Detection of carbapenemases, AmpC and ESBL genes in *Acinetobacter* isolates from ICUs by DNA microarray

Fakhur Uddin [1], Timothy D McHugh[2], Kerry Roulston[2], Gareth Platt[2], Taseer Ahmed Khan[3] and Muhammad Sohail[1]*

[1]Department of Microbiology, University of Karachi, Karachi-75270, Pakistan, [2]Centre for Clinical Microbiology, University College London, UK, [3]Department of Physiology, University of Karachi, Karachi-75270, Pakistan

*Author for all correspondence: msohail@uok.edu.pk

Abstract

The accumulation of multiple inherent and acquired resistance mechanisms in *Acinetobacter* sp. results in emergence of "pandrug resistant" strains which is one of the major concerns in healthcare sectors worldwide. Surveillance of the carbapenemase/ extended-spectrum β-lactamases (ESBLs) genes in *A. baumannnii* by phenotypic methods is challenging especially in developing countries, like Pakistan. In this context, a novel microarray (CT 103XL Check-MDR) assay was used for simultaneous detection of genes encoding clinically important carbapenemases and ESBLs. The results were compared with the phenotypic methods including MHT, Rapidec Carba NP, EDTA+DDST and Rosco (KPC/MBL). The results of the microarray were also confirmed by PCR. All of the strains of *A. baumannii* (47) were resistant to imipenem and meropenem, while the other species of *Acinetobacter* were sensitive. Microarray and PCR results showed presence of OXA-23 in all the isolates of *A. baumannii* while 36.17% also harbored PER. Rosco kit test showed 100% sensitivity to detect carbapenemases but exhibited low specificity to classify them. Rapidec Carba NP test has 100% sensitivity and specificity to detect the carbapenemases when compared with microarray. Sensitivity and specificity of microarray assay were 100% for *bla*-genes in comparison to PCR. This reveals that Check-MDR CT103 XL assay is an accurate method for the identification of ESBLs and carbapenemase genes in *A. baumannii* in comparison to the other methods.

Key words: *A. baumannii*, Carbapenemase, ESBL, MHT, Microarray, OXA-23, Rosco,

1. Introduction:

Prevalence of extended-spectrum β-lactamases (ESBLs), cephalosporinase (AmpCs) and carbapenemases producing Gram-negative bacilli (GNB) is rapidly increasing and limiting the therapeutic options (Bush and Jacoby, 2010; Livermore, 2009). These β-lactamases are the common mode of resistance to β-lactam antibiotics (Penicillins, Cephalosporins and Carbapenems). The genes encoding these resistance determinants along with some other genes are also responsible for resistance to non-β-lactam antimicrobials (Aminoglycosides, Quinolones, etc). The coexistence of resistance determinants makes a pathogen multidrug resistant (MDR) strain. Although, *Acinetobacter* sp. is not common in the community acquired infections (CAIs), but can cause life threating infections of lower respiratory tract, blood stream, urinary system and skin (Dally et al., 2013). The MDR and carbapenem-resistant *A. baumannii* (CRAB) strains are prevalent in intensive care units (ICUs) and hence can cause healthcare-associated infections (HAIs) with higher rates of mortality and morbidity. Moreover, due to its ability to avidly acquire transferable genetic resistance elements in addition to intrinsic resistance mechanism, pandrug resistant (PDR) *A. baumannii* strains have also been reported (Doi et al., 2009). A variety of acquired β-lactamases, carbapenemases and other resistance determinants are identified in *A. baumannii* (Peleg et al., 2008). Non-availability of safe and effective therapeutic agents against PDR *A. baumannii* (Talbot et al., 2006) renders the situation grave. Consequently, *A. baumannii* has been included in the most common and serious MDR pathogens group, ESKAPE, i.e. *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* sp. (Russo et al., 2009).

Clinical microbiology laboratories rely upon cost-effective, rapid, accurate and sensitive protocols to detect and characterize β-lactamases in resistant microbes. Most laboratories focus on cost-effective phenotypic assays, including selective indicator based media, antimicrobial susceptibility testing (AST) systems with detection of MICs, followed by employing confirmatory tests for enzyme expression such as modified Hodge test (MHT) and Carba NP test (Cunningham et al., 2016). Accumulation of huge genetic resistance elements and masking effects of some of the genes have made the interpretation of the results complicated, consequently false results are reported. This can deceive to provide appropriate selection of antimicrobial agent and results in treatment failure (Braun et al., 2014). Standard PCR and gene sequencing are the gold

standard for identification of resistance determinants, but their accuracy remains uncertain for simultaneous detection of more than one gene of a resistance determinant (Naas et al., 2012).

