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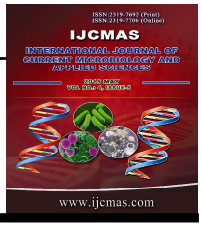

Original Research Article

Clonal Diversity of *Acinetobacter baumannii* Mediated by Carbapenem Resistance in Saudi Arabian Hospitals

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Acinetobacter baumannii, Carbapenem resistance, Saudi Arabia, Prevalence

A B S T R A C T

To investigate the clonal diversity of multi-drug resistant *A. baumannii* associated with carbapenem resistance in hospitals of Saudi Arabia based in Riyadh city. Sixty-two non-repetitive strains of *A. baumannii* from different specimens, collected from King Faisal Specialist Hospital and Research Centre (KFSHRC) in Riyadh were included in the study. The isolates were identified by the Vitek compact II system. Multiplex PCR using primer for $bla_{\text{OXA-51}}$ combined with primers for $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$ and $bla_{\text{OXA-58}}$ was employed. The resistance pattern of the tested isolates was determined by Vitek 2 compact system and the minimum inhibitory concentrations of imipenem, meropenem, tigecycline and colistin were determined by Etest strips. The clonal diversity of the isolates was investigated by PFGE. Carbapenem resistance was considerably high. Sixty-one out of 62 (98.4%) and 58/62(93.5%) were resistant to imipenem and meropenem, respectively. All isolates were susceptible to colistin but resistance to tigecycline was observed in 9/62 (14.5%). The prevalence of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$, bla_{VIM} and bla_{SPM} were 32 (51.6%), 15 (9.3%), 55 (89%) and 37 (60%), respectively. None of the isolates had $bla_{\text{OXA-58,}}$ bla_{IMP}, bla_{SIM} or bla_{GIM}. IS*Aba1* and IS*Aba2*, were 53 (85%) and 1(1.6%) respectively, while IS*Aba3* and IS*18* were not detected. PFGE results showed that the tested isolates were clustered in twenty two groups. Clone 10 and 17 were the dominants clones containing 7 and 9 isolates respectively were from six hospitals followed by clone 14 and 18 containing 5 and 6 isolates respectively were from 6 hospitals. It is concluded that $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$, bla_{VIM} and bla_{SPM} were the most prevalent genes in the carbapenem resistant *A. baumannii* isolates under investigation while IS*Aba1* was the most common insertion sequence with bla_{VIM} emerging as the chief culprit. Early recognition of the epidemic clone is very helpful to prevent its dissemination by application of strict infection control measures.

Introduction

Acinetobacter baumannii, in recent years, has been emerged as an important pathogen associated with a wide range of infections causing significant health problems globally.

In immunocompromised patients[1] Carbapenems are often used as the drugs of last resort for treating severe *A. baumannii* infections; however, their use is increasingly becoming challenged due to the emergence of β-lactamase molecular classes B and D capable of hydrolysing carbapenems.[2]The emergence of carbapenem resistance in *A. baumannii* has been reported worldwide across different continents.[3]A variety of mechanisms have been conclusively proven to be responsible for carbapenem resistance in *Acinetobacter*, that include modification or reduced expression of porins, over expression of efflux pump and production of carbapenemases.[1]

β-lactamases with carbapenem hydrolysing activity produced by *Acinetobacter* include Class B metallo- β-lactamase and Class D oxacillinase.[4] Several OXA carbapenemase genes in carbapenem resistant *Acinetobacter* have been reported so far.[2] The commonly found genetic lineages include OXA-23 like, OXA-24/40 like, OXA-58 like and OXA-51 like. The former three are acquired whereas the latter is a naturally occurring intrinsic gene that requires presence of insertion sequence IS*Aba*1 upstream of the gene. Insertion sequence IS*Aba*1 provides a promoter sequence that enhances expression of OXA-51 like gene.[5]

