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Molecular Analysis of Oxacillinase Genes in MDR strain of *Acinetobacter baumannii* and Comparative evaluation of Vitek 2 and Broth micro dilution method for Colistin Susceptibility testing isolated in clinical sample from North India

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ABSTRACT

Background: Performances of the Colistin antimicrobial susceptibility testing (AST) systems of *Acinetobacter baumannii* vary depending on the manufacturer, and data on Colistin-resistant *A. baumannii* are limited. We evaluated the VITEK2 and Sensititre systems to determine Colistin resistance and minimum inhibitory concentration (MIC) for *A. baumannii* isolated from a clinical microbiology laboratory. A total of 88 clinical *A. baumannii* isolates were tested, including 14 Colistin-resistant *A. baumannii*. ASTs were performed using the VITEK2 according to the manufacturer's instructions. Reference MICs for Colistin were determined using the manual broth micro dilution method (BMD). The results of AST methods were compared with the BMD results.

Material Methods: Clinical samples received for bacterial culture and antimicrobial sensitivity testing were inoculated and incubated for 24-48 hours at 37°C. For blood culture incubation was done for 5 days in blood culture bottle. Bacterial identification and antimicrobial sensitivity were done by Vitek®2, bioMérieux. Antimicrobial Sensitivity of *A. baumannii* isolated was recorded and multi drug resistant isolates were preserved for Polymerase chain reaction (PCR) to detect the presence offbeat lactam Oxacillinase blaOXA 51 and blaOXA58 genes.

Result: In total 2644 samples were processed during the study period from 30th April, 2022 to 20th October, 2024. 920 samples were culture positive. Out of 920 bacterial isolates, 88 were *A. baumannii* and 84 out of these were Carbapenem Resistant *A. baumannii* (CRAB). 54 CRAB isolates were for PCR and all showed presence of blaOXA 51 genes while none of the isolates harbored blaOXA 58 genes.

Conclusion: There is an increased isolation rate of *A. baumannii* from clinical samples and more than 95% isolates of *A. baumannii* are CRAB. All the CRAB isolates tested by PCR showed presence of blaOXA 51 genes and none had the presence of blaOXA 58 genes. Management of *A. baumannii* infections is a challenge. In the current scenario proper implementation of Hospital Infection Control policies and adherence to antimicrobial stewardship policies as preventive strategies should be our priority.This study highlights that the high frequency of isolation of Carbapenem-resistant Acinetobacter baumannii strain in high-risk infectious wards requires an accurate application of methods for detecting susceptibility to antibiotics, in particular to Colistin, so as the correct therapeutic approach. As the frequency of resistance Colistin is low, it can be used as an easily available drug for Carbapenem resistance Acinetobacter baumannii strains which are susceptible to Colistin.

Keywords: *Acinetobacter baumannii, Colistin, Antimicrobial Susceptibility Test, Broth Micro Dilution, Hospital-Acquired Pneumonia*

1. INTRODUCTION

Acinetobacter baumannii are Gram-negative coccobacilli involved in the etiology of nosocomial infections and manifesting an important antibiotic resistance. A. baumannii is the cause of a wide range of clinical manifestations, including hospital-acquired pneumonia (HAP), followed by urinary tract infections (UTIs), bloodstream infections (BSIs), and surgical site