DNA microarray assay is capable of detecting a variety of β-lactamase encoding (*bla*) genes simultaneously in relatively short period of time. Several microarray based methods for detection of resistance markers in different bacterial species are available (Dally et al., 2013). Studies employing DNA based microarray assays which target a number of β-lactamase encoding genes suggest that such platforms may be used to detect resistance genes for MDR strains of *A. baumannii,* as well (Cunningham et al., 2016; Bogaerts et al., 2012). In this study, a novel array Check-Points carbapenemases, ESBLs and AmpCs Microarray (Check-MDR CT103XL; Check-Points B.V., Wageningen, the Netherlands) was used to detect all the previously targeted genes, including ESBLs, AmpCs and carbapenemases, in a single reaction vial. This test detected the genes for carbapenemases (GES-2,4-6,13-15, 17, 18, 20, 21, GIM-1, IMP-1, 3, 8, 10, 13, 19, 20, 24-26, 30, KPC-1-17, NDM-1-10, OXA-23like- 23, 27, 49, 73, 146, 165-171, 225, 239, OXA-24like-24-26, 33, 40, 72, 139, 207, OXA-48like, 58, 96, 164, SPM-1, VIM-1-6, 8-38), CTX-M (CTX-M-1 group, CTX-M-I like, CTX-M-3like, CTX-M-2 group, CTX-M-8-&-25 group, CTX-M-9 group, CTX-15like, CTX-32-like)TEM ESBLs genes (TEMwt, 104k, R164S, R164C, R164H, & TEM G238S), SHV ESBLs (SHV wt, G238S, G238A, & E240K), Other ESBLs (VEB-1-8,PER-1, 2- 6,BEL-1-3;GES-1, 3, 7-12, 17, 19, 22) and AmpCs variants (CMY I/MOX, ACC, DHA, ACT/MIR, CMY II, FOX). Beside this assay we have used the phenotypic methods, double disc diffusion method for ESBL (Clavulanic Acid) and MHT, Rapedic CARB NP for detection of carbapenemases, inhibitor based disc diffusion method for MBL, KPC, and OXA carbapenemases (Rosco Kit/ KPC/MBL), MBLs (EDTA+ double disc diffusion test), and in-house PCR.

2. Materials and Methods

2.1 Place and Period of the Study

This study was conducted in the Department of Microbiology, Basic Medical Sciences Institute (BMSI), Jinnah Postgraduate Medical Centre (JPMC), Karachi, in collaboration with the Surgical, Medical Units of Jinnah postgraduate Medical Centre Karachi (a tertiary care hospital with more than 1500 beds) and Centre for Clinical Microbiology, UCL Royal Free Hospital

London, UK. The clinical isolates were collected during March, 2014 to February, 2015 from JPMC.

2.2 Sample size and nature

A total of 47 *Acinetobacter baumannii* were collected from various clinical specimens including tracheal aspirates, urine, blood, pus and stool were collected. The single isolate per patient was included, repeated samples from same patient were not considered.

2.3 Identification of bacteria

Isolates were initially identified by Microscopic and cultural characteristics and further analyzed by API 20NE (Biomeriux, France). The identification of *A. baumannii* was confirmed by Matrix-Assisted Laser Desorption Ionization–Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) using a MALDI BrukerMicrolex^{TM} with Maldibiotyper^{TM} software (Microflex, BrukerDaltonics). Antimicrobial sensitivity of the isolates was evaluated by disc diffusion test. Minimum inhibitory concentration (MIC) for Carbapenems (ERT, IMP and MEM) and Colistin (CT) was determined with Etest (BioMerieux, Lyon, France). CT MIC was also performed by the microbroth dilution. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2016).

2.4 Phenotypic Detection of ESBLs

2.4.1 Double Disc Synergy Test

The isolates were screened for the ability to resist multiple drugs by adopting a modified double disc synergy test. The antibiotic discs of Ceftazidime (30µg), Ceftriaxone (30µg), Cefepime (30µg), Aztreonam (30µg) and Amoxicillin/clavulanic acid (20/10 µg) were used as described previously (Litake et al., 2015).

