Prevalence of carbapenem resistance genes in *Acinetobacter baumannii* isolated from clinical specimens obtained from an academic hospital in South Africa

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Acinetobacter baumannii is an important cause of hospital-acquired infections. The occurrence of carbapenem resistance that is caused by the carbapenem-hydrolysing class D β-lactamases and the metallo-β-lactamases (MBLs) limits the range of therapeutic alternatives in treating *A. baumannii* infections. In this study, two multiplex polymerase chain reactions were performed to screen for both carbapenem-hydrolysing class D β-lactamases and MBL genes in 97 clinical isolates of *A. baumannii*. Oxacillinase (OXA)-51 had a prevalence of 83% (81/97), and OXA-23 had a prevalence of 59% (57/97). One isolate was positive for an MBL [Verona integron-encoded metallo β-lactamases (VIM)]. Therefore, continuous surveillance and monitoring of *A. baumannii* is crucial because of the high prevalence of antibiotic resistance genes.

Introduction

Acinetobacter baumannii is an opportunistic pathogen and a common cause of hospital-acquired infections, especially in intensive care units.[1,2] Patients are often hospitalised with severe illnesses when they are exposed to multidrug-resistant *A. baumannii*, an opportunistic pathogen, which has become more prevalent in the past 20 years.[3,4]

Acinetobacter spp. possesses mechanisms of resistance to all existing antibiotic classes, as well as a great propensity for developing mechanisms of drug resistance rapidly.[1] The bacteria produce naturally occurring AmpC β-lactamases, as well as naturally occurring oxacillinases (OXAs) with carbapenemase activity.[5] Resistance in *A. baumannii* has been traced to plasmids, transposons and the bacterial chromosome.[1] Carbapenemases that are found in the bacterium include those that belong to the class D family of serine-β-lactamases (OXAs) or the imipenemase (IMP)/ Verona integron-encoded (VIM) class B family of metallo-β-lactamases (MBLs).[6,7]

Carbapenem-hydrolysing class D genes are widespread over multiple continents, as opposed to the relatively rare class B MBL genes of *A. baumannii*.[8,9] Currently, class D carbapenemases are classified into four subgroups: the OXA-23 group (plasmid-encoded), the OXA-24 group (chromosomally encoded), OXA-51 (chromosomally encoded) and OXA-58 (plasmid-encoded).[10] Resistance to carbapenems is often associated with reduced drug uptake because of porin deficiency and reduced affinity for the drug owing to the modification of penicillin-binding proteins by mutations.[11,12] The biochemical activities of many of the OXA enzymes are yet to be characterised.[13]

In 2007, a study by Koh et al in Singapore showed that 91% of *A. baumannii* isolates were carbapenemase producers, carrying the bla_{OXA-23} gene.[13] Initially, the OXA-51-like enzymes were thought not to contribute to the antibiotic resistance of *A. baumannii* to β-lactams.[14] However, Turton et al found that in *A. baumannii* isolates with OXA-51 as the sole carbapenemase, carbapenem resistance was associated with an insertion sequence, IS*Aba*I.[15] It is suggested that this promotes the hyperproduction of β-lactamases.[15]

To date, six types of MBLs have been identified in *A. baumannii*.[7,16] These MBLs are IMP-like, VIM-like, Seoul imipenemase (SIM-1), Sao Paulo (SPM-1), German imipenemase (GIM-1), New Delhi (NDM-1) and NDM-2.[7,17-20] MBL enzymes require the presence of metal ions to be active.[16] Broadly, MBLs can be divided into those that are mobile and those that are normally carried by the bacterium on its chromosome.[21] Most MBLs are carried on a number of genetic elements that aid in the movement from one genetic apparatus to another.[21] Generally, MBLs have stronger carbapenem-hydrolysing activity than the OXA-type β-lactamases.[22]

The aim of this study was to evaluate and optimise multiplex polymerase chain reaction (M-PCR) assays for the rapid differentiation of the four subgroups of the OXA genes and the subgroups of the MBL genes of *A. baumannii*. The study did not include primers for the NDM-1 and NDM-2 genes. It is important for surveillance and epidemiological purposes to be aware of the circulating antibiotic resistance genes in important healthcare-associated pathogens such as *A. baumannii*. The knowledge of the genes is of paramount importance to understand their origin and can aid in the establishment of control measures.