infections (SSIs), which occur preferentially in patients admitted to intensive care units (ICUs), where they have five to ten times higher risk of acquiring hospital infections [1) Several predisposing factors have been identified, including previous antibiotic therapy, which can alter normal bacterial flora and select resistant strains. Furthermore, cross transmission among patients and the hospital environment are the most likely sources of infection. (2) A number of A. baumannii isolates are highly resistant to most of the clinically available antibiotics [3]. Carbapenems have been used for decades as the antibiotics of choice in the clinical management of the infections caused by this organism. Yet, resistance to carbapenems emerged, leaving very few therapeutic options [4) Class D (OXA-type) carbapenemases are the main enzymes responsible for this resistance. The OXA carbapenemases found in A. baumannii complex species belong to four subgroups: OXA-23-like, OXA-24-like, OXA-51-like, and OXA-58. The OXA-51-like subgroup is intrinsic to A. baumannii, occurring in most or all of the strains (5) recently, Colistin has become one of the major therapeutic options in the management of Carbapenem-resistant A. baumannii infections. However, Colistin resistance has rapidly emerged in A. baumannii after reintroduction of this drug into the clinical practice(4) To complicate the picture, observations have recently been made about the congruity of the results of the susceptibility profiles and the values of the minimum inhibitory concentration (MIC) obtained by automated diagnostic devices and diffusion methods(6) These observations were assumed on the basis of the discontinuity of the diffusion of Colistin in agar, which often renders untrustworthy the reading of the inhibition halos and of all the interpretations related to the diffusion method (for example E-test® and various semiautomatic systems). As confirmation of this problem, a caveat for the interpretation of the susceptibility tests obtained through these techniques has been issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), with the indication to perform sensitivity tests using the micro dilution method, considered as a reference method [7-10]

One mechanism of Colistin resistance involves reduced Colistin binding to the bacterial outer membrane due to the charge modification of the lipopolysaccharide (LPS) moiety [13]. A second mechanism is the complete loss of lipid A, which also prevents the interaction of the antibiotic with the bacterial outer membrane [14) In this work, a collection of well-characterized, multidrug-resistant A. baumannii isolates obtained from clinical specimens of infected or colonized patients was used to compare the performances of both commercial and in-house prepared Colistin susceptibility tests. Substantial discrepancies between an automated testing method and both agar diffusion and in-house broth micro dilution methods were observed. Our findings highlight the need for confirmative Colistin susceptibility testing by the broth microdilution method, especially in the case of borderline MICs, which are increasingly frequent among multidrug resistant A. baumannii, so as to ensure a consistent therapeutic approach.

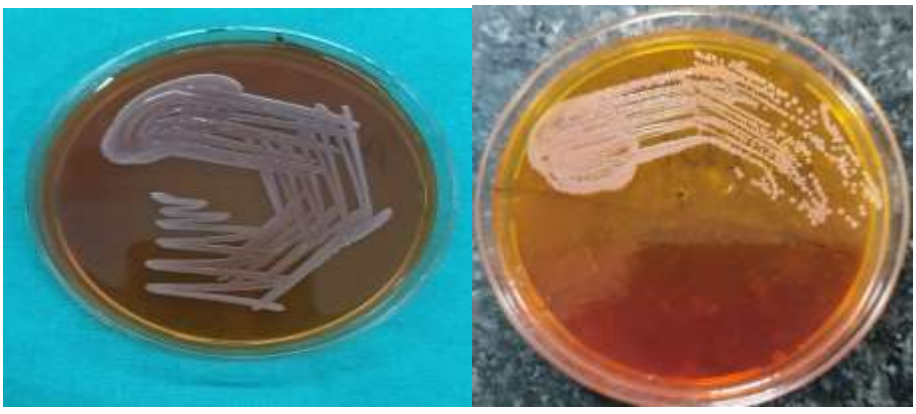
2. MATERIAL METHODS

Acinetobacter baumannii isolates were collected from a clinical microbiology laboratory of a Cadmus Clinical Laboratory. In total 2644 samples were processed; 920 samples were culture positive. Out of 920 samples 88 samples were Acinetobacter baumannii and out of 88 Acinetobacter baumannii 84 isolates were Carbapenems Resistant Acinetobacter.

Inclusion Criteria: The study included clinical specimen such as Blood, Pus, Sputum, Plural Fluid, Throat swab that yield Acinetobacter growth. Strain identified as Acinetobacter baumannii and resistant to two antibiotics Imipenem and Meropenem were included.

