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Detection of Efflux Pumps by mdeA gene, a Chromosomally-Encoded from Multidrug Staphylococcus aureus isolates

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ABSTRACT

Objective: Isolation and Identification of S.aureus and study their susceptibility, study mdeA gene, a Chromosomally-Encoded Multidrug Efflux Pump, from Staphylococcus aureus in patient of Baquba city. Materials and Methods: A total of 25 clinical specimens of Staphylococcus aureus isolates were collected from Baquba Teaching Hospital between June,1, 2022 to August,1, 2022. The isolates were identified based on their cultural characteristics, microscopic examination of cells, and biochemical tests. Antibiotic susceptibility testing was performed using an antibiotic diffusion disc assay to determine the sensitivity of the isolates to Staphylococcus aureus. The mdeA gene from S. aureus was also studied using the PCR technique.

Results: The results show that the Staphylococcus aureus distributed as (4)(16%) samples from blood, 4(16%) samples from urinary tract infections, 6(24%) from wound infections , 2(8%) samples from burn infections, 3(12%) from ear swabs and 6(16%) samples from nasal swabs. The collected isolates were initially diagnosed in hospitals as Staphylococcus aureus that resistant to methicillin formed 100% ,The result revealed that MRSA isolates appeared completely resistant (100%) to Ceftazidime and Cefepime, the results showed that the resistance rate of MRSA isolate to Amikacin, Genetamicin and Kanamycin was 10\18(55.6%), 12\18 (66.6%)and 16 (88.9%)respectively and quinolone antibiotic such as Levofloxacin 14/18(77.7%), Norfloxacin 12/18 (66.6%) , Ofloxacin 12/18(66.6 %) ,Ciprofloxacin4/18(22.2%). The presence of the mdeA gene in the isolates was verified using uniplex PCR, which is considered the gold standard approach. A solitary band was detected at a specific molecular weight of 677 (bp). Our findings demonstrated that (16) MRSA isolates Thus, the prevalence of mdeA in patients of Baquba hospital was (88.8%). The remaining 2(12.2%) isolates were mdeA-negative. Staphylococcus aureus exhibits a significant level of resistance to quinolone antibiotics.

Conclusion: This resistance is further enhanced by the presence of the model gene in the isolates, contributing to bacterial resistance against the antibiotics often used in our local area.

Keywords: *Staphylococcus aureus, mdeA gene, PCR Detection, Ethidium Bromide*

1. INTRODUCTION

Staphylococcus aureus is a type of bacteria that has a spherical shape and stains purple when tested using the Gram staining method. It is usually found on the skin and mucosal membranes. This bacteria is responsible for most infections that occur in hospitals. Methicillin-resistant *S. aureus* (MRSA) strains are particularly problematic as they are resistant to many antibiotics and pose a significant threat to public health [1]. *S. aureus* infections typically exhibit a positive correlation with individuals who have impaired immune systems and those who are hospitalized [2]. The clinical emphasis of *S. aureus* virulence factors are enzymes, toxins and surface proteins that result in the rapid development of drug resistance [3]. *S. aureus* strains often resist to many types of antibiotics. At present, MRSA has become a severe problem in hospitals and a main clinical importance a global public health concern worldwide [4]. *S. aureus* is a highly adaptive, widespread and multipurpose pathogenic bacteria that colonizes skin and mucous membrane of the anterior nares, pharynx, perineum, gastrointestinal tract and genitourinary tract[5]. Staphylococci can cause disease by spreading broadly and multiplying in tissues, as well as by producing numerous extracellular chemicals. Some of these compounds are toxic while others are enzymes [6]. The MdeA protein belongs to the MFS family of efflux pumps and consists of 479 amino acids, arranged in 14 transmembrane helices. This 52 kDa protein actively transports fluoroquinolone antibiotics out of the cell and has a relatively weak binding affinity[7]. Effluxes exhibiting limited sequence similarity with *MdeA* include *QacA* (with a similarity of 23%), *EmrB* from *E. coli*, LmrB from B. subtilis, and *FarB* from Neisseria gonorrhoeae[8]. Quinolones are the predominant antibiotics utilized in clinical settings to treat various bacterial infections, and certain quinolones demonstrate exceptional