A clone of *A. baumannii* with OXA-23 like gene was first reported from a clinical isolate in Scotland in 1985, paradoxically even before the introduction of carbapenem itself, but since then this plasmid borne gene has disseminated across the globe.[6] Clonal outbreaks of carbapenem-resistant and OXA-23 producing *A. baumannii* have been reported in many countries, such as Bulgaria, People's Republic of China, Brazil, Iraq, Afghanistan and French Polynesia.[7]

Other frequently detected carbapenem hydrolyzing oxacillinase enzymes among *A. baumannii* include the subgroups OXA24/40 and OXA-58. OXA-24/40 was first detected in a highly carbapenem resistant strain isolated in Spain.[8] OXA-58, which share <50% amino acid sequence with other oxacilinases, was first identified in France in 2005.[9]There are reports of OXA-58-like enzyme from Argentina, Kuwait and Southern England.[10]Carbapenem resistance in *A. baumannii* in Saudi Arabia has been reported to be significantly high.[11,12] Multi drug resistant nosocomial *A. baumannii* has been reported in several hospitals in Saudi Arabia.[12]The aim of this study was to investigate the clonal diversity of carbapenem resistance in *A. baumannii* in a major Saudi Arabian Hospital and to establish the extent of dissemination of the common clonal lineages in Saudi Arabia.

Material and Methods

Bacteriological processing

Sixty-two isolates of *A. baumannii* were collected from non-repetitive specimens from King Faisal Specialist Hospital and Research Centre (KFSHRC) in Riyadh between January 2011 and April 2011.The identity of *A. baumannii* was established by the Vitek Compact II system. Overall, the Vitek II GN kits for identification of Gram negative bacteria identifies more than 97% of isolates correctly up to the species level[13] and detection of $bla_{\text{OXA-51-like}}$ gene was done by PCR.[14]

Susceptibility testing

Disc diffusion method for testing antibiotic susceptibility was performed and minimum inhibitory concentrations (MICs) were determined for imipenem, meropenem, tigecycline and colistin as per the British

Society for Antimicrobial Chemotherapy (BSAC) guidelines.[15]

Polymerase chain reaction (PCR)

Multiplex PCR was performed using primer for *bla*$_{OXA-51}$combined with primers for *bla*$_{OXA-23}$, *bla*$_{OXA-24/40}$ and *bla*$_{OXA-58.}$ The former was used to confirm identity of the isolate as well, as this is an intrinsic gene. The amplification process was initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 25s, 52°C for 40s and 72°C for 50s, and a final elongation at 72°C for 6 minutes.[14]The primers used were F- (5'-TAA TGC TTT GAT CGG CCT TG-'3) and R- (5'-TGG ATT GCA CTT CAT CTT GG-'3) to amplify a 353 bp fragment of the intrinsic OXA-51 like genes and these primers were combined with six new primers to amplify fragments of OXA-23-like 501bp F- (5'-GAT CGG ATT GGA GAA CCA GA-'3) and R- (5'-ATT TCT GAC CGC ATT TCC AT-'3), OXA-40/24-like 246 bp F- (5'-GGT TAG TTG GCC CCC TTA AA-'3) and R- (5'-AGTTGA GCG AAA AGG GGA TT-'3) and OXA-58-like 599 bp F- (5'-AAG TAT TGG GGC TTG TGC TG-'3) and R- (5'-CCC CTC TGC GCT CTA CAT AC-'3) genes. The primers were evaluated individually using control strains and then in a multiplex format.[14]Metallo β-lactmases coding genes were amplified as previously described.[16]

Molecular typing by PFGE

Sixty two clinical isolates of *A. baumannii* were typed by PFGE analysis as previously described. DNA obtained from bacteria was digested using Apa1 restriction endonuclease (Promega, Southampton, UK), and DNA fragments were separated on 1% agarose gel in 0.5× TBE buffer using the CHEF-RDII apparatus (Bio-Rad, UK). The conditions used were the following: pulse time 5-35s at field strength of 6v/cm for 24h at 37C. The gel was stained by ethidium bromide and then the digital images were captured by Gel doc2000 (Bio-Rad, UK). All isolates were analyzed using Bionumerics software version 6.5. Isolates that clustered together with a similarity of >85% were considered to belong to the same PFGE type [17].

