Detection of *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub> metallo-β-lactamase genes by Multiplex PCR among carbapenem resistant *Acinetobacter baumannii* strains isolated from ventilator associated pneumonia in ICU patients

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**ABSTRACT**

This study was undertaken to examine the microbial causes of ventilator associated pneumonia (VAP) among adult intensive care unit (ICU) patients of Al-Ansar Hospital, Medina, KSA and to detect the prevalence of metallo-β-lactamase (MBL) in carbapenem-resistant *A. baumannii*. Microorganisms obtained from different respiratory specimens in patients diagnosed with VAP between January 2011 to January 2014 were included in the current analysis. Standard laboratory methods were used to identify microorganisms, and a standardized susceptibility test was performed. Vitek 2 compact identification system (BioMerieux) using GP-67 and GN-291 cards were also used to confirm the identification. Screening for the detection of MBL in A. baumannii strains was done by disc potention test with EDTA impregnated-impenem and meropenem discs and confirmed by multiplex PCR. A total of 208 clinical isolates of bacteria including Gram negative bacteria constituted 150 (72.1%) of VAP, while Gram positive bacteria constituted 58 (27.9%) of isolates. The most common pathogen associated with VAP was *Acinetobacter* spp. (26.9%). Patients with primary VAP associated with *Acinetobacter* spp. were more likely to have nasogastric intubation, sedation and they were on mechanical ventilator for more than 15 days. Out of the 56 strains of A. baumannii isolated form VAP patients, 35 were carbapenem-resistant; of these, 20 were positive for MBL production by potentiated disk test, 17 were positive for *bla*<sub>VIM</sub> by multiplex PCR but none of our strains were positive for *bla*<sub>IMP</sub>. In conclusion, gram-negative pathogens are responsible for most of VAP cases in our patients. *A. baumannii* is the most common pathogen associated with VAP, especially recurrent cases. Carbapenem resistance in A. baumannii is mainly mediated by MBL production. The common MBL gene is *bla*<sub>VIM</sub>.

**Keywords:** Recurrent ventilator-associated pneumonia, *Acinetobacter baumannii*, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, multiplex PCR.

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**INTRODUCTION**

Ventilator-associated pneumonia (VAP) continues to complicate the course of 8 to 28% of patients receiving mechanical ventilation and accounts for 15 to 25% of all types of intensive care unit (ICU) acquired infections (Kleven et al., 2007). Hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) are important causes of morbidity and mortality, with mortality rates approaching 62% (Rosenthal et al., 2010). Increasing drug resistance rates among Gram negative pathogens that frequently cause VAP may compromise treatment and result in prolongation of hospital stays, inflation of health care costs and further increase in hospital mortality (Amin, 2009).

*Acinetobacter* spp. have become major pathogens in hospital-associated infections, especially in critical care settings such as ICUs. They can survive in the hospital environment for long periods and have a remarkable propensity to develop resistance to multiple classes of
Antimicrobial agents.

*Acinetobacter baumannii* is the most clinical important species of the *Acinetobacter* genus, as it is responsible for over 90% of infections and it is often involved in outbreaks (Murray and Hospenthal, 2008; Ecker et al., 2006).

The broad-spectrum β-lactam antibiotics, carbapenems were introduced by 1985 and have been for years the most important agents for the treatment of infections caused by multidrug resistant *A. baumannii* (MDRAB). However, today carbapenem resistance is more frequently reported worldwide, attributable mainly to Ambler class D carbapenemases and class B metallo-β-lactamases (MBLs) (Poirel and Nordmann, 2006).

Carbapenem-resistant isolates of *A. baumannii* are usually resistant to all classes of antimicrobials, and show susceptibility to tigecycline and colistin (Ecker et al., 2006; Poirel and Nordmann, 2006). The resistance of *A. baumannii* to carbapenems can be mediated by one of the resistance mechanisms that are known to occur in bacteria, including enzymatic inactivation, active efflux of drugs, and modification of target sites. The production of carbapenem-hydrolyzing β-lactamases is the most common mechanism responsible for carbapenem resistance in *A. baumannii* (Poirel and Nordmann, 2006). Among them, bla_mbl and bla_vim are the most common types of MBLs with worldwide distribution. VIM (Veronese Imipenemase) enzymes have been grouped into three main clusters designated VIM-1, VIM-2, and VIM-7. To date, VIM-2 is more widely spread among *P. aeruginosa* isolates, whereas VIM-1 is normally confined to *Enterobacteriaceae* isolates. The IMP gene has at least 27 unique variants differing by up to 22% amino acid sequence divergence (between IMP-9 and IMP-19) that exhibit important structural and functional differences from each other or from enzymes of other genes. IMP-type MBL determinants are mostly found in *P. aeruginosa*, *A. baumannii* and *Enterobacteriaceae* isolates (Kouda et al., 2009; Walsh et al., 2005).