Exclusion Criteria: Samples that yield gram Positive bacteria, Pseudomonas and Enterobacterales and were excluded from the study.Additionally, strains susceptible two Carbapenem antibiotics Imipenem and Meropenem were also excluded.

Isolation and Identification of*Acinetobacter Baumannii:*The samples were cultured on agar media (blood agar and MacConkey Agar, bioMérieux,) and incubated for 24 h at 37 °C. The bacterial colonies were identified by a semiautomatic biochemical method (ID-GN cards, Vitek®2, bioMérieux)



(a) **(b)**

Figure 01:Colony characteristics of *A. baumannii on Culture media* (a) MacConkey Agar (b) 5% Sheep Blood Agar

Figure 02

Figure 02: A Bactec™ Blood Culture Bottle after positive growth. Bacteria produce Carbon dioxide as a byproduct of metabolism. The sensor at the base of the bottle changes color in presence this gas which in turn is detected by the BacT/Alert 3D machine.

Figure 03

Figure 03: Antimicrobial Sensitivity Card of Vitek®2 system. A semiautomatic diagnostic modality, it is based on the principle of colorimetric. The machine matches the color change in each of the 64 blocks on the card with the database and gives the sensitivity result in Minimum inhibitory concentration values.

The MIC of various antibiotics tested (Cefaperazone Sulbactam, Cefepime,Ceftazidime, Amiakcin,Piperacillin/tazobactam, Imipenem, Meropenem, Gentamicin, Ciprofloxacin, Levoflaxacin, Minocycline,and trimethoprim/sulfamethoxazole) was assayed by automated methods on isolated colonies of *A. baumannii*, resuspended in 0.45% NaCl solution at an inoculums concentration of 0.50 McFarland (MF). In the first instance, antimicrobial susceptibility profiles were tested using the Vitek®2 system (AST N-406 card, bioMérieux) according to the manufacturer's instructions. Reference MICs for Colistin were determined using manual BMD according to CLSI guidelines. The BMD MIC test range was 0.25 µg/mL to 256 µg/mL. Susceptibility results for Colistin were interpreted according to the CLSI guidelines as follows: ≤2 µg/mL indicated susceptibility, and ≥4 µg/mL indicated resistance (13) For BMD and Sensititre quality control, *Escherichia coli* ATCC 25922 (MIC 0.25–2 µg/mL) and *Pseudomonas aeruginosa* ATCC 27853 (MIC 0.5–4 µg/mL) were tested according to CLSI guidelines and manufacturer's instructions [13].

Determination of Colistin by Broth Dilution Method (BDM):

The Minimum inhibitory concentration (MIC) of Colistin against Acinetobacter baumannii was determined using the broth micro dilution method dilution according to clinical and laboratory standard institute (CLSI) guidelines (CLSI2024).Initially, 96-well assay plates were prepared with serial half dilution of Colistin, and furthermore, a fresh culture of Acinetobacter baumannii (10^3 CFU/ml) was added to each well.The plate were incubated on at37c. The last columns of the plates served as "no antibiotics "and full growth controls. (20)

Figure 04: Determination of Colistin by Broth dilution method

Molecular detection of Oxacillinase genes: Isolates of *A. baumannii* resistant to both Imipenem and Meropenem were further stored in glycerol nutrient broth for PCR to detect the blaOXA genes. Consent was taken from patients or their attendants whose sample yielded CRAB isolates during the dispersal of their report. For PCR, bacterial DNA was extracted using Xploregen Bacterial gDNA Column filter Based Extraction Kit®. Extraction process was performed as per the manufacturer protocol. PCR master mix constituted of 1) High-Fidelity Taq DNA Polymerase, 2) 2.5mM dNTPs. 3) 3.2mM MgCl2, 4) PCR Enzyme Buffer, 5) Forward Primer, 6) Reverse Primer, 7) 10X Taq DNA polymerase Assay Buffer, 8) RNAse Free water. Separate PCR was performed forblaOXA 58 and blaOXA 51. The blaOXA 51 was 641 base pair (bp) and blaOXA 58 was 453 bp long.