Results and Discussion

Sixty-two clinical isolates of *A. baumannii* were collected and identified using Vitek Compact II system. High level of carbapenem resistance was observed among the *Acinetobacter* isolates. Antibiotic sensitivity testing and MIC determination was performed by disc diffusion test as per BSAC guidelines. Sixty one isolates (98.4%) were resistant to imipenem. Of these, fifty-six (90.3%) were also resistant to meropenem as well (Table 2). Two strains were intermediate (MIC>4-8mg/L) and another two were sensitive (MIC≤ 0.5mg/L) (Table 1 and Figure 1). All isolates were susceptible to colistin (MIC≤ 0.5 - 1mg/L). Four isolates demonstrated resistance to tigecycline (6.5%) while six isolates were intermediate susceptible (Table 1 and Figure 1).

Multiplex PCR was performed to detect OXA-like genes in order to find out genetic basis of carbapenem resistance. Thirty-two carbapenem resistant isolates possessed *bla*$_{OXA-23}$ gene while OXA-40 gene was found in 15 isolates (Table 2). None of the organisms had OXA-58. An isolate was sensitive to imipenem, tigecycline and colistin while intermediate sensitive to meropenem was also found to be harbouring *bla*$_{OXA-23}$ (Figure 1). On the other hand, *bla*$_{VIM}$ and *bla*$_{SPM}$ were detected in 55 and 37 isolates, respectively, while *bla*$_{SIM}$, *bla*$_{IMP}$ and *bla*$_{GIM}$ were absent (Table 2). Figure 1

showed the PFGE dendrogram of the 62 *A. baumannii* tested isolates.

PFGE was applied to study the clonal diversity and relatedness of the tested isolates. The discrimination power of PFGE technique was express by dice coefficient via BioNumerics software version 6.5. Figure 1 shows the clustering of Twenty-two clones of PFGE comprise the ID number (isolate code), sex, locations, β-lactamase of OXA-23, OXA-40, insertion sequences (IS*Aba1*, IS*Aba2*, IS*Aba3* and IS*18*) and MBLs (VIM, SIM, GIM, IMP and SPM).

All detected PFGE patterns demonstrate the genetic similarity coefficient ranged from 65% to 100%. Epidemic isolates that clustered together with a similarity of more than 85% were considered to present the same PFGE type. Clone 10, 14, 17 and 18 included 7, 5, 9 and 6 PFGE types, respectively, with genetic similarity ranges from 85 to 100%, 88 to 100%, 89 to 100% and 87 to 100% respectively, and have shared 3, 2, 3 and 4 cities, respectively, as shown in Figure 1. The clonal diversity of the twenty-two clones and the mechanism of resistance in relation to the cities from which the isolates obtained were collected. Twelve, five and four different clones were detected in 12, 8 and 7 isolates from Riyadh, Breedah and Almadinah, respectively Table 5.