This study was undertaken to examine the microbial causes of VAP among adult ICU patients of Al-ansar Hospital, Medina, KSA and to detect the prevalence of MBLs in carbapenem-resistant *A. baumannii*, which is one of the common organism associated with VAP.

**MATERIALS AND METHODS**

**Setting**

The current study was conducted at adult ICU of Al-ansar Hospital, Medina, KSA. Al-ansar Hospital an approximately 200-bed tertiary care facility that provides health care services to Saudi patients, Hajj and Omera patients. The ICU at Al-ansar Hospital is a 26-beds covered 24 h/7 days a week and admits approximately 950 patients /year. The nurse-patient ratio is 1:1.

**VAP definition**

Diagnosis of Ventilator Associated Pneumonia (VAP) was based on pneumonia that occurred 48 h after endotracheal intubation, with the following diagnostic criteria: new or progressive infiltrates, consolidation, or cavitation on chest X-ray, with one of the following: (a) new onset purulent bronchial secretions with leukopenia (white blood cells <4 × 10⁹/l) or leukocytosis (>12 × 10⁹/l), or core temperature >38.5 without other cause, (b) significant positive culture from endotracheal aspirate or bronchoalveolar lavage (BAL). Both primary (first) and recurrent (second or third) episodes of VAP during the same hospitalization were included in the analysis. Recurrent VAP was diagnosed using the same criteria as primary VAP only after an evidence of clearance of the previous VAP.

**VAP surveillance**

On a daily basis, data were collected prospectively from all the patients admitted in the ICUs. Data were gathered according to the CDC-NNIS and CDC-NSHNI definitions for DA-HAI (Horan et al., 2008) and the methodology followed was as proposed by INICC (Rosenthal et al., 2008).

**Microorganisms**

Microorganisms obtained from different respiratory specimens from patients diagnosed with VAP between January 2011 and January 2014, were included in the current analysis. Specimens examined included endotracheal aspirate and BAL. Specimens were processed in a quantitative manner with a cut-off of 10⁵ CFU/ml. The isolates were identified by conventional methods (Clinical Laboratory Standards Institute, 2010). All isolates were non-duplicate. *Escherichia coli* ATCC 25922 was used as quality control strain.

**Susceptibility testing**

Susceptibility testing was performed using amikacin, gentamicin, tobramycin imipenem, meropenem, azertanom, ceftazidime, piperacillin, piperacillin/tazobactam, cefepime, ticaricillin, tigacycline, tetracycline, and ciprofloxacin (BBL chemical, USA) by disk diffusion method according to NCCLS guidelines (Clinical Laboratory Standards Institute, 2010). Vitek compact identification system (bioMéreix) cards was also used to confirm the identification using GP-67, and GN-291 cards and for antimicrobial susceptibility testing using AST –P580 and AST –N232 cards.

**MBL production**

Gram negative bacilli isolates were subjected to antibiotic susceptibility testing by Kirby Bauer disk diffusion method as per the CLSI guidelines (Hemalatha et al., 2005). Acinetobacter isolates resistant to imipenem and meropenem were subjected to screening for detection of MBL that was done by disc potention test with EDTA impregnated impenem discs and EDTA impregnated meropenem discs and confirmed by multiplex PCR (Hemalatha et al., 2005).

**Disc potentiation test method**

*Acinetobacter* spp, with resistant to imipenem, meropenem were inoculated into Mueller Hinton plates (opacity adjusted to 0.5 MacFarland). The plates were inoculated using cotton-tipped applicators. A 0.5 M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA, 2H₂O in 1000 ml of distilled water and
Table 1. PCR: genes, primer sequences, and amplicons size.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5'-3')</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIM</td>
<td>F: 5'-GAT GGT GTT TGG TGG CAT A-3'</td>
<td>390</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>R: 5'- CGA ATG CGC AGC ACC AG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP</td>
<td>F: 5'-GGA ATA GAG TGG CTT AAY TCT C-3'</td>
<td>188</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCA AAC YAC TAS GTT ATC T-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Multiplex PCR assay for bla\textsubscript{VIM} and bla\textsubscript{IMP} genes. Agarose gel electrophoresis showing: Lane L 100 to 1000 base pair (bp) ladder. Lane 11 to 20 are all positive for bla\textsubscript{VIM} gene (390 bp) except lane 11,16. None of the samples were positive for bla\textsubscript{IMP} gene (188 bp).

