Microbes and Infectious Diseases

Original article

Expression of metallo-β-lactamase genes in carbapenem resistant *Acinetobacter baumannii* isolates from intensive care unit patients

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A R T I C L E I N F O

A B S T R A C T

Background: *Acinetobacter baumannii (A. baumannii)* is a Gram-negative, aerobic coccobacillus. It causes life-threatening nosocomial infections, particularly in immunocompromised patients. Carbapenem-resistant *A. baumannii* are prevalent worldwide mostly caused by carbapenemase synthesis. Metallo β-lactamases (MBLs) include imipenemase (IMP), New Delhi metallo- β-lactamases (NDM) and Verona integrin metallo- β-lactamase (VIM). We aimed to investigate the prevalence of MBLs genes expression in carbapenem-resistant *A. baumannii* isolated from ICU patients as a reliable method for reducing the morbidity & mortality of these patients. **Methods:** Using conventional methods, 87 *A. baumannii* isolates were identified from 103 ICU patient specimens. Metallo β-lactamases were detected phenotypically in imipenem-resistant strains using a combined disc test (CDT). Real-time PCR was used to quantitate the expression of the *blaIPM*, *blaNDM* & *blaVIM* genes. **Results:** Imipenem resistance was identified in 82.8% of patients. Combined disc test was positive in 44.4% of imipenem-resistant isolates. For metallo-lactamases gene expression, *blaVIM* had a higher median value than *blaNDM* and *blaIPM* (0.5, 0.1 and 0 respectively). Combined disc test was found to have a statistically significant relationship with both *NDM* and *VIM* gene expression, which was considerably higher in isolates with positive CDT. Both *NDM* and *VIM* gene expression had a statistically non-significant correlation with CDT value, but their expression had a statistically significant negative correlation with CD zone of inhibition value. **Conclusion:** The expression of *blaVIM* and *blaNDM* genes is directly correlated with the level of MBLs production and the level of these enzymes can be detected phenotypically depending on its negative correlation with the CD zone of inhibition diameter.

Introduction

Acinetobacter is a genus that contains around 50 species, some of them are pathogenic, despite the majority are not. *Acinetobacter baumannii* (*A. baumannii*) then *A. calcoaceticus* & *A. lwoffii* are considered the commonest pathogenic

species. *A. johnsonii* and *A. haemolyticus* were observed to be pathogenic in certain occasions. *A. baumannii* can be molecularly distinguished by the presence of *blaOXA-51* as it cannot be detected in other species [1]. *Acinetobacter baumannii* is

considered one of the most frequent elements of hospital-acquired infections [2].

The emergence of *A. baumannii* as a compelling nosocomial pathogen is attributed to its resistance to disinfectants, dehydration & large scale of antimicrobial [3]. In intensive care units (ICU), *Acinetobacter* species have been considered main cause of opportunistic infections [4,5]. A fatal association has been developed between pathogenicity and antimicrobial resistance which invades hospitals due to the prosper *A. baumannii* resistance to essential antimicrobials [6]. They are classified as an ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), carbapenem-resistant *A. baumannii* is recognized as a major WHO crucial priority pathogen that necessarily needs new therapies [7].

Nowadays, a considerable fraction of *Acinetobacter* isolates are carbapenem-resistant *A. baumannii* (CRAB), extensively drug-resistant (XDR) or pandrug-resistant (PDR) *A. baumannii* [8]. It is estimated that in some areas of the world, carbapenem resistance rates are more than 90% [9], and for the highest prevalent CRAB infections i.e., bloodstream infections (BSI) and hospital-acquired pneumonia (HAP), the death rate may reach 60%. The rates of carbapenem-sensitive *A. baumannii* infections are considerably lower than the rates of CRAB infections. The existing antimicrobials to treat infections caused by CRAB (tigecycline, polymyxins, and occasionally aminoglycosides) are far from ideal therapy alternatives due to their pharmacokinetic characteristics and increasing resistance rates [3].

For severe bacterial infections, carbapenems are utilized as effective antimicrobials. These agents are characterized by being effective against a wide range of microorganisms and are not hydrolyzed by several β- lactamases involving extended spectrum beta-lactamases (ESBLs). Hospitals with an elevated prevalence of ESBLs, consume more carbapenem antibiotics [10].