2.4.2 Phenotypic Detection of Carbapenemases and their classes

Carbapenemases were detected by four phenotypic methods, modified Hodge test, Rapidec Carba NP (Biomeriux, France), EDTA double disc synergy test (DDST) and KPC/MBL (Rosco) using *P. aeruginosa*/*Acinetobacter* kit. KPC/MBL test was performed and interpreted in accordance to the manufacturer's instructions with slight modifications as MacConkey agar was replaced with CLED agar. The EDTA+IMP/MEM DDST were used in accordance with the method described earlier (Song et al., 2015).

2.5 DNA Extraction

Automated DiaSorin LIAISON® Ixt (DiaSorinS.p.A. - Share Capital € 55.948.257 R.E.A. 180729) was used to extract genomic DNA from single colony of *A. baumannii* by following the manufacturer's instructions for Gram-negative bacteria.

2.6 DNA Microarray

The presence of clinically relevant carbapenemases, ESBLs, CTX-M and AmpCs-encoding genes was detected by Check-MDR CT103XL microarray kit (15LTN0384) designed by Check-Points Health BV (Wageningen, Netherlands).

2.7 Detection of β-lactamase genes by PCR

The results of microarray were confirmed by manual PCR. The primers *blaVIM* F-GGTGTTTGGTCGCATATCGC, R-CCATTCAGCCAGATCGGCATC; *blaIMP* F-GGAATAGAGTGGCTTAATTC, R-CAACCAGTTTTGCCTTACC; *blaNDM*, F-CACCTCATGTTTGAATTCGCC, R-CTCTGTCACATCGAAATCGC; and *blaOXA-23like* genes F-AAGCATGATGAGCGCAAAG, R-AAAAGGCCCATTTATCTCAAA for carbapenemases genes and *blaVEB* F-GTTAGCGGTAATTTAACCAGATAG, R-CGGTTTGGGCTATGGGCAG; and *blaPER* F-GCTCCGATAATGAAAGCGT, R-TTCGGCTTGACTCGGCTGA for ESBLs genes were used as previously described (Wolter et al., 2009; Kaase et al., 2011; Dallenne et al., 2010; Senkyrikova et al., 2013; Chen et al., 2010; Jiang et al., 2005). The PCR mixture (20µl) containing MasterMix (Qiagen) HotStarTaq Plus DNA Polymerase (10µl), forward primer (0.5µl), reverse primer (0.5µl), DNA sample template (2µl), loading dye (2µl) was prepared and programmed as initial activation at 95^{o}C for 15 min (1 cycle); followed by 35 cycles for denaturation of DNA template at 94^{o}C for 30 sec; annealing temperature was 50-65^{o}C for 30 sec; the primer extension at 72^{o}C for 1 min and final primer extension at 72^{o}C for 10 min. The product was observed by agarose gel and identified by gel scanning system (G-BOX, SYNGENE).

2.8 Statistical analysis

The data was initially punched on Microsoft excel and imported to the SPSS version 16. The frequencies were analyzed and represented in percentages. Diagnostic test evaluation for

sensitivity and specificity was calculated by statistical calculator (https://www.medcalc.org/calc/diagnostic_test.php)

3. Results

All the isolates of *A. baumannii* were obtained from indoor patients. Most of these patients were neonates. Tracheal aspirate was the most common sample (28/47) for the isolation of *A. baumannii* followed by blood (17/47) and urine (2/47).

The resistance pattern against commonly used antibiotics in *A. baumannii* is presented in Table 1. *A. baumannii* isolates were 100% resistance to all β-lactam antibiotics except for ampicillin/sulbactam, whereas, the resistance to aminoglycosides and ciprofloxacin was 91% and 95%, respectively. The resistance to tetracycline was lower (36%) in comparison to the other antibiotics, while colistin was found as the most effective drug as none of the isolates of *A. baumannii* showed resistance to it.

Among various methods employed to screen antibiotic resistance determinants in *A. baumannii*, the double disc diffusion method did not give any positive result for ESBLs. All the strains of *A. baumannii* were found to be positive for carbapenemases by Rapidec Carba NP. The positive and negative predictive value of Rapidec Carba NP was 100% for detection of OXA-23 carbapenemase in *A. baumannii*; the sensitivity and specificity of this method were also 100%. With 87.23% sensitivity, EDTA+IMP DDST method detected MBLs in 6 cases (12.76%). The results of Rosco kit indicated presence of MBL in 37 isolates among which 10 were OXA producers; none of the isolates was found AmpCs producer. The sensitivity and specificity of Rosco for carbapenemases detection was 100%, whereas, sensitivity to differentiate OXA-carbapenemases was 21.28%. Although, the MHT showed poor sensitivity, its specificity was 100% for the detection of carbapenemases.