**Statistical analysis**

The data were analyzed using Package for Social Sciences 19.0 for Windows" (SPSS-19) software (SPSS Inc., Chicago, IL, USA). Data were expressed as frequencies, percents. Chi-square and Fishers exact test used for comparisons of categorical data. A p value < 0.05 was considered statistically significant.

**RESULTS**

During the study period, a total of 2650 cases were admitted to the ICU; of these, 1720 cases were on ventilator and 239 cases were diagnosed as VAP. About 15% of the VAP cases were diagnosed mainly based on clinical signs and symptoms with no pathogen identified. A total of 208 pathogens were identified during the study; 170 (92.1%) were associated with primary VAP and 38 (7.9%) were associated with recurrent VAP. Gram negative and Gram positive bacteria constituted 150 (72.1%) and 58 (27.9%), respectively, of 208 clinical bacterial isolates recovered from patients diagnosed with VAP. The isolates were obtained from tracheal aspirates (90%) and BAL (10%) as all our patients are intubated (Table 2).
The most common pathogen associated with VAP was *Acinetobacter* spp. (26.9%), followed by *Pseudomonas aeruginosa* (21.1%), *Staphylococcus aureus* (16.8%), *Klebsiella* spp. (11%), MRSA (8.6%), *Proteus* spp. (6.1%), *E. coli* (2.9%), *Streptococcus pneumoniae* (2.4%), *Stenotrophomonas maltophilia* (1.9%), and lastly *Serratia* spp. (0.9%). Gram negative pathogens were slightly more encountered in recurrent VAP (78.3%) than in primary VAP (71.2%), but the difference was not statistically significant (Table 2). *A. baumannii* was the only pathogen significantly associated with recurrent VAP compared to primary VAP (42.1% vs. 23.5%, p = 0.03). Patients with primary VAP associated with *A. baumannii* were more likely to have nasogastric intubation (p = 0.03), sedation (p = 0.03), and to be mechanically ventilated more than 15 days (Table 3). Fifty six strain of *A. baumannii* were isolated form VAP patients. Out of them, 35 were carbapenem-resistant; of these, 20 were positive for MBL production by potentiated disk test. MBL-positive *A. baumannii* strains were 100% resistant to ceftazidime, aztreonam, piperacillin, piperacillin/tazobactam, cefepime, tigecycline, tetracycline, and ciprofloxacin, while the resistance rates were 95% for amikacin, imipenem and meropenem and 65% for both tobramycin and levofloxacin (Table 3).

Detection of *bla*\textsubscript{VIM} and *bla*\textsubscript{IMP} genes by PCR

Among 20 *A. baumannii* isolates, *bla*\textsubscript{VIM} gene was
detected by multiplex PCR in 17 isolates (61.5%) as shown in Figure 1. However, bla IMP gene was not detected in any of the isolated A. baumannii strains (Table 4).

**DISCUSSION**

The incidence of nosocomial pneumonia in ventilated patients is high, ranging from 7% to more than 40%. Such HAI s prolong hospital stay and contribute to ICU patient mortality (Jones, 2010; Trouillet et al., 1998). *Acinetobacter* spp. are aerobic, Gram negative coccobacilli that can survive in moist and dry conditions for a prolonged period, so it often leads to nosocomial outbreaks (Manikal et al., 2003; Paterson, 2006).

Our study reports the bacterial causes of VAP among patients of a general adult ICU over a period of 3 years and. *A. baumannii* was the most common VAP pathogen (26.9%), followed by *P. aeruginosa* (21.1%) and *S. aureus* (16.8%). The 3 pathogens together are associated with almost two-thirds of all VAP cases. The current findings are in accordance with previous studies as *Acinetobacter* spp. was the most common isolated pathogen (21 to 36%) in six studies (Arabi et al., 2008). Meanwhile, different data were reported from western and several developing countries. Data from 55 ICUs between 2002 and 2005 in eight developing countries showed that *P. aeruginosa* and *Enterobacteriaceae* were the most common isolated pathogens (26% each), followed by *Acinetobacter* spp. (20%) (Rello et al., 2002). On the other hand, reports from the USA and Europe showed that *S. aureus* (23 to 32%)
P. aeruginosa (16 to 22%) were the leading causes of VAP, while Acinetobacter spp. was responsible for only 4 to 8% of VAP cases (Jones, 2010; Hidron et al., 2008). The high association of Acinetobacter spp. to VAP in our study can be explained by that our VAP patients were exposed to more prolonged use of ventilator (44.1% more than 15 days) than western patients (Rello et al., 2002) and lack of strict application of standard infection control precautions for surfaces and equipment in our ICU, that are insufficient to control Acinetobacter spp., which is particularly known for its resistance to disinfectants and long survival on dry surfaces (Barchitta et al., 2009; Weber et al., 2010).