Table 1: Primers Sequence

Oligo Name	**Sequence (5`à 3`)**	**Tm (°C)**	**GC- Content**
blaOXA 58Forward	5' AGTATTGGGGCTTGTGCT 3'	43	50.00%
blaOXA 58Reverse	5' AACTTCCGTGCCTATTTG 3'	41	44.44%
blaOXA 51 Forward	5'AACAAGCGCTATTTTTTATTTCAG 3'	44	29.17%
blaOXA 51 Reverse	5' CCCATCCCCAACCACTTTT 3'	46	52.63%

Result:In (Table 2) total 2644 samples were processed, 920 samples were culture positive. Out of 920 bacterial isolates, 88 were (9.56%). isolates were identified to all clinical samples.

Table2: Prevalence of Acinetobacter baumannii in all clinical samples		
Total Sample	Acinetobacter baumannii	Percentage
920	88	9.56 %

In (Table3) total 2644 samples were processed; 920 samples were culture positive. Out of 920 bacterial isolates, 88 were *A. baumannii* and 84of these were CRAB.

Table 3: Prevalence of Carbapenem Resistant Acinetobacter baumannii (CRAB) in clinical samples.		
Total Sample	CRAB	Percentage
88	84	95.45%

The *A. baumannii*was isolated from various clinical specimens collected from patients indicating colonization or potential infection. The most common specimen sources (Table 4) were Sputum (31.81%), and Endotracheal aspirate (30.68 %), followed by Tracheal Aspirate (11.36%), blood (6.81%), Broncho alveolar Lavage (5.68%),Pus (4.54%), Tip (2.27%), Tracheotomy aspirate (2.27%),and other less frequent sources such as Plural Fluid, Semen, Throat Swab and Urine(each 1.136%).

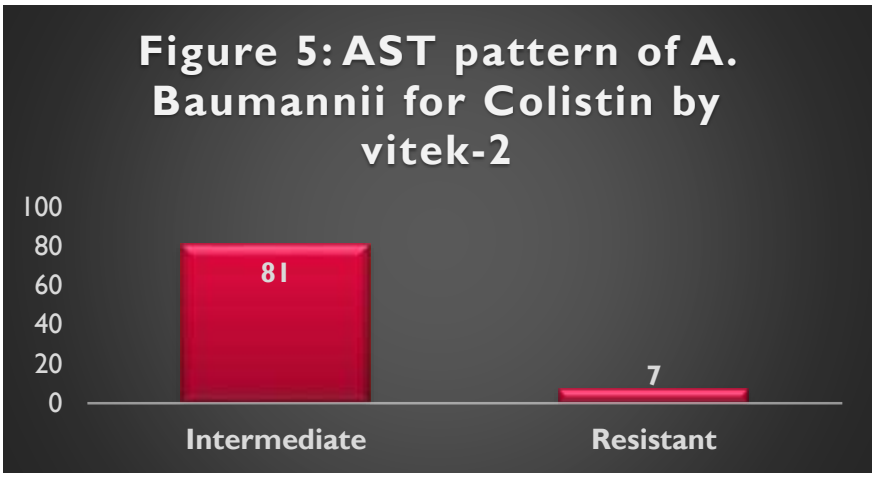
Table 4:Distribution of *A. baumannii* isolates from various clinical specimens with gender-wise breakdown			
Specimen	**Number of isolates**	**Male**	**Female**
Sputum	28	21	7
Endotracheal Aspirate	27	17	10
Tracheal aspirate	10	8	2
Blood	6	3	3
Broncho Alveolar Levage	5	1	4
Pus	4	3	1
Tip	2	2	0