Materials and methods

Bacterial isolates

A total of 97 consecutive *A. baumannii* isolates were collected from the Diagnostic Division of the Department of Medical Microbiology, National Health Laboratory Service, Pretoria, between February and April 2008. The *A. baumannii* isolates were collected and analysed without prior knowledge of patient information, hospital ward and specimen type. The isolates were analysed after routine diagnostic tests had been performed. Isolates were identified as *Acinetobacter baumannii* and underwent susceptibility testing using the Vitek® 2 Automated System (bioMérieux, France).

DNA extraction from Acinetobacter baumannii isolates

Automated whole cell DNA extraction was carried out using the MagNaPure LC Compact® (Roche, Germany), according to the manufacturer's protocol. The extraction was performed using 400 µl of overnight broth culture of *A. baumannii*. After extraction, the final elution volume was 100 µl of pure *A. baumannii* DNA. The extraction results were evaluated by performing DNA gel electrophoresis using Elite® 300 power pack (Wealtec, South Africa) on a 1% agarose gel (Whitehead Scientific, Brackenfell, Cape Town) that contained 0.5 µg/ml ethidium bromide (Promega, Madison, USA), using 1 µl of eluent to verify the presence of DNA in the extraction product. A ready-to-use 100 bp molecular weight marker was included as a reference for each gel. The extracted DNA was stored at -20°C until required for further analysis.

M-PCR assays for antibiotic resistance genes in Acinetobacter baumannii

Two M-PCRs were performed on each isolate. The first M-PCR assay (M-PCR I) was performed to screen for the presence of the OXA group genes (OXA-23, OXA-24, OXA-51 and OXA-58). The second M-PCR assay (M-PCR II) screened for the presence of the MBL genes (IMP, VIM, SIM, SPM and GIM).

The M-PCR assays were performed using the Qiagen® Multiplex PCR 1000 kit (Promega, Madison, USA) according to the manufacturer's instructions. A 25 µl final reaction mixture consisted of 12.5 µl Master Mix HotStart Taq® (Qiagen, Germany), 4 µl sterile deionised water (Promega, USA), 1 µl primer mix, 5 µl Q-Solution® (Qiagen, Germany) and 2.5 µl DNA template. The primers used in this study were described by Woodford et al[23] and Ellington et al[24] (Table I).

Multiplex polymerase chain reactions were performed using the Perkin Elmer GeneAmp System 9600® (Lab Centraal BV, Haarlem, The Netherlands). The amplification conditions included an initial activation step (HotStarTaq® DNA Polymerase) at 95°C for 15 minutes; 35 cycles of 94°C for 30

Table I: Nucleotide sequences of the primers used for the M-PCR I[23] and M-PCR II[24]

Primer*	Oligonucleotide sequence (5'-3')	Concentration (µM)	Amplicon size (bp)	Specificity
OXA-23F OXA-23-R	5'-GATCGGATTGGAGAACCAGA-3' 5'-ATTTCTGACCGCATTTCCAT-3'	2 µM	501 bp	OXA-23
OXA-24-F OXA-24-R	5'-GGTTAGTTGGCCCCCTTAAA-3' 5'-AGTTGAGCGAAAAGGGGATT-3'	2 µM	246 bp	OXA-24
OXA-51-F OXA-51-R	5'-TAATGCTTTGATCGGCCTTG-3' 5'-TGGATTGCACTTCATCTTGG-3'	2 µm	353 bp	OXA-51
OXA-58-F OXA-58-R	5'-AAGTATTGGGGCTTGTGCTG-3' 5'-CCCCTCTGCGCTCTACATAC-3'	2 µM	599 bp	OXA-58
IMP-F IMP-R	5'-GGAATAGAGTGGCTTAAYTCTC-3' 5'-CCAAACYACTASGTTATCT-3'	2 µM	188 bp	IMP
VIM-F VIM-R	5'-GATGGTGTTTGGTCGCATA-3' 5'-CGAATGCGCAGCACCAG-3'	2 µM	309 bp	VIM
GIM-F GIM-R	5'-TCGACACACCTTGGTCTGAA-3' 5'-AACTTCCAACTTTGCCATGC-3'	2 µM	271 bp	GIM-1
SPM-F SPM-R	5'-AAAATCTGGGTACGCAAACG-3' 5'-ACATTATCCGCTGGAACAGG-3'	2 µM	477 bp	SPM-1
SIM-F SIM-R	5'- TACAAGGGATTCGGCATCG-3' 5'-TAATGGCCTGTTCCCATGTG-3'	2 µM	570 bp	SIM-1