Variable mechanisms of carbapenem resistance have been reported, **(A)** efflux pump, **(B)** diminished membrane permeability as a consequence of reducing the synthesis of the outer membrane porins (OMP), which consequently leads to loss of OMP and **(C)** generation of carbapenemases [11,12]. Carbapenemases production are primarily responsible for CRAB

clinical isolates. According to β- lactamases AMBLser classification, carbapenemases belong to three distinct molecular classes: (i) class A carbapenemases e.g. Imipenem-hydrolysing β-lactamase (IMI) and *Klebsiella pneumoniae* carbapenemase (KPC), (ii) class B carbapenemases or the commonly-named metallo-β-lactamases (MBLs) e.g. imipenemase (IMP), New Delhi metallo- β-lactamases (NDM) and Verona integrin metallo- β-lactamase (VIM) and (iii) class D carbapenemase or the commonly named OXA- type carbapenemases e.g. OXA- 23, OXA- 40, OXA- 48, OXA- 51, OXA- 58 and OXA- 181 [1]. The lateral transfer of genes producing carbapenem hydrolyzing enzymes that are included in either AMBLser class B (metallo-ß-lactamases) or class D (oxacillinases) is the main cause of *A. baumannii* carbapenem resistance [13, 14].

Because it is difficult to detect carbapenem resistance by traditional disc diffusion method, the Clinical and Laboratory Standards Institute (CLSI) has recommended different inhibition-based tests like Modified Hodge Test (MHT), double- disk synergy test & combined disc test as routine phenotypic methods for carbapenemase detection. These tests for detecting MBLs take advantage of the enzyme's need for Zn++, inhibiting its action with chelating chemicals like EDTA [15]. Furthermore, a reliable polymerase chain reaction (PCR) can be used to detect the most common genes in carbapenemase positive clinical isolates [16].

As a result, the current study studied the prevalence of MBLs genes expression among CRAB in ICU patients as a reliable method to reduce ICU patient morbidity and mortality.

Material and Methods

This cross-sectional study was conducted in Medical Microbiology and Immunology Department and Scientific and Medical Research Center of Zagazig University, Faculty of Medicine, Zagazig University from January 2021-June 2021. One hundred and three specimens were taken randomly from patients in ICU in Zagazig University Hospitals under complete aseptic conditions. Specimens were obtained from suspected *A. baumannii* infections at any site in the body. They were considered eligible after matching the following inclusion criteria: Immuno-compromised patient, hospital-acquired infections, previous laboratory investigation detecting *A. baumannii*, indwelling catheter, endo-tracheal intubation. On the other hand, the exclusion criteria

were: Patient refusal, Previous laboratory investigation detecting organisms other than *A. baumannii*. A careful clinical history was taken, reports referred by every patient's physician including diagnosis and previous antibiotic treatment, and reports of previous laboratory investigations were considered.

Written consents were taken from relatives about the nature and the objectives of the study. There was no risk or harm to the participants in the study. The data about the patients were treated with confidentiality. Approval for this study was taken from Medical Microbiology Department & the Institutional Review Board (IRB), Faculty of Medicine, Zagazig University (No. 6533 -2-12-2020).

Bacterial isolates

Using conventional methods, 87 *A. baumannii* strains were identified from 103 isolates and verified using API 20 NE **(Bio-Mérieux)**. Kirby-Bauer disc diffusion test was used to identify antibiotic sensitivity for Imipenem [17]. Clinical and Laboratory Standards Institute guidelines were used to interpret the results.

Combined disc test (CDT)

This test was used for the phenotypic detection of carbapenemases. Each imipenem resistant strain was inoculated on a Müller-Hinton agar plate using a 1:10 dilution of 0.5 McFarland suspension. Imipenem (10 g) **(Oxoid, Basingstoke, United Kingdom)** and imipenem/EDTA (10/930 g) **(Lioflichem s.r.I, Italy)** discs were put in each plate at a spacing of at least 20mm between 2 discs centers. The inhibition zones around both discs were compared after 18–24 hours of incubation at 37°C. Increasing inhibition zone by 7mm with imipenem /EDTA disc more than inhibition zone around imipenem disc alone was regarded as an indication to MBL generation [18].

