentamicin, amikacin, and cefoperazone (68 %, 64 %, 54 %, 56 %, 56 %, and 64 % respectively).

ESBL-producing strains may play an essential role in the early recognition of infection control according to age and sex. Most of the ESBL-producing isolates were found to be in the age group of 41-50, and >50 years, while none of the ESBL-producing strains were belong to the age group 11-20 years (0.00%).

The frequency of ESBL-producing isolates from different types of clinical samples is shown in Figure 1. Aseptic precautions were taken during the isolation of *Acinetobacter* spp. Among the clinical samples, urine (38 %) was the frequent source followed by blood (26 %), pus (14 %), skin (10 %), respiratory secretions (6 %), and sputum (6 %) respectively. The highest percentage of ESBL production was detected in the urine (12 %) followed by blood (6 %), sputum, and pus 2 % respectively (Figure 1). Furthermore, strains from respiratory secretions and skin did not produce any ESBL.

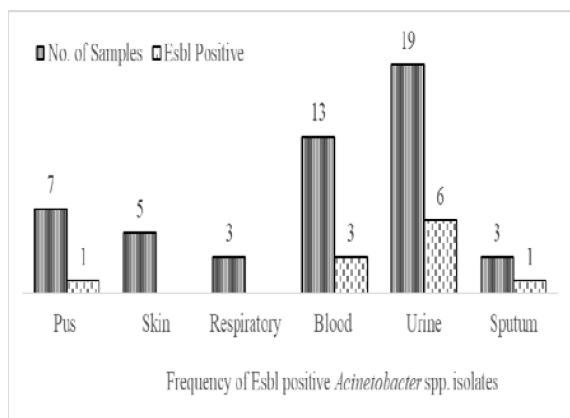


Figure 1. Frequency of ESBL-producing isolates from different types of samples.

ESBL-positive isolates were confirmed by the Double Disk Synergy Test (DDST). The inhibition zone between disks of AMC30 and CAZ30 were superimposed, and this synergy (Figure 2) confirmed the presence of ESBL production by the specific isolates. The occurrence rate of ESBL-producing strains of *Acinetobacter* spp. is shown in Figure 1.

However, among the fifty isolates of *Acinetobacter* spp., none of the isolates produced MBL.

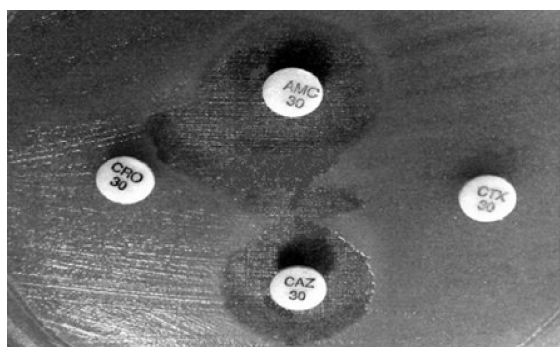


Figure 2. Double Disk Synergy Test showing ESBL production

The Close and Rodrigue method was performed for plasmid profile analysis, and the commercial extraction kit was used to detect plasmid DNA. The similarity of Plasmid DNA was found in both described methods. In Figure 3, the left lane was done by the Close and Rodrigue method, and the right lane was done by the commercial extraction kit (QIAprep spin kit) for a better observation. The presence of both chromosomes and plasmids was observed in *Acinetobacter* spp. under 0.7 % gel electrophoresis (Figure 3).

Out of the fifty *Acinetobacter* spp., fourteen isolates were found to harbor plasmid. Few strains were found to carry a maximum of four plasmids (Figure 4). The size of the plasmids varied from 1.8 to 14kb (1.8, 2.2, 3.4, 4.6, 5, 8 and 14 kb) under 0.7 % gel electrophoresis. Plasmid occurrence rate (POR) was 28 % (fourteen isolates) among the studied isolates, and thirty-six (72 %) strains did not harbor any plasmid DNA (Table 3).

Table 3. Plasmid occurrence rate (POR) in *Acinetobacter* spp. isolates.