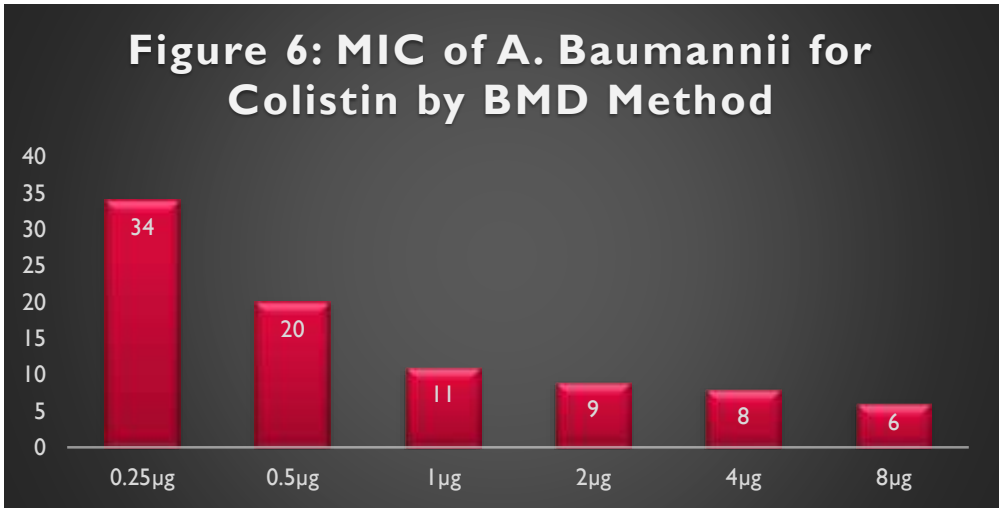
Tracheotomy aspirate	2	0	2
Plural Fluid	1	0	1
Semen	1	1	0
Throat Swab	1	1	0
Urine	1	0	1
Total	**88**	**57**	**31**

Table 5:Distribution of *A. baumannii* isolates from different wards

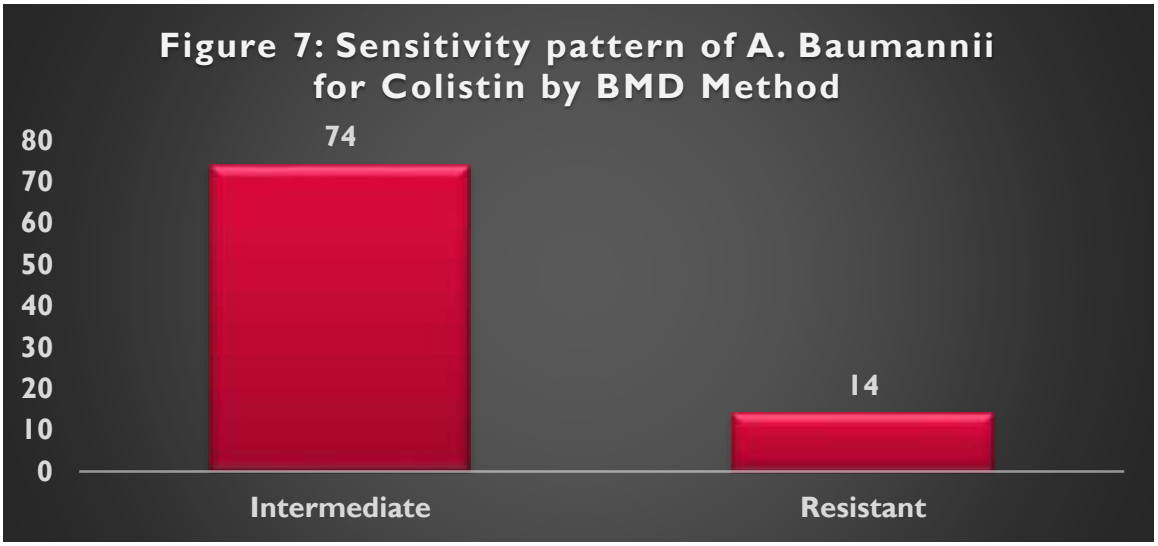
Ward	**Number of isolates (n=88)**	**Percent**
ICU	49 Isolates	55.68%
Medicine Ward	25 Isolates	28.40%
Surgery Ward	10 Isolates	11.36%
Obstetrics and Gynecology	4 Isolates	4.54%