Real-time PCR

RNA extraction of bacterial RNA was done after 24 hours of cultivation of each isolated strain in a fluid medium using **Fast HQ RNA Extraction Kit** following manufacturer instructions.

Reverse transcription to get cDNA from extracted RNA was performed using **TOPscript™ cDNA Synthesis Kit**. The volume of material needed for each reaction was calculated by adding 2 μl RT Buffer, 1 μl Reverse Transcriptase (200 units/μl), 2 μl dNTP Mixture (2 mM each), 1 ng~5 μ g Template RNA, 1 μl Random hexamer Primer (50 μM~100

μM), 0.5 μl RNase Inhibitor (40 units/μl), and sterile water (RNase free) up to 20 μl. The reaction mixture was incubated at 50°C for 60 min, then at 95°C for 5 min to inactivate the reaction.

Real-time polymerase chain reaction with the SYBR Green PCR master mix **TOPreal™ (SYBR Green)** kit was used to quantify the expression of the metallo-lactamase genes NDM, IMP, VIM, and 16s rRNA housekeeping gene, primers used are listed in **(Table 1)**. For each reaction 20 μl mixture was prepared by adding 10 μl TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX), 1 μl template DNA, 1 μl (5~10 pmol μl) forward primer, 1 μl (5~10 pmol μl) reverse primer and sterile water (RNase free) up to 20 μl. Real-time PCR reaction was done using, real-time thermal cycler **(Stratagene Mx3005p, Agilent Technologies, Germany)**. Thermal cycling condition was done as follows: an initial activation step of 15 min at 95°C to activate HotStar Taq DNA Polymerase, followed by 50 cycles of denaturation at 95°C for 10 sec, then annealing at 60°C for 15 sec, and extension at 70°C for 30 sec. Relative expression of MBLs genes NDM, IMP, VIM was calculated compared to rRNA housekeeping gene expression, ΔCt method was used, ΔCt = (Ct-target gene – Ct-16s rRNA) depending on the detected threshold cycles for each target gene and 16s rRNA control, for each sample.

Statistical analysis

Analysis of data was done using the SPSS statistics software (version 23, IBM, NY, USA). Statistically significant value was considered when p- value≤0.05.

Results

The participants· age in this study ranged from 18-65 years old (mean 39,069 years). Most of them were male patients with a percentage (62%). All the study patients stayed in the hospital for more than 7 days. About 60% of the studied specimen were derived from endotracheal aspirate and 40% from surgical site infections (SSI). Approximately 29% of the study patients received Meronem and vancomycin **(Table 2)**.

Antimicrobial susceptibility

▪ **Imipenem susceptibility and CDT of the studied patients:**

Concerning the imipenem susceptibility, the percentage of patients with imipenem resistance was 82.8%. Combined disk test was done for patients with imipenem resistance **(Figure 1)** and the results elucidated that 44.4% were positive for CDT.

Combined disk test was interpreted as positive for MBL if the difference between inhibition zones of imipenem /EDTA disc and imipenem disc was ≥7mm **(Figure 1)**.

Metallo-β-lactamases genes expression:
Concerning MBLs genes expression values, the NDM gene expression folds ranged from 0 to 609.1 with median 0.1 and the mean value was 64.5±161.045, whereas the VIM gene expression folds ranged from 0.1 to 46.4 with median 0.5 and the mean value was 4.9±12.14 and no value was detected to IMP gene expression as shown in **(Figure 2)**.

A statistically significant association between positive and negative results of CDT and the gene expression folds of both NDM and VIM genes was found. NDM and VIM genes expression was significantly higher in those who had positive CDT results as shown in **(Table 3).** However, there was a statistically non-significant correlation between CDT values (the difference between inhibition zones of imipenem /EDTA disc and imipenem disc) and both NDM and VIM gene expression folds as shown in **(Table 4)**. Moreover, a statistically significant negative correlation between CD zone of inhibition diameter value and both NDM and VIM gene expression folds was reported as shown in **(Table 4)** and **(Figure 3)**.

Table 1. Primers used in real-time PCR.