No. of Plasmid DNA	Isolates No.	POR%
0	36	72
1	3	6
2	4	8
3	3	6
4	4	8
5	0	0

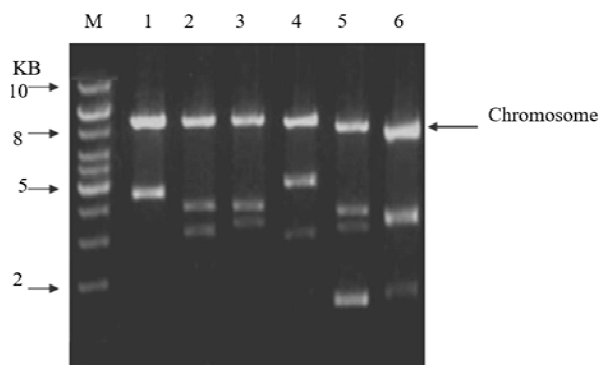


Figure 3. Comparison of the two methods used in this study

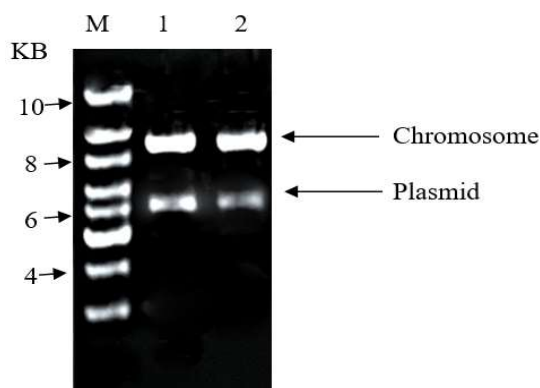


Figure 4. Agarose (0.7 %) gel electrophoresis of plasmid DNA

4. Discussion

The prospective sources of ESBL associated with an infection, its spread, and colonization of humans have been studied considerably over the past few years (Walther *et al.*, 2018). The highest occurrence of antibiotic resistance in this study was exhibited by the carbapenems and cephalosporins groups of antibiotics (Table 1). This is in agreement with another recent study in Pakistan (Sohail *et al.*, 2016). The frequency of ESBL-producing isolates of *Acinetobacter* spp. from different types of clinical sources is shown in Figure 1. In this present study, urine showed the highest number (37.58 %) as an ESBL producer compared with other clinical sources of isolates; this finding is an agreement with another recent study in 2017 (89 %) (Park and Kim, 2017). The DDST is one of the most common and confirmative tests to detect ESBL production based on the synergistic effects of clavulanate and ceftazidime in the presence of clavulanic acid (Figure 2). Both DDST and E-test showed similar results regarding the ESBL production (Table 2). The production of ESBL confers resistance at the various levels to expanded-

spectrum cephalosporins (cefotaxime and ceftazidime) and aztreonam, but typically not to the cephamycins and carbapenems (Shaikh *et al.*, 2015). Recently, a substantial increase in the occurrences of ESBL-related infections has noted been throughout the globe.

This study showed that the ESBL-producing *Acinetobacter* spp. were 11 out of 50 (22 %) isolates by both phenotypic and E-test. The outcome of the current research has a similarity with another study by Owlia *et al.*, (Owlia *et al.*, 2012) where it was reported that 21 % of *Acinetobacter* spp. produced ESBL. However recent studies done in Bangladesh reported a lower rate (9.09 %), five out of fifty-five *Acinetobacter* spp. isolates were ESBL-positive (Paul *et al.*, 2017) and 7.24 % of ESBL production among *Acinetobacter* spp. Isolates (Dhillon and Clark, 2012). Similarly, another study performed in Saudi Arabia reported that 8.1 % of *Acinetobacter* strains isolated from burns' patients were ESBL-producers (Al-Tawfiq and Anani, 2009). A number of studies showed a higher occurrence for ESBL production, 44 % out of 147 *Acinetobacter* spp. in 2010 (Hashemizadeh *et al.*, 2010), 46 % in 2001 (Vahaboglu *et al.*, 2001), 23 % by Soudeiha *et al.*, (Soudeiha *et al.*, 2018), 21.74 % in 2018 (Hafiz *et al.*, 2018) and 54.6 % (Yong *et al.*, 2003). In addition, Pournajaf and his colleagues in 2018 showed 78 % of isolates to produce ESBL using Double Disk Synergy Test, and 83.5 % of the isolates to produce ESBL using E-test (Pournajaf *et al.*, 2018); these findings are closer to those by another study reporting 88 % (88/100) of ESBL production (Goyal *et al.*, 2018). There was a significant association between ESBL production based on sex and age in this present study. The highest frequency of ESBL was reported in 18.18 % of the isolates in the age group of 21-40 years in male and 36.37 % in females. The prevalence of ESBL production was significantly different between males and females in all age groups and was observed to be 27.27 % and 72.73 % respectively.

In the present study, after screening among all the *Acinetobacter* spp. isolates, none of the isolates produced the MBL gene, and this is an agreement with a recent study in 2014 (Al-Agamy *et al.*, 2014). But a number of studies showed MBL productions, 39 % (42 out of 108) isolates in 2012 (Owlia *et al.*, 2012), 10 % in 2007 (Pino *et al.*, 2007), and fifty strains (96 %) were found to be MBL-positive by the modified Hodge test in 2015 (Aksoy *et al.*, 2015). However, the presence of MBL in *Acinetobacter* spp. was less common (Irfan *et al.*, 2011; Shoja *et al.*, 2017).