GIM: German imipenemase metallo-β-lactamases, IMP: imipenemase metallo-β-lactamases, OXA: oxacillinase, SIM: Seoul imipenemase metallo-β-lactamases, SPM: Sao Paulo metallo-β-lactamases, VIM: Verona integron-encoded metallo-β-lactamases

*: All primers were manufactured by Inqaba Biotech, South Africa

seconds, 60°C for 90 seconds and 72°C for 90 seconds; and a final extension step at 72°C for 10 minutes, as recommended by the manufacturer.

A 2% agarose gel which contained 0.5 µg/ml ethidium bromide was used to perform gel electrophoresis using Elite 300® power pack in order to screen for successfully amplified amplicons from the PCR reactions. The loading dye that was used was the Fermentas® 6X orange loading dye solution (Fermentas UAB, Lithuania). The ready-to-use 100 bp molecular weight marker (Promega, Madison, USA) was used as standard to determine the sizes of the bands that were obtained. The amplicons of the PCR reactions were visualised using an ultraviolet light box (UV Products, South Africa).

Results

There were a total of 20 antibiotics against which the susceptibilities were regularly tested. The nine antibiotics that were most commonly tested were amikacin, cefepime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, meropenem and piperacillin-tazobactam. The overall percentage of resistance to the tested antibiotics was amikacin (5%), cefepime (62%), ceftazidime (45%), ciprofloxacin (65%), colistin (0%), gentamicin (58%), imipenem (59%), meropenem (63%) and piperacillin-tazobactam (60%).

M-PCR I showed that 59% (58/97) of the *A. baumannii* isolates were positive for OXA-23, 83% (81/97) were positive for OXA-51 and 3% (3/97) were positive for OXA-58 (Figure 1). None of the isolates was positive for OXA-24.

All except one (96/97) of the isolates (isolate number 36) were negative for all MBL genes in M-PCR II. Isolate number 36 was positive for the VIM-like gene, as well as for OXA-23 and OXA-51.
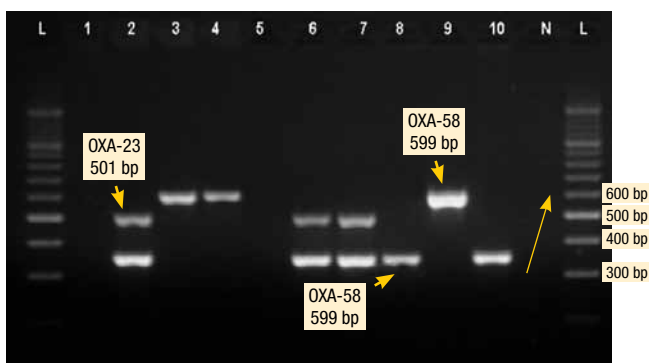
M-PCR I simultaneously amplified OXA-23 (501 bp), OXA-51 (353 bp) and OXA-58 (599 bp). Lanes 1 and 5 were negative for all of the OXA genes. Lanes 2, 6 and 7 were positive for OXA-23 and OXA-51. Lanes 3, 4 and 9 were positive for OXA-58 only. Lanes 8 and 10 were positive for OXA-51 only. Lane "N" was the negative control. Lanes marked "L" represented the O'Range Ruler™ 100 bp DNA Ladder

Figure 1: Results obtained after M-PCR I, using a 2% agarose gel for the detection of OXA genes in *A. baumannii* isolates

Discussion

The general resistance of the *A. baumannii* isolates varied from 45-65% for the tested antibiotics, except that of amikacin (5%) and colistin (no resistant isolates were detected in this study). In comparison to the report by Bamford et al, the overall resistance to various antibiotics of *A. baumannii* isolates from the Steve Biko Academic Hospital increased between 17% and 39% in the period 2008-2010.[25] Bamford et al reported overall resistance for each antibiotic as: 28% for amikacin (a 23% increase from 2008), 85% for cefepime (a 23% increase from 2008), 84% for ceftazidime (a 39% increase from 2008), 65% for ciprofloxacin (no change from 2008), 79% for gentamicin (a 21% increase from 2008), 80% for imipenem (a 31% increase from 2008), 80% for meropenem (a 17% increase from 2008) and 95% for piperacillin-tazobactam (a 35% increase from 2008).[25] The differences in susceptibility are most likely because of the different sampling strategies that were employed by the two studies.