Primer name	Sequence
NDM – F	5´ GGT TTG GCG ATC TGG TTT TC 3´
NDM – R	5´ CGG AAT GGC TCA TCA CGA TC 3´
IMP – F	5´ GGA ATA GAG TGG CTT AAY TCT C 3´
IMP – R	5´ CCA AAC YAC TAS GTT ATC T 3´
VIM – F	5´ GAT GGT GTT TGG TCG CAT A 3´
VIM – R	5´ CGA ATG CGC AGC ACC AG 3´
16s rRNA housekeeping gene - F	5´ TCA GCT CGT GTC GTG AGA TG 3´
16s rRNA housekeeping gene – R	5´CGT AAG GGC CAT GAT G 3´

Table. 2. Baseline data of the studied patients.

	N=87	**%**
Gender:		
Female	33	37.9
Male	54	62.1
Age (year):		
Mean ± SD	39.069 ± 12.342	
Range	18 – 65	
Specimen:		
Endotracheal aspirate (ETA)	52	59.8
Surgical site infection (SSI)	35	40.2
Current antibiotic:		
Meronem, ciprofloxacin	14	16.1
Meronem, gentamycin	13	14.9
Meronem, vancomycin	25	28.7
Meronem	20	23.0
Vancomycin, tigecycline	15	17.2
Length of hospital stay:		
>7 days	87	100.0

Table 3. Relation between metallo- β-lactamases gene expression & CDT results of studied patients.

Parameter	CDT**		Test	
	Negative	**Positive**	Z	*p*
NDM **Median (Range)**	0.1 (0 – 58.4)	21.9 (0 – 609.1)	-2.12	0.034*
VIM **Median (Range)**	0.5 (0.1 – 46.4)	3.6 (0.2 – 6.8)	-2.342	0.019*

p*<0.05 is statistically significant, **Z= Mann Whitney test, data is represented as median and range. **CDT positive (imipenem /EDTA zone of inhibition – imipenem zone of inhibition ≥ 7 mm)

Table 4. Correlation between metallo- β-lactamases gene expression & (CDT and CD zone of inhibition diameter value of the studied patients.

	CDT		CD zone	
	r	***p***	**r**	***p***
NDM	0.059	0.62	-0.345	0.003*
VIM	0.121	0.312	-0.322	0.006*

p*<0.05 is statistically significant, **r =correlation value.