The menace of multidrug resistant organisms including pan drug and carbapenem resistant *Acinetobacter* has thrown new challenges to medical fraternity.[1] No part of the world is left untouched with this problem and Saudi Arabia is no exception to it. In fact, with a vibrant economic and raised standard of life, Saudi Arabia has been able to provide an excellent class healthcare delivery system. As a consequence of this, it has to face the aforementioned problem too. In this study, a considerably high level of carbapenem resistance among *A. baumannii* isolates was observed (96% against imipenem versus 93% against meropenem). Studies on carbapenem resistant *A. baumannii* in Saudi Arabia have also cited extremely high percentage resistance[11,12,18-23]. Four strains (14%) were also resistant to tigecycline. Recent studies have reported an increased resistant to this antibiotic which has further narrowed down the therapeutic options.[24]The incidence of OXA type β-lactamase mediated carbapenem resistance among *A. baumannii* has been rising and several different types of OXA-like genes have been identified until now.[2] The $bla_{\text{OXA-23}}$ gene, first characterized in Scotland, has been increasingly reported worldwide. *A. radioresistens* was recently identified as the progenitor of the $bla_{\text{OXA-23}}$-like genes.[6] Clonal outbreaks of carbapenem-resistant and OXA-23–producing *A. baumannii* have been reported in many countries, such as Brazil[25], China (GenBank accession number AY554200)[26], Korea[27], the United Kingdom[5] and Singapore (GenBank accession number AY795964). OXA-24/40 and OXA-58 are the other commonly occurring carbapenem hydrolyzing class D β-lactamases. OXA-58, a plasmid-borne carbapenemase, has been seen in several countries including France, England, Argentina, Spain, Turkey, Romania, Austria, Greece, Scotland, and Kuwait.[1] The intrinsic gene OXA-51 of *A. baumannii*, might also be responsible for carbapenem resistance, if it is accompanied with IS*Aba*1 promoter gene upstream.[5]46% (13/28) of isolates tested by us harbored$bla_{\text{OXA-23}}$, whereas 41% (11/28) had $bla_{\text{OXA-24/40.}}$ $bla_{\text{OXA-58}}$, the other common OXA-like gene was not detected in any isolate. We reviewed previous reports of bla_{OXA} gene prevalence among carbapenem resistant *A. baumannii*in Saudi Arabian hospitals.

Studies conducted on the genes responsible for carbapenem resistant *A. baumannii* in Saudi Arabia have shown that OXA-23 genes are the leading genes prevalent. The prevalence of $bla_{\text{OXA-23}}$ gene in Saudi Arabia has been in the range of 38-72.5%. The genetic clone has disseminated considerably in this country as shown in Table 3. *A. baumannii* strains bearing OXA-23 gene has also been reported to be the chief cause of carbapenem resistant worldwide. $bla_{\text{OXA-23}}$ geneis now endemic in several hospitals of England[28]. In Greece, the $bla_{\text{OXA-23}}$ gene mediated carbapenem resistance has been found to be 72.4%[29].

Other parts of Middle East do have the presence of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24/40}}$ carrying *A. baumannii*. Iraq, Bahrain, and United Arab Emirates have been found with such β-lactamase producing *A. baumannii* strains[6]. 26/44 (59.1%) strains harboured $bla_{\text{OXA-23}}$ gene in a study from Turkey[30]. There are studies testifying to the occurrence of such strains of *A. baumannii* in South East Asia and Pacific region as well. Jeon et al. (2005) have reported that 36/52 or 69.2% of hospital isolates of *A. baumannii* in Republic of Korea had $bla_{\text{OXA-23}}$.[27] Researcher in South Korea have found it to be 77%.[31] Very high prevalence of $bla_{\text{OXA-23}}$ gene bearing *A. baumannii* - 28/32 (87.5%), was found in a study of clinical isolates from Sydney Hospital, Australia.[32] Similar higher prevalence has also been reported from Singapore (91%)[33] and Hong Kong (100%)[34].

OXA-24/40 family of OXA genes could be seen in 41% isolates in this study. OXA-24/40 in clinical isolates of *A. baumannii* has been reported in significant number.[31] Previous studies in Saudi Arabia have also documented the presence of *A. baumannii* strains carrying this gene as show in Table 4.

No strains were positive for OXA-58 in our study. High prevalence of OXA-58 (47.8%) from different cities in Europe has been reported by Marque et al. (2005) [35]. Much higher prevalence of $bla_{\text{OXA-58}}$ (100%, n=19) has been reported from Turkey[36]. However, $bla_{\text{OXA-58}}$ is a less commonly found gene in comparison to the other genes[31].

The remaining carbapenem resistant isolates (14% imipenem and 11% meropenem) had no OXA genes in them. Their resistance to carbapenem could be because of OXA-51 itself- an intrinsic OXA gene in *A. baumannii* over expression of which is known to be a reason for carbapenem resistance[2] or perhaps the presence of Class B metallo β-lactamase which we didn't attempt to identify.