The results of M-PCR I suggested the presence of three resistant profile types of *A. baumannii* that were circulating in the Steve Biko Academic Hospital (previously the Pretoria Academic Hospital). The first type was positive for both OXA-23 and OXA-51 (58%). The second type was positive for OXA-51 alone (25%). The third type was positive for OXA-58 (3%). Two of the three isolates that were positive for OXA-58 were also positive for OXA-23.

Since 1985, the OXA-23 genes have been reported and have been described in the UK, East Asia and South America.[9] In this study, the prevalence of OXA-23 in clinical isolates of *A. baumannii* was 59% (58/97). This was lower than the results that were obtained in a study by Mendes et al in 2008.[26] These researchers found that the prevalence of OXA-23 was 66.5% in the Asia-Pacific nations (India, China, Thailand, Korea, Hong Kong and Singapore).[26] In 2008, a study conducted by Feizabadi et al in Iran reported a prevalence of 36.5% for OXA-23 genes in clinical isolates of *A. baumannii*.[27] In contrast to these studies, Zhou et al[28] and Wang et al[29] reported OXA-23 to be present in 94% and 97% of imipenem-resistant *A. baumannii* from Chinese hospitals. In the work published by Merkier and Centrón in 2006, OXA-23 was detected in 3% (6/194) of isolates in Argentina between 1982 and 2005.[30] It is clear from the aforementioned results that the worldwide prevalence of OXA-23 genes in clinical isolates of *A. baumannii* varies by region.

Eighty-three per cent (81/97) of the clinical isolates of *A. baumannii* tested positive for OXA-51. This is lower than the findings of similar studies as the OXA-51 gene can be used as a marker for the *A. baumannii* species.[31] The isolates without an OXA-51 gene might have been misidentified as *A. baumannii*, but this was not followed-up. This highlights the need to confirm the identity of *A. baumannii* isolates as true *A. baumannii* isolates, and not merely *A. baumannii-A. calcoaceticus* complex isolates.

The OXA-58 gene has been reported in isolates of *A. baumannii* scattered throughout Argentina, Australia, Europe, Kuwait and the UK.[32] In this study, the prevalence of the OXA-58 gene in clinical isolates of *A. baumannii* was found to be 3% (3/97). This is lower than the prevalence found in a study conducted by Feizabadi et al where 15% of clinical isolates of *A. baumannii* tested positive for OXA-58 genes.[27] In a study conducted by Mendes et al in China, Thailand and India in 2008, 12% of *A. baumannii* isolates were found to be OXA-58 positive.[26]

The OXA-24 genes were first described in 1996. Strains harbouring this gene have been known to cause epidemic outbreaks in Spain and Portugal.[9] In this study, no OXA-24 genes were detected in any of the clinical isolates of *A. baumannii*.

The plasmid- or chromosomally encoded OXA-23 cluster is distributed in Europe, the USA, Australia, China, Korea, Vietnam, Singapore, Brazil, Pakistan and Noumea, while the OXA-24 (mostly chromosomally encoded, except for OXA-40 which is plasmid-encoded) cluster is distributed in the USA and the European countries of Belgium, France, Portugal and Spain.[33] The OXA-51 cluster is chromosomally encoded, is used as a marker to identify *A. baumannii* and is distributed worldwide, while the OXA-58 cluster is plasmid- or chromosomally encoded and has been reported in the UK, Australia, the USA, Argentina, Pakistan, Kuwait and some European countries (Austria, Belgium, France, Greece, Italy, Romania, Spain and Turkey).[33]

However, it is important to note that in a recent publication by Chen et al, 49.6% (58/117) of *A. baumannii* isolates that were obtained from 10 Taiwanese hospitals contained the OXA-51-like gene on a plasmid which conferred increased rates of resistance to imipenem and meropenem.[34] This phenomenon can be explained by the increased gene expression because of the higher copy number on the plasmids.[34]