The rate of VAP recurrence in our study (23.5%) was nearly the same as that reported in a meta-analysis done by Siempos et al. (2008) (26.8%) (Siempos et al., 2008). A. baumannii was the only pathogen significantly associated with recurrent VAP in our patients. Supporting our finding, Chastre and Fagon (2002) report, which showed that a high rate of VAP relapse was associated with primary infection by Acinetobacter spp. (Chastre and Fagon, 2002).

In our investigation, the majority of A. baumannii occurred among patients with age more than 50 (82.5%). Age more than 55 was reported by El-Ageery and Al-Hazmi (2014) as a co-factor for the acquisition of A. baumannii (El-ageery and Al-hazmi, 2014).

In our study the nasogastric intubation, use of sedation and prolonged use of ventilator were significantly associated with A. baumannii infection in VAP cases. This finding was also reported by Aiman et al. (2013) and Balkhy et al. (2014) both studies were carried in Saudi Arabia. As the commonly encountered organism with VAP (especially recurrent type) in this study was carbapenem-resistant A. baumannii. The prevalence of MBL (bla_vim and bla_imp) were investigated by phenotypic and molecular methods.

In the present study, the majority of the isolates (95%) were carbapenem resistant. High resistance rates to carbapenems have been observed in previous studies, ranging from 75 to 100% for imipenem and from 61 to 77% for meropenem (Bonnin et al., 2013; Mohamed and Raafat, 2011; Nasr and Attalah, 2013). The resistance to imipenem reflects a problem that might be described as countrywide. In the Middle East and North Africa, the occurrence of imipenem-resistant A. baumannii is recognized with alarm. The emergence of A. baumannii strains with increased carbapenem resistance in this area of the world may be due to the extensive misuse of carbapenems (Al-Agamy et al., 2014).

The most prevalent mechanism of carbapenem resistance in A. baumannii is the enzymatic degradation by carbapenem-hydrolyzing b-lactamases, MBL, mostly VIM and IMP, has been reported sporadically in some parts of the world (Alm El-Din et al., 2014). Nevertheless, in the present study, none of the A. baumannii isolates harbored bla_imp but bla_vim was discovered in 85% of A. baumannii isolates. Lower rate of detection was reported by other researchers, 15.38%, 16.28 and 46.55% were reported in a study conducted by Alam El Din et al. (2014), Karthika et al. (2009) and Amudhan et al. (2011), respectively.

The absence of bla_imp and bla_vim in 3 out of our 20 tested MBL positive A. baumannii strains could be either due to presence of unidentified MBL genes, limitation of the primer set used either with regards to picking up the variant IMP/VIM gene or because of presence of MBL genes other than IMP/VIM, presence of other enzymes (OXA like Ambler class D carbapenemase Amp C β-lactamases) or other mechanism of carbapenem resistance, namely loss of prions, increase in efflux pump activity alteration in penicillin binding proteins (PBPs) (Singh et al., 2009).

In conclusion, Gram negative pathogens are responsible for most of VAP cases in our patients. A. baumannii is the most common pathogen associated with VAP, especially recurrent cases. Carbapenem resistance in A. baumannii is mainly mediated by MBL production. The common MBL gene is bla_vim.

RECOMMENDATIONS

1. Our ICU should continue to actively screen for Acinetobacter spp. in all admitted patients, shorten the duration of ventilation, minimize sedation, encourage oral gastric rather than nasogastric intubation, and improve currently implemented infection control measures, including environmental disinfection.

2. The development of simple and inexpensive screening methods to detect MBL production in microbiology laboratories is crucial for optimal treatment of patients, particularly critically ill and hospitalized patients, and to control the spread of resistance.

Limitation of the study

In this study, only A. baumannii strains were subjected to multiplex PCR for detection of the common MBL resistance genes (bla_vim and bla_imp), because they are the most common organisms isolated from VAP patients. Other commonly isolated organisms, such as P. aeruginosa, were not included due to limited resources.

Ethical approval

Adherence to Saudi Arabia regulations concerning the welfare of human subjects was maintained throughout the study. The participant’s rights, privacy, health, and well-being were safeguarded through informed consent forms that they asked to read and sign if they agreed to participate in the study.
REFERENCES