anti-MRSA activity both in laboratory settings and within living organisms[9]. The appearance of methicillin-resistant (MRSA) in hospital-acquired infections as a potential pathogen can deal with these antimicrobial agents[10]. Infections caused by *S. aureus* are difficult to be treated because of it can develop and acquire resistance to multiple antibiotics [11]. Resistance can be achieved through antibiotic target modification, *Staphylococcus aureus* is aopportunistic bactrria . Humans seem to have little resistance to the surface colonization of *S. aureus*, so these bacteria can easily colonize the skin and nose)[12]

2. MATERIALS AND METHODS

Isolation and identification of bacterial isolates

A total of 25 clinical specimens were collected from various sources, including (nose, blood, urinary tract infection and wounds) from patients in Baqubah City between June 1, 2022, to August 1, 2022. After obtaining a single colony of isolated bacteria, the isolates were identified depending on phenotypic colony characteristics, in different biochemical tests oxidase, coagulase, and catalase [13]. To determine the potential resistance of *Staphylococcus aureus* isolates to 12 different antibiotic types from various classes, 18 isolates were subjected to the antibiogram testing in accordance with the guidelines of Clinical and Laboratory Standards Institute(CLSI, 2020) and this assay could be preferable achieved by widespread Kirby-Bauer disk diffusion technique was carried out by using disks (Bioanalyse, Turkey) on Mueller Hinton agar The zone inhibition ruler was used to measure the inhibition zones in (mm) and the results were compared with the National committee for CLSI, 2020. To identify the genotype, the *S. aureus* isolates were tested for the presence of *medA* using PCR. All of the isolates were found to be MRSA.

Determination of Ethidium Bromide Minimum inhibition concentration (MIC).

The EtBr-agar cart wheel method [14] was utilized in this test specifically to determine whether efflux pumps were present. In the following manner:

Both sets of measurements were carried out by the parameters established by the CLSI [15], and the MIC value of EtBr was obtained in duplicate. Every culture was applied onto TSA plates that contained varying concentrations of EtBr, (0.25, 0.5, 1, 1.5, 2 mg/ml). The plates were placed in an incubator at a temperature of 37°C for a duration of 16 hours. After this time, the lowest concentration of EtBr that caused the bacterial mass to emit fluorescence under UV light was measured. The plates were subsequently placed in an incubator at a temperature of 48°C for an additional 16 hours. Following this, the lowest concentration of EtBr that resulted in fluorescence was measured and compared to the smallest concentration of EtBr that produced fluorescence at a temperature of 37°C.

Genomic DNA extraction

The Promega DNA extraction kit was utilized to extract the genomic DNA of the 14 MRSA isolates. The lysozyme enzyme was added at a concentration of 30 µg/ml. Overnight, bacterial colonies were cultured in brain heart infusion broth at 37 °C. 1ml of bacterial growth allowed to grow overnight was centrifuged at a speed of ten thousand revolutions per minute for five minutes. All extraction procedures were carried out per the manufacturer's instructions, and the solutions provided were incorporated into the process. An additional step was performed, which involved treating the bacterial cells with lysozyme for one hour before the extraction procedures.

Polymerase chain reaction procedure

The presence of *mdeA* efflux pump genes in the eighteenth MRSA isolates was assessed using the specified primer: forward 5´- **AACGCGATACCAACCATTC** -´3 and reverse 5´- **TTAGCACCAGCTATTGGACCT** -´3 for to produce 677 bp fragment **(16**.) Tables (1,2, and 3).