Antimicrobial Susceptibility of Colistin:(Figure 5) Susceptibility tests performed with the Vitek®2 system. (92.04%) of the isolates wereColistin Intermediate and (7.95%) were resistant.





Susceptibility of Colistin by BMD Method:(Figure 7) Susceptibility tests performed with the BMD. (84.09%) of the isolates wereColistin Intermediate and (15.90%) were resistant.



Comparative Analysis of Colistin Susceptibility Using Different Tests: Antibiotic susceptibility testing by Vitek®2 showed that 7/88 *A. baumannii* isolates were resistant to Colistin (MIC ≥ 4 µg/mL). therefore, a discordant interpretation between the two methods was found in 7/88 (7.95%) isolates (To determine if there were any inaccuracies in reporting the Colistin MIC values by Vitek®2, due to causes strictly referable to the instrument, Colistin susceptibility was tested for all 88 A. *baumannii* isolates by the broth micro dilution method, considered as the gold standard. The broth micro dilution test showed that 14/88 isolates (15.90%) were resistant to Colistin. Furthermore, 7/88 (7.95%) of the isolates showed discordance between the values obtained with Vitek®2 and the micro dilution method with discrepant MIC values of more than two dilution factors.

Molecular characterization of oxacillinase genes

Of the 84 MDRO *A.baumannii*, 84 were tested for the OXA51 and OXA58 genes by the PCR. All the isolates had presence of OXA51 980bp and 1 isolate tested positive for OXA58 843gene.