Chi-square test was used to compare the locations and OXA-23 among them and the value of chi-square was 29.86 with p-value 0.019, which had significant value. To determine which location made that difference, Post-hoc analysis was performed as follows: when converted to a z-score, the standardized residual (2.0) was greater than the critical value (1.96), supporting a specific finding that among survey respondents who were in Breeda. There were more who had [OXA-23 (-)] than would be expected as shown in Table 4. The same test was used to compare the locations and OXA-40 among them, and the value of chi-square was 34.24 with p-value 0.005, which had significant value. In addition, when converted to a z-score, the standardized residual (4.6) was greater than the critical value (1.96), supporting a specific finding that among survey respondents who were in Breeda. There were more who had [OXA-23 (+)] than would be expected.

Table.1 MIC values and level of resistance among *A. baumannii* isolates

Antibiotics	No. of resistant isolates	MIC range (ml/L)	%
Imipenem	61	>16	98.4
Meropenem	58	>16	93.5
Tigecycline	9	4 – 8	14.5
Colistin	0	--------	0

Table.2 Prevalence of different carbapenemases in the tested isolates

Gene	Number of isolates (%)
$bla_{\text{OXA-23}}$	32 (51.6)
bla_{OXAcc}	15 (24.2)
bla_{VIM}	55 (88.7)
bla_{SPM}	37 (59.6)
bla_{SIM}	0 (0)
bla_{IMP}	0 (0)
bla_{GIM}	0 (0)

Table.3 Prevalence of bla_{OXA} genes in Saudi Arabia

Reference	Number of isolates (%) harboring		
	$bla_{\text{OXA-23}}$	$bla_{\text{OXA-24/40}}$	$bla_{\text{OXA-58}}$
Al-Arfajet al. (2011)[12]	29/40 (72.5)	18/40 (45)	15/40 (37.5)
Ribeiro et al. (2012)[22]	16/27 (46.3)	1/27 (3.7)	ND
Shehata et al. (2012)[20]	45/118 (38)	ND	ND
Present study	32/62 (51.6)	15/62 (24.1)	0/62 (0)

ND: Not Determined

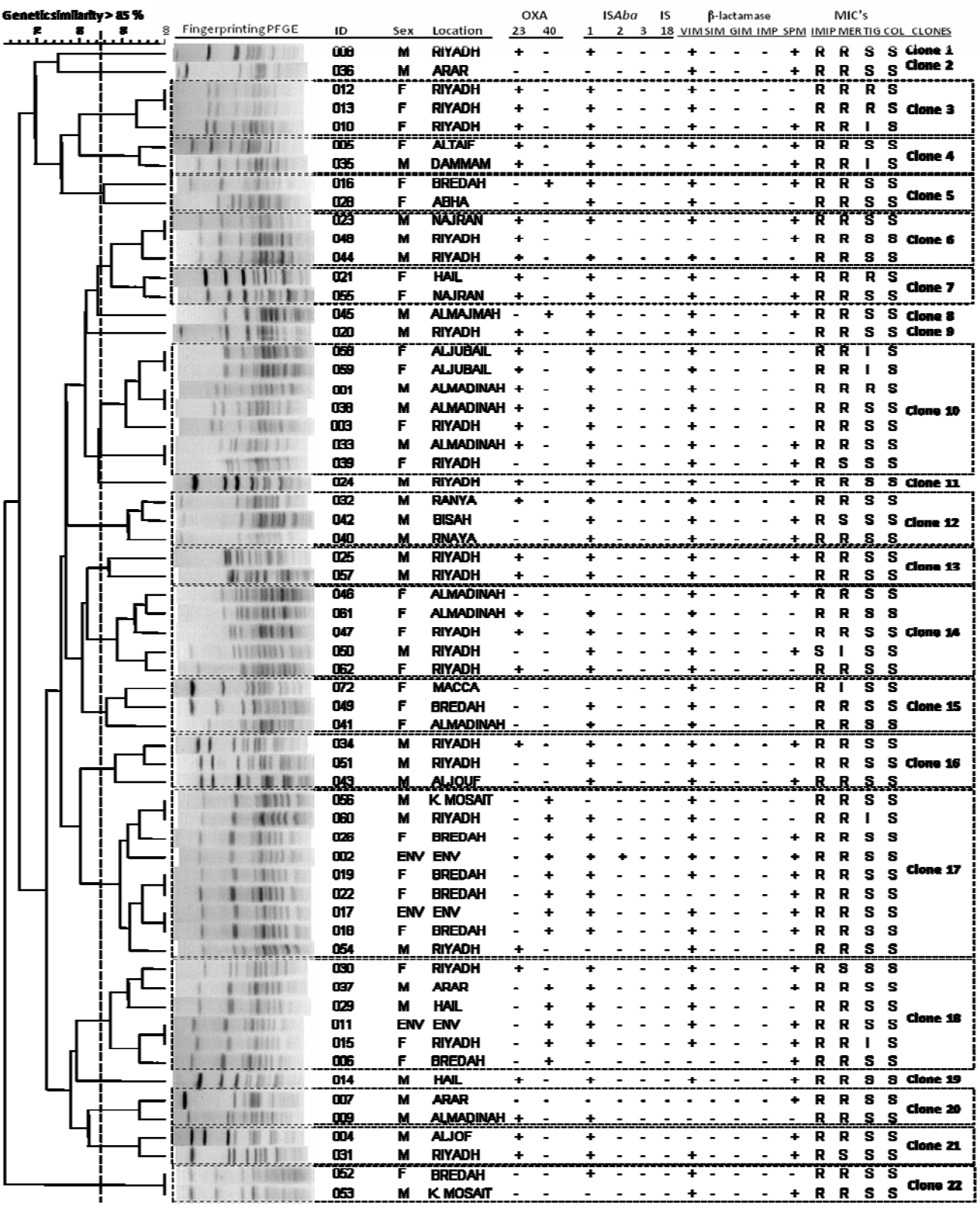
Table.4 Relation between location and gender with the prevalence of different components