Table 1:Primers and their proper volumes for PCR reaction

Name of target gene	Type of reaction	Type and volume of master mix	F primer 10 pmol	R primer 10 pmol	*N.F.W*	Volum and concentration of template	Final volume
***mde*A**	Uniplex PCR	10 µl*	1 µl	1 µl	6 µl	2 µl 40ng)(	20 µl

Table 2: Illustrating the PCR program

Amplified genes	Initial denaturation	Denaturation	Annealing	Elongation	Final extension

***mde*A**	95ºC/ 5 min,	94ºC/30 Sec	61 ºC/30 Sec	72ºC/1 min	72ºC/7 min
	one cycle	30cycle	30cycle	30cycle	one cycle

Table 3: Conventional PCR primers used to detect efflux pump *medA* gene

Primer' Name	**Primer sequences 5`……..3`**	**Annealing Tem °C**	**Product size (bp)**	**Ref.**
***mde*A- F**	**AACGCGATACCAACCATTC**	**61**	**677**	**[16]**
***mde*A- R**	**TTAGCACCAGCTATTGGACCT**			

Table 4:Minimum Inhibition Concentration of Ethidium Bromide/mdeA PCR Detection

***mde*Achromosomal efflux pump gene**	**EtBrMICmg**	**Isolate number**
-	0.25	1
+	0.25	2
+	0.25	3
+	0.25	4
+	2	5
+	2	6
+	2	7
+	2	8
+	2	9
+	2	10
+	2	11
-	2	12
+	2	13
+	2	14
+	2	15
+	2	16
+	2	17
+	2	18

Statistical analysis

The results were reported as mean values with standard error (SD). Statistical analysis was conducted using SPSS 26 (SPSS Inc., Chicago, USA). Significance was determined at a P-value below 0.05. The Chi-square test assessed significance when comparing percentages with probabilities of 0.05 and, 0.01.

3. RESULTS

This study was conducted in Baquba teaching hospital in Diyala governorate over a continuous two-month period from June 1, 2022, to August 1, 2022. It was a prospective, descriptive, and investigative study. The eighteenth clinical isolates were

identified as *Staphylococcus aureus* using routine biochemical testing. All of the isolates were grown on primary isolation and selective media. In addition to other biochemical testing, Gram staining, catalase, and oxidase tests were performed on each of the outcomes of the Gram staining. Upon examination, it was observed that every single Staphylococci isolate had a definite zone of hemolysis encircling the colonies. This particular outcome is classified as β-hemolysis by the classification

Table 5: Results of cultural and microscopically properties as well as biochemical tests.

Biochemical test	Result
Mannitol fermentation	100% positive, characterized by a change in the color of the medium from pink to yellow.
Blood hemolysis	100% (beta hemolysis)
Coagulase	100% Clot formation(+)
Oxidase	100% No purple color (-)
Catalase	100% bubbles (+)
Gram stain	100% Gram positive cocci
Motility test	100% negative (non-motile)

To enhance focus on the danger. Previous studies on MRSA have shown that there is a notable disparity in the occurrence of the infection across different regions, both within individual countries and between different countries.

Distribution of *S. aureus* among clinical samples was different according to the source and percentage of isolation, it could be said the percentage of *S.aureus* among the clinical samples were varied According to the source of the samples as the table 6

Table 6: Distribution of Staphylococcus aureus according to source of isolation

Source of isolation	***S. aureus* isolates**	**Percentage of *S. aureus* from total isolates 25**
UTI	4	16
Blood	4	16
Wounds infection	6	24
Burn infection	2	8
Nasal carriage	6	24
Ear infection	3	12
Total isolates	25	100

The results of susceptibility test obtained from 18 MRSA isolates showed different antibiograms. The summary of multiple antibiotics resistance profiles for isolate identification is shown in table (7).

Table (7): Antimicrobial susceptibility results and *p-value* of 18 MRSA isolates

Antimicrobial agent	**No.(%) of isolate**	
	R	**S**
Cefoxitin	18 (100)	0 (0)
Oxacline	18 (100)	0 (0)

Ceftazidime	18 (100)	0 (0%)
Cefepime	18 (100)	0 (0)
Imipenem	5(27.8)	13(72.3)
Kanamycine	16 (88.9)	2 (12.1)
Genetamicin	12 (66.6)	6 (33.4)
Amikacin	10(55.6)	8 (44,4)
Ciprofloxacin	4