The results of microarray (CT 103XL Check-MDR) revealed that OXA-23 was the only cause (47/47) of carbapenem resistance in *A. baumannii*; KPC, NDM, VIM, IMP, OXA-48-like, GES, GIM, SPM, OXA-24-like and OXA-58-like were not detected. Moreover, all the isolates were negative for the CTX-M groups and AmpCs variants. No variant of VEB and SHV was detected, whereas genes for PER (17/47), TEMwt (2/47) and GES (1/47) were present (Table 2).

The conventional PCR analyses showed that all the carbapenem resistant *A. baumannii* strains harbored bla_{OXA-23} gene but were negative for bla_{VIM}, bla_{IMP}, and bla_{NDM}, genes. The most prevalent ESBL in these bugs was PER 36.17% (17/47). The product size of amplified PER and OXA-23 genes from carbapenem resistant *A. baumannii* was 520 (Fig. 1A) and 1066 bp (Fig.1B) respectively.

4. Discussion

Rapid molecular identification of carbapenemase genes in Gram-negative bacteria and exploring prevalence of such genes at local health care setting is the fundamental step for infection control and prevention. Moreover, such findings will help in making a policy for prophylactic treatment of critically ill patients. Phenotypic tests for the detection of carbapenemases, ESBLs, CTX-M and AmpCs are not well established and their sensitivity and specificity are compromised especially for *A. baumannii* (Braun et al., 2014). The poor detection of ESBLs by phenotypic method can be attributed to the masking effect due to the production of carbapenemases (over expression of blaOXA-51 and AmpC) by these isolates. The most prevalent ESBL genotype in the given setting was bla_{PER} i.e. 36.17% in CRAB that lies in between the global prevalence from 46% in Turkey to 54.6% in South Korea (Farajniaet al., 2013).

Data available from Pakistani health care centers regarding prevalence of PDR *A. baumannii* and their resistance mechanisms to β-lactam antibiotics is limited as diagnostic laboratories mainly rely on phenotypic detection with poor sensitivity and specificity. Considering the limitations of phenotypic methods, the study was conducted to detect carbapenemases in *A. baumannii* by using a DNA based microarray assay (CT 103XL, Check-MDR). The method allowed rapid and simultaneous detection of prevalent and important *bla* genes for carbapenemases, ESBLs, CTX-M and AmpCs in *Acinetobacter*. Thus, the assay bears an edge over multiplex PCR and other techniques to detect several *bla* genes in a single reaction (Cuzon et al., 2012). The method is reliable, accurate with good sensitivity and specificity for the detection and identification of various β-lactamses in Gram-negative bacilli (Braun et al., 2014).

OXA-23 producing *A. baumannii* has been reported frequently in many countries including China and South Korea (Jean and Hsueh, 2011), suggesting a worldwide dissemination (Fu et al., 2010) of these markers. In present study OXA-23 carbapenemase was detected in all CRAB isolates by microarray and PCR, indicating that the most common mode of resistance to

carbapenems in our region is OXA-23. Similar results have previously been documented from Pakistan (Irfan et al., 2011), showing endemic nature of OXA-23.

The results obtained by Rosco kit and DDST+EDTA did not comply with genotypic results; the false positive MBLs may be appeared due to the effect of EDTA on cell wall of *A. baumannii* as described earlier (Boran et al., 2015; Aksoy et al., 2015) and chelators (EDTA or MPA) may inhibit the dimers formation by OXA-23 (Martins et al., 2013). The results of double disc inhibitor (EDTA+IMP) based test have higher specificity (87.23%) than combined disc methods to differentiate OXA from MBLs. Similar findings were reported by Hansen et al (2013).