Variables	Location			Gender		
	Chi-square value	df	P-value	Chi-square value	df	P-value
OXA-23	29.86	16	**0.019**	1.224	1	0.269
OXA-24/40	34.24	16	**0.005**	1.740	1	0.187
IS*Aba1*	26.15	16	0.052	0.661	1	0.488
VIM	16.9	16	0.392	2.278	1	0.205
SPM	22.95	16	0.115	0.680	1	0.410
Imipenem resistance	1.665	16	1	0.858	1	1
Meropenem resistance	47.1	32	**0.042**	0.048	2	0.976
Tigecycline resistance	35.97	32	0.287	2.917	2	0.185

Table.5 Profiles of *A. baumannii* tested isolates

Hospital location (Area)	Clone diversity	Mechanism of resistant	No. of isolates (%) (*n=62*)
RIYADH	1, 3, 6 9, 10, 11 13, 14, 16 17, 18, 21	OXA-23, OXA-40, IS*Aba1*, VIM and SPM	22 (13.6 %)
BREDAH	5, 15, 17 18, 22	OXA-40, IS*Aba1*, VIM and SPM	8 (5.0%)
ALMADINAH	10, 14, 15, 20	OXA-23, IS*Aba1*, SPM and VIM	7 (4.4%)
ARAR	2, 18, 20	OXA-40, IS*Aba1*, VIM and SPM	3 (1.9%)
HAIL	7, 18, 19	OXA-23, OXA-40, IS*Aba1*, VIM and SPM	3 (1.9%)
ENV	17, 18	OXA-40, IS*Aba1*, VIM and SPM	3 (1.9%)
NAJRAN	6, 7	OXA-23, IS*Aba1*, VIM and SPM	2 (1.2%)
ALJUBAIL	10	OXA-23, IS*Aba1*, VIM and SPM	2 (1.2%)
RANYA	12	OXA-23, IS*Aba1*, VIM and SPM	2 (1.2%)
ALJOUF	16, 21	OXA-23, IS*Aba1*, VIM and SPM	2 (1.2%)
K. MOSAIT	17, 22	OXA-40, VIM and SPM	2 (1.2%)
ALTAIF	4	OXA-23, IS*Aba1*, VIM and SPM	1 (7.8)
DAMMAM	4	OXA-23, IS*Aba1* and SPM	1 (1.6)
ABHA	5	OXA-40, VIM and SPM	1 (3.1)
ALMAJMAH	8	OXA-40, IS*Aba1*, VIM and SPM	1 (1.6)
BISHAH	12	IS*Aba1*, VIM and SPM	1 (1.6)
MACCA	15	VIM	1 (1.6)