Figure 1. Combined Disk Test: positive results on the right and negative result on the left.
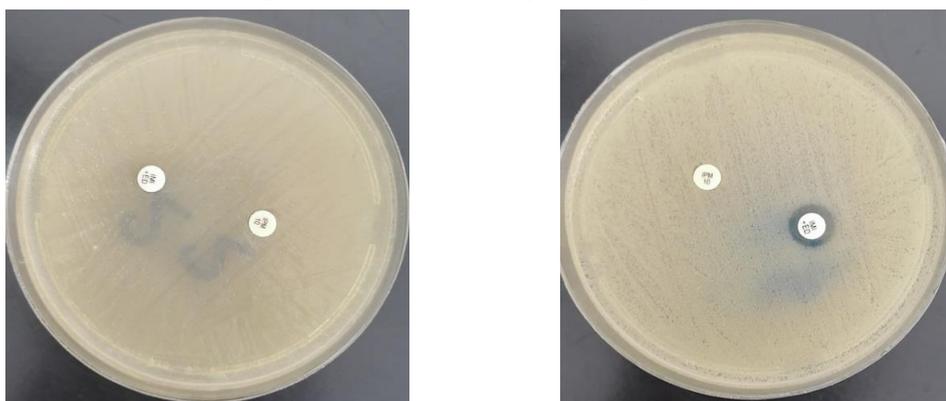
Figure 2. Simple bar chart showing median of metallo-β-lactamases gene expression among the studied patients.
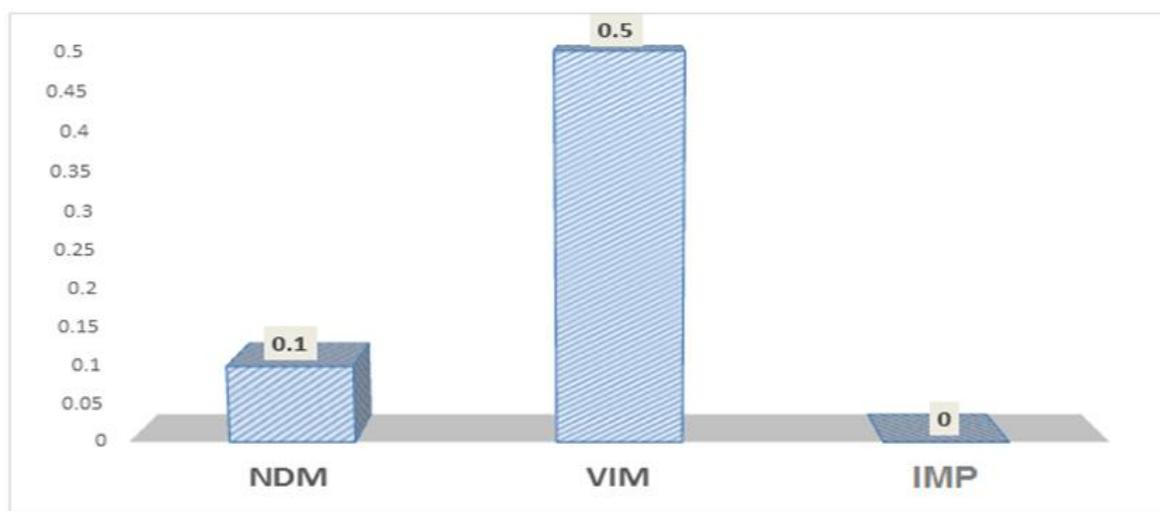
Figure 3. Scatter matrix showing significant negative correlation between CD zone of inhibition diameter value and NDM, VIM genes expression value.
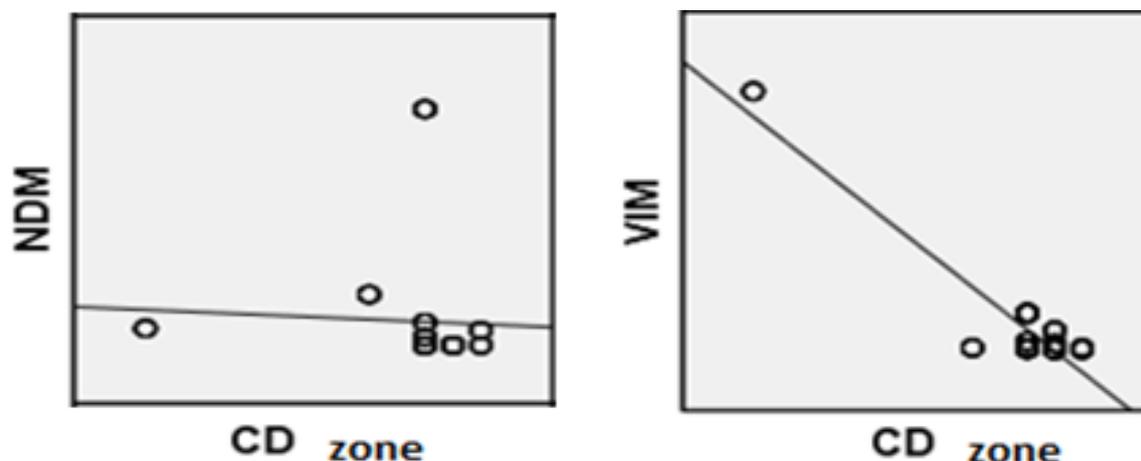
Discussion

Acinetobacter baumannii, is a critical pathogen as it frequently results in life-threatening health care-associated infections, particularly among immune-compromised patients in ICUs [19, 20]. In this work, *A. baumannii* strains were isolated from ICU patients. Most strains were detected in endotracheal aspirate and SSI specimens (59.8% and 40.2 percent, respectively). In agreement with our results, multiple studies have demonstrated that infection of the respiratory system is the most common form of *A. baumannii* nosocomial infections followed by wound infections [21, 22, 16].

At present, multidrug-resistant *Acinetobacter* infections are preferred to be treated with carbapenems [23], however, an elevated resistance rate to carbapenems has been reported worldwide, critically restricting the therapeutic choices. CRAB has long been regarded as concerned health problem, principally in economically developing nations [24]. Imipenem resistance was found in 72 of the 87 A.baumannii isolates (82.8 %). This high prevalence of imipenem resistance is consistent with several studies [5.20, 25, 26]. Other investigations in Egypt have revealed a higher prevalence of CRAB, reaching 90% in some cases [27-29], implying that Egypt has the highest CRAB prevalence in the region [30].