The MHT had poor sensitivity and specificity to detect the carbapenemases in *A. baumannii* because the OXA-type producer shows weak synergistic images when there is low production of carbapenemases (Bonnin et al., 2012; Indhar et al., 2017). The reported low rates of carbapenemase activity are challenging with low sensitivity and interpretation of its results can be subjective for the identification of the clover-leaf indentation (Kateete et al., 2016). In comparison to MHT, Rapidec Carba NP had better sensitivity and specificity to detect the carbapenemases in *A. baumannii*. Furthermore, the later is substantially rapid (2h) compared to the former (18-24h). These findings are in agreement with previous study from Pakistan (Indhar et al., 2017). However, it suffers with the disadvantage that it cannot differentiate between classes of carbapenemases which is possible with Rosco kit test. Rosco kit test showed a sensitivity of 100%, but it failed to differentiate between various classes of carbapenemases in *A. baumannii*. Furthermore, modifications and revised interpretations for inhibitor-based tests (combined disc methods) are required for confirmation of MBL-production in *A. baumannii*.

In this study, phenotypic screening tests showed the production of metallo-β-lactamases, which could not be confirmed by the genotypic techniques (PCR and microarray CT103XL). The data obtained from microarray and PCR of each strain was correlated with its phenotype that was challenging task as a distinct correlation was difficult to be established when several resistance encoding genes (with masking effect) were detected in a single isolate (Poulou et al., 2014). The masking effect of OXA-23 was evidently exhibited by the strains that carried *bla*PER resistance determinant; therefore, double disc diffusion method could not detect the presence of both the markers. False-negative results are regarded as more critical than a false-positive result due to therapeutic failure. The results of CT 103 XL microarray showed the high accuracy for the

detection of β-lactamases in *A. baumannii*. The similar findings have been reported by Cunningham et al (2016) and Juiz et al (2014) from microarray technique for characterization of resistance mechanism in MDR Enterobacteriaceae (MDRE) and *Pseudomonas aeruginosa*. Despite of its higher cost microarray based method provides more information regarding the resistance mechanisms in comparison to phenotypic methods, therefore, it should be included in the studies detecting antimicrobial resistance, particularly for MBL.

5. Conclusion

It is concluded that CT103XL Check-MDR is an accurate test that can simultaneously detect multiple targets for antimicrobial resistance in carbapenem-resistant *A. baumannii* (CRAB). The phenotypic methods such MHT have low sensitivity for screening of the carbapenemases and ESBLs detection in CRAB. OXA-23 and PER are the most common mechanisms of resistance against β-lactam antibiotics.

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Table 1 Antimicrobial resistance pattern of *A. baumannii* isolated in this study (n=47)

Antibiotics	Total No. (%)
PIP	47 (100)
PZT	47(100)
SAM	41 (87.23)
CRO	47(100)
CAZ	47(100)
FEP	47(100)
IMP	47(100)
MEM	47(100)
TOB	44 (93.61)
CN	43(91.48)
AK	42(89.36)
CIP	45(95.74)
TE	17(36.17)
SXT	38(80.85)
CT*	00 (00)

*CT, was performed by the Etest (BioMerieux, Lyon, France) and microbroth dilution method.

Table 2. Frequency of ESBL and carbapenemase types in *A. baumannii* detected by Rosco kit, modified Hodge test and microarray (CT 103XL, Check-MDR, n=47)

Tests	CPs* Positive (%)	MBL	OXA	OXA-23like	ESBLs TEM	ESBLs PER	ESBLs GES	Total (%)
ROSCO phenotypic test	47 (100)	37(78.73)	10(21.27)	-	-	-	-	47 (100)
EDTA+IMP	6 (12.76)							
Modified Hodge Test	6 (12.76)	-	-	-	-	-	-	47 (100)
Rapedic Carba NP Test	47 (100)	-	-	-	-	-	-	47 (100)
Microarray (CT 103XL, Check-MDR)	47 (100)	00	47 (100)	47 (100)	2 wt (4.25)	17(36.17)	01(2.12)	