During M-PCR II, one of the isolates (isolate number 36) tested positive for the VIM-like gene, as well as for OXA-23 and OXA-51, indicating that this was a highly drug-resistant isolate of *A. baumannii.* Antimicrobial susceptibility testing with the Vitek 2 Automated System® confirmed that isolate number 36 was resistant to gentamicin, ceftazidine, piperacillin-tazobactam, ciprofloxacin, imipenem, cefepime and meropenem. This isolate was found to be sensitive to colistin and amikacin only. This was the only isolate that was detected in this study that harboured the VIM-like gene which corresponds with the highly resistant nature of this *A. baumannii* isolate. In 2008, Nordmann and Poirel stated that the detection of VIM enzymes in *A. baumannii* was not a common event.[35] The first-ever detection of the VIM-1 gene in *A. baumannii* was reported by Tsakris et al in 2006.[36]

Generally, the prevalence of the MBL genes is low within isolates of *A. baumannii*, as illustrated in a study by Mendes et

al, who found the prevalence to be 0.8% (2/188) in Taiwan.[26] In 2003, research by Oh et al found that there was a 13% prevalence of MBLs in isolates of *A. baumannii*.[37] These isolates were found to have decreased susceptibility to imipenem and ceftazidime.[37]

However, in 2005, a study by Altoparlak et al found that 33% of imipenem-resistant isolates of *A. baumannii* from a burn wound unit in a hospital in Turkey were MBL producers.[38] In 2011, Peymani et al reported the phenotypic detection of MBL producers in 49% (31/63) of *A. baumannii* isolates in a tertiary hospital in Iran using the MBL E-test.[39] The VIM gene was detected in 29% (9/31) and the IMP gene in 61% (19/31) of the MBL-producing isolates, highlighting the increase in MBL genes in Iran.[39]

In this study, five classes of MBL genes were screened for, of which VIM, previously found in China, and IMP, primarily detected in South Korea, are detected most frequently in isolates of *A. baumannii*.[9] In the case of MBL producers, there is high-level resistance to carbapenems and other β-lactams, except aztreonam, and often also associated resistance to aminoglycosides and fluoroquinolones.[35] This has a serious impact on the drugs that are available for treatment, limiting treatment to the polymyxins.[35] OXA-producing isolates, such as the OXA-51-producing isolates, hydrolyse carbapenems weakly, and are not active against extended-spectrum cephalosporins.[40]

There were 13 isolates in which none of the MBL or OXA genes were detected. Upon investigation of these isolates, it was found that nine were still multidrug-resistant *A. baumannii* and four were susceptible to all other tested antibiotics. This was a disturbing finding in that it showed that there are mechanisms of conferring resistance in *A. baumannii* other than the OXA and MBL genes. Such mechanisms may include diminished permeability of the cell membrane (3% as permeable as *Escherichia coli*), as described in a study by Quale et al.[41] No further investigation into possible misidentification or determination of the antibiotic resistance mechanisms was conducted on these isolates. Thus, the possibility that the isolates were not true *A. baumannii* isolates cannot be excluded.

As the genetic relatedness of the *A. baumannii* isolates was not determined, the high prevalence of the OXA-23 and OXA-51 genes could be because of the spread of a single clone. This highlights the importance of infection control to prevent the spread of this highly resistant healthcare-associated pathogen. Another limitation of the study was the possible inclusion of more than one isolate per patient which limited the study to laboratory-based surveillance, rather than truly representing the prevalence rates of these carbapenemase genes. Unfortunately, the minimum inhibitory concentration (MIC) values for each of the different antibiotics of the studied isolates were not obtained. This was an additional limitation as no comparison could be made of the genes and the MIC values.

Conclusion

On completion of this study, it is evident that the OXA group genes were prevalent in clinical isolates of *A. baumannii* from the Steve Biko Academic Hospital in Pretoria. The prevalence rates of 59% and 83% for OXA-23 and OXA-51 respectively, were similar to the globally reported prevalence of OXA genes. MBL genes were not prevalent in the clinical isolates of *A. baumannii* that were surveyed in this study. Continuous research and surveillance is necessary to monitor the prevalence and spread of antibiotic-resistance genes that are associated with *A. baumannii* in clinical settings. Future research should include confirmation of the identity of all *A. baumannii* isolates, possibly through detection of the OXA-51 gene, determination of the genetic relatedness of circulating *A. baumannii* isolates (to establish whether infections are caused by single or multiple clones), as well as comparisons of phenotypic and molecular antibiotic resistance patterns.

Conflict of interest

None of the authors has any commercial or other association that may pose a conflict of interest with this study.

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