Different mechanisms of carbapenem resistance in *A. baumannii* have been described [31].

The synthesis of carbapenemases is regarded to be the most important resistance mechanism [32]. The Modified Hodge Test, CarbAcineto NP test & Carbapenem inactivation method (CIM), boronic acid disc test, and CDT are among the phenotypic procedures employed.

In this work, a phenotypical screening of carbapenem-resistant bacteria for carbapenemase production was done using CDT, positivity was identified in 44.6 percent of the imipenem resistant isolates. According to **John and Balagurunathan**, CDT is one of the most reliable procedures for identifying AMBLser class B MBLs production with a high positive rate [33-35]. **Sachdeva et al**. reported that the CDT has 97.95% sensitivity and 96.11% specificity when compare with E-Test for the detection of MBL in *P. aeruginosa* [34]. Moreover, **Deopa et al.** reported that CDT is the best screening tests followed by MHT for the detection of MBL in *P. aeruginosa* [35] . On the contrary, other studies have shown a lower positivity rate by CDT [15, 36].

The phenotypic detection of carbapenemases is characterized by the fact that it is inexpensive, simple to execute, and does not require elaborate or expensive equipment; yet it is restricted by its low sensitivity and specificity. As a result, the gold standard for establishing the sensitivity of various phenotypic approaches is PCR screening of several genes involved in carbapenem resistance and some insertion sequences [18].

Enzymatic pathways are assumed to be the major determinants that regulate the development of

carbapenem resistance in Gram-negative bacteria, including *A. baumannii* [37]. Carbapenem-hydrolyzing class D- lactamases (CHDLs) are the main carbapenemases, followed by metalloenzymes (MBLs) such *blaNDM, blaIMP, and blaVIM* [38]. Conventional PCR methods were used to detect the genotype of MBLs in the majority of the published data.

In the current study, we demonstrated the MBLs gene expression (*blaNDM*, *blaVIM*, and *blaIMP*) in CRAB using RT-PCR as a more reliable method. We found that *blaVIM* gene expression was higher than *blaNDM* and *blaIMP* gene expression cannot be detected. Previous studies investigated the gene expression of other carbapenemases [20, 24]. CRAB strains overexpress *blaOXA- 23/ blaOXA-24*, according to **Nguyen et al.** [24]. Furthermore, in CRAB **Wong et al**. discovered that high expression of the *blaOXA- 58* gene increases enzyme concentration in the periplasmic space and its extracellular release, resulting in effective carbapenem hydrolysis [20].

The present study found a statistically significant association between CDT results (positive/negative) as simple phenotypic method in detection of MBLs producing *A. baumannii* and *blaNDM*, *blaVIM* genes expression, so it can be used as a reliable easy screening test for MBLs genes expression. In contrast, there was a non-significant correlation between CDT values and both *blaNDM* and *blaVIM* genes expression values confirming that CDT is only a qualitative test for MBLs production and cannot determine the level of gene expression.

The current study found a novel statistically significant negative correlation between the CD zone of inhibition diameter value and both *blaNDM* and *blaVIM* gene expression values. This indicates that the increase in the level of MBLs gene expression directly results in decreasing the zone of inhibition diameter around CD. This could be attributed to the elevation of MBLs enzymes which consume the chelating EDTA in the combined disc and lead to a decrease in the zone of inhibition diameter around it. Therefore, the expression of *blaVIM* and *blaNDM* genes is directly correlated to the level of MBLs production. To conclude, this study has proved that measuring the zone of inhibition diameter around CD containing imipenem/EDTA (10/930 μg) for imipenem resistant *A. baumannii* strains that produce MBLs can be used as a simple, cheap, and trusted phenotypic method that indicates level MBLs genes expression.

Conclusion

Metallo-β-lactamases generation is primarily responsible for carbapenem resistance in *A. baumannii* isolates from ICU. For identification of MBLs generation, CDT is considered an easy, qualitative, and reliable test. The expression of *blaVIM* and *blaNDM* genes is directly correlated to the level of MBLs production and the level of these enzymes could be easily phenotypically detected depending on its negative correlation to CD zone of inhibition diameter.

Conflict of interest

No conflict of interest was declared by the authors.

Contributors and authorship

All authors have made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, and revising it critically for important intellectual content. Finally, they have approved the version to be submitted.

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