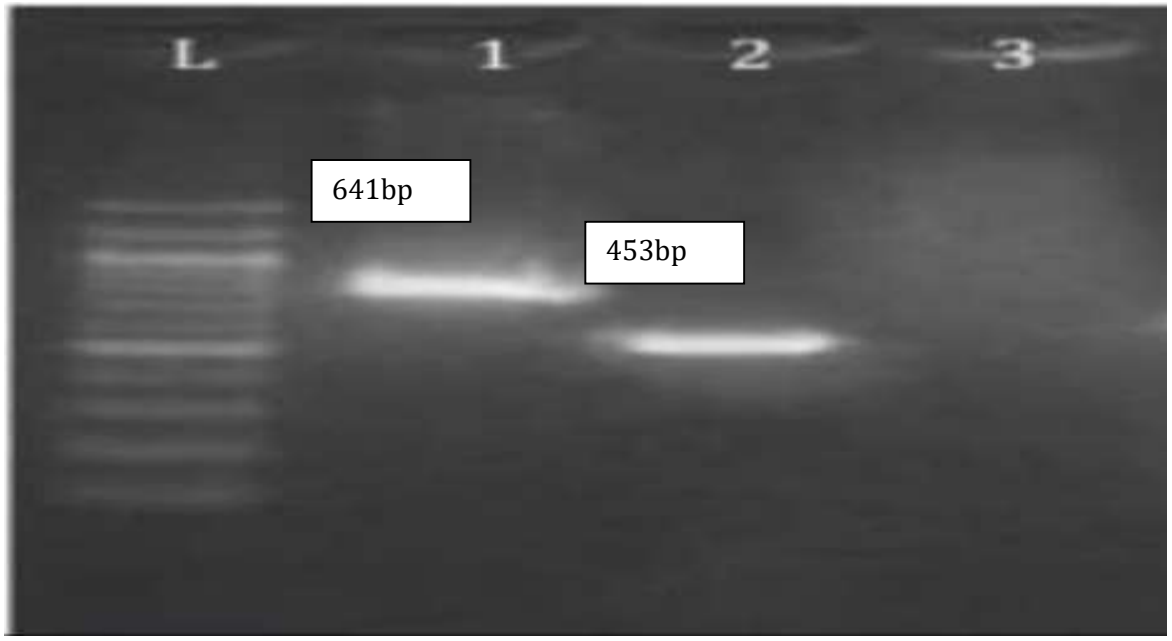


Figure 4: L-Base pair Ladder,

1- Positive control for 641 bp,

2- Positive control for 453 bp

3- Negative Control

4- Sample PCR product of blaOXA51

5- Sample PCR product of blaOXA58

3. DISCUSSION

The persistence in hospital environments and the extraordinary ability to rapidly acquire resistance to new antibiotics rendered problematic the treatment and eradication of infections caused by multidrug-resistant (MDR) A. baumannii strains [14]. In this dramatic situation, Colistin often represents the last-resort (lifesaving) drug for the treatment of infections sustained by A. baumannii . However, since 1999 there has been evidence of the growing frequency of nosocomial A. baumannii isolates that showed resistance to Colistin . A progressive decline in susceptibility to β-lactams antibiotics in A. baumannii has been documented over the past several decades . Carbapenems resistance was mainly attributed to the spread of genes encoding for oxacillinase (blaOXA-like). In the early 2000s, OXA-58 oxacillinase were the most widespread in Italy and neighboring countries, but their prevalence has drastically decreased over the years, with the onset of OXA-23, which gradually replaced OXA-58 . The problem of the clinical management of patients affected by PDR A. baumannii strains poses a number of questions about the correct execution and interpretation of Colistin susceptibility tests, especially agar diffusion tests (e.g., E-test®, disk-diffusion) and automated systems (e.g., Vitek®2). The relative instability of the maintenance of concentration gradients of Colistin in agar plates has raised a caveat against the use of such systems, both manual and automated. In fact, isolates that were considered susceptible to the action of the antibiotic resulted resistant when tested with reference tests, such as broth dilution methods . Inappropriate Colistin susceptibility testing can lead to misinterpretation of the results and, consequently, to an inadequate antibiotic therapy . Excessive use of antibiotics, especially Carbapenems, contributes to the emergence of resistance in A. baumannii. In our study, antibiotic susceptibility testing showed that all isolates were resistant to most antibiotics, except Colistin, and a high rate of resistance of imipenem .In conclusion, this study highlights that the high frequency of isolation of XDR and PDR A. baumannii isolates in high-risk infectious wards requires an accurate application of methods for detecting susceptibility to antibiotics, in particular to last-resort antibiotics like Colistin, so to ensure a correct therapeutic approach.

4. CONCLUSION

Based on the results of this study, we advise laboratories not to trust gradient tests for Colistin susceptibility testing and to use broth micro dilution methods for this purpose. Hence, Vitek 2is not reliable for Colistin susceptibility testing. There are several commercial broth micro dilution tests available and in principle they perform well. This study highlights that the high frequency of isolation of Carbapenem-resistant Acinetobacter baumannii strain in high-risk infectious wards requires an accurate application of methods for detecting susceptibility to antibiotics, in particular to Colistin, so as the correct therapeutic approach. As the frequency of resistance Colistin is low, it can be used as an easily available drug for Carbapenem resistance Acinetobacter baumannii strains which are susceptible to Colistin. The main aim of this study was to achieve a baseline background to determine the resistant pattern of oxacillinase producing *A. baumannii* in clinical samples. Result from this study affords valuable knowledge to perform further large prospective study to identify other mechanisms involved in Carbapenem resistance in *A. baumannii*.

Conflicts of Interest: There is no any conflict of interest associated with this study.

Consent to participate: There is consent to participate.

Consent for publication: There is consent for the publication of this paper.

Authors & contributions: Author equally contributed the work.

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