In the nosocomial pathogens, the transfer of antibiotic resistant genes causes complications in patients' treatment (Beige *et al.*, 2015). In recent years, several protocols have developed to simplify the isolation of plasmid DNA. Most of these rapid procedures are invariably contaminated with chromosomal DNA. The detection of plasmids depends on the adequately used methods (Nazmul MHM *et al.*, 2016). For the plasmid profile analysis, the Close and Rodriguez method (1982) with modification and the plasmid extraction kit (QIAprep spin kit) were used to extract and purify plasmid DNA from the bacterial isolates. The most purified plasmids without contamination with genomic DNA was detected by the plasmid DNA extraction kit (QIAprep spin kit) compared to the Close and Rodriguez method. Plasmid DNA extraction kit could even purify small-sized plasmids. The reproducible recovery of all

plasmid DNA was seen by the plasmid DNA extraction kit in *Acinetobacter* spp.

In the present study, the supercoil DNA marker (molecular weight markers) was used to estimate the plasmid size of *Acinetobacter* spp. in each gel. Plasmids were detected from fourteen strains, and the overall sizes of the plasmid DNA ranged from the lowest 1.8 kb to the highest 14 kb, while another study (Sadeghifard *et al.*, 2011) observed the sizes of the plasmid DNA to range from the lowest 1kb to the highest 21kb. A survey in KSA showed that five *A. baumannii* isolates harboured plasmids among nineteen isolates which were not less than 2.71kbp in molecular weight (Selim and Hagag, 2013). The results of the current study are also in agreement with another recent study in Malaysia which showed that 40 % of the isolates harbored plasmid DNA ranging from 1.8kb to 8kb (Nazmul MHM *et al.*, 2016). Cameranesi *et al.* reported 9kbp to slightly more than 23 kbp (Cameranesi *et al.*, 2018) plasmids, whereas a pK50a (79.6 kb) plasmid was found by Wibberg *et al.*, 2018. Besides, according to a study in 2014, plasmids were found in eighty out of eighty-eight isolates (90.9 %) with one being of a 7.30kbp molecular weight (Ali *et al.*, 2014). Some researchers have reported a much lower rate of plasmid detection 42 % of the tested strains (Koeleman *et al.*, 2001), seventeen isolates (34 %) carried plasmids with molecular sizes of > 20kbp (Eftekhar *et al.*, 2018), and the isolates contained a molecular weight of a plasmid ranging between 2 to > 25kb (Saranathan *et al.*, 2014). Thirty-six isolates (72 %) did not carry any plasmids in the current study which is in agreement with other studies in 2016 (60 %) (Nazmul MHM *et al.*, 2016) and 2018 (11 %); the isolates did not carry any plasmid (Salto *et al.*, 2018).

These fourteen isolates appear to harbor one or more plasmids with the maximum number of four plasmids. Various plasmid profiles were determined where four isolates owned single plasmids, and five strains carried four plasmids, while four and one isolates had two and one plasmids respectively. Plasmid values are essential for antibiotic resistance studies as they can specify or identify the plasmid-mediated resistance gene in addition to in typing different bacterial strains. The total plasmid occurrence rate (POR) was (28 %) when using the Close and Rodriguez method with modification (1982) and the QIAprep spin kit. This finding is in agreement with Saranathan and his colleagues who detected fifty isolates (94.5 %) in 2014 (Saranathan *et al.*, 2014), and plasmids were found in 107 out of the 112 (95.5 %) strains (Sadeghifard *et al.*, 2011).

A number of studies around the world have shown the involvement of plasmids in multidrug resistance (Botts *et al.*, 2017; Mathers *et al.*, 2015; Porse *et al.*, 2016; Weber *et al.*, 2015). Among the gram-negative bacteria, plasmids-encoding genes are transferred to the variety of microbial agents by transmission, and this can lead to serious public health hazards. In addition, the β -lactam resistance genes in the plasmid are supported by plasmid DNA analysis. Some of these genes may characterize the chromosome-based mechanisms of resistance. According to the present study, the involvement of ESBL, MBL, and plasmid association may play an essential role in resistance. The differently sized plasmids found in the current investigation might be involved in multidrug resistance. Further studies are required to find out the association of

these plasmids in multidrug resistance and β -lactamase-producing strains. Moreover, specific infection control practices and regular monitoring of antimicrobial properties of microorganisms, need to prevent the spread and outbreaks of ESBL and MBL-producing bacteria.

5. Conclusion

The incidence of infections due to ESBL and MBL gene-producing bacteria is becoming more and more common in hospitals and the community. Rapid recognition and characterization of different types of resistance might minimize the spread of bacterial infections, and can help select the right choice of medication. Among all the clinical isolates of *Acinetobacter* spp., plasmids may have an additional mechanism for resistance, but there is still a need for further investigation to confirm these results. The current study might be helpful in the treatment and prevention of disease by seeking to improve the efficiency of antibiotics.

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The authors declare no conflict of interest in conducting this study.

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