***CPs, Carbapenemases**

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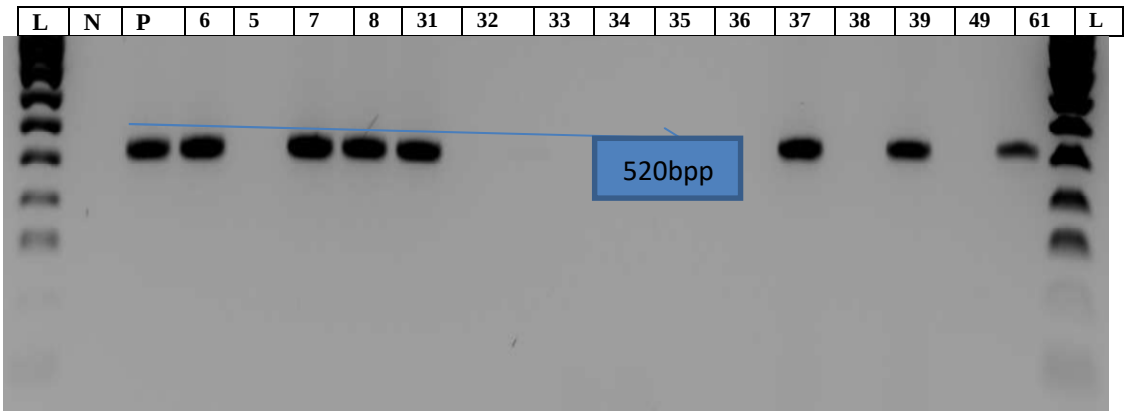

Figure. 1A PCR amplification of *bla*$_{PER}$ gene (520 bp) *A. baumannii* isolates. L, DNA ladder (100 bp), N, negative control, P, positive control, the sample 6, 7, 8, 31, 37, 39, 61 positive, while 5, 32, 33, 34, 35, 36, 38 and 49 samples were negative

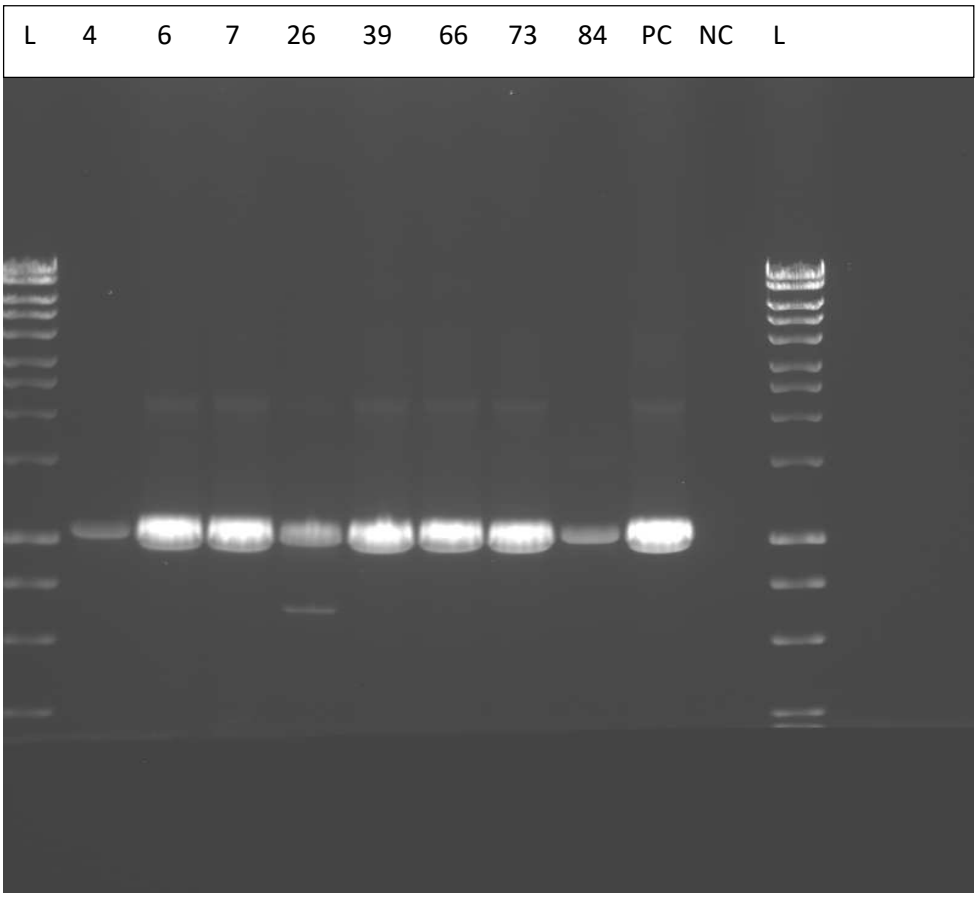

Figure. 1B Arms PCR gel image of *bla*$_{OXA\text{-}23like}$ has expected size of **(**1066 bp). L, DNA Ladder (1kb), NC, Negative control, PC Positive Control, samples # 4, 6, 7, 26, 39, 66, 73, and 84 of *A. baumannii*