Figure.1 PFGE patterns of *A. baumannii*. Abbreviations: R, resistance; S, susceptible; I, intermediate, IMIP, imipenem; MER, meropenem; TIG, tigecycline; COL, colistin



The same procedure was applied to compare the locations and meropenem resistance among them. Chi-square value was 47.1 with p-value 0.042, which had significant value and the standardized residual (5.2) was greater than the critical value (1.96), supporting a specific finding that among survey respondents who were in Macca. There were more who had [Meropenem (I)] than would be expected. On the other hand, there was not any significant value between Gender and any other variable (Table 4).

PFGE is one of the most important discriminatory methods of *A. baumannii* and many other pathogens[37-39.] It is an efficient tool for determining the genetic relationship between strains isolated and certain epidemiological situation[39]. In the current work, the genetic similarity in PFGE among its own different types is very high (87 to 100%). The clonal diversity revealed two types of clones that cause an epidemic: monoclonal and polyclonal as mentioned in Table 5.

The polyclonal model showed that it is the most common clone appears in 9 of 17 cities in the Kingdom. Two types of epidemic PFGE mainly caused these types of polyclonal outbreaks. In addition, Riyadh is the capital city of Saudi Arabia, which affected by 12 different clones of 17, and Bredah is the capital city of Al-Qaseem province had also affected by five different clones. Thus, it shows that both cities were affected an explosive outbreak however at different times.

Whereas, the monoclonal model has affected eight cites (Jubail, Ranya, Altaif, Dammam, Abha, Almajmah, Bisha and K. Mosait), and each of these cities had only one clone. Therefore, it is depicted that it is present in the minority and that could reflect the coexistence of sporadic and epidemic clones[40] too. Indeed, it leads to a conclusion that all of these cities might have low levels of hospital infection control that may help in these outbreaks. Likewise, it appeared that clone 18 has been detected in hospitals of Riyadh, Bredah, Arar, Hail, and while clone 10 has been found from the environment in three cities Riyadh, Almadinah and Aljubail (Table 5).

Henceforth, this study has pointed out that the transmission of an existing clone from one hospital to another could originate a new outbreak at these hospitals and that eventually affects health care workers. And during transmission, the possibility of *A. baumannii* transmission is highly recognized [41-43] that reflects the reappearance of certain clones within these hospitals. Consequently, it reinforces persistence of endemic from this pathogen in patients, hospital and environments, which, could be a major risk factor in future outbreaks.

Thus, it is highly recommended that these isolates should to be compared with different strains on other countries through typing and β-lactamase gene sequencing. The further research would establish whether there is a particular bacterial clone present and is associated with particularly to the kingdom of Saudi Arabia.

The result of the current study revealed that $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$, bla_{VIM} and bla_{SPM}were the most common β-lactamases conferring carbapenem resistance to *A. baumannii*.

It is concluded that $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$, bla_{VIM} and bla_{SPM} were the most prevalent genes in the carbapenem resistant *A. baumannii* isolates under investigation while IS*Aba1* was the most common insertion sequence with bla_{VIM} emerging as the chief culprit. Early recognition of the epidemic

clone is very helpful to prevent its dissemination by application of strict infection control measures. Moreover, the current study provided significant data regarding the clonal diversity of carbapenem resistant *A. baumannii* in different cities in Saudi Arabia. Detection of the certain clone in different cities reflects the horizontal transmission of carbapenem resistance. Strict infection control measures should be applied to prevent such type of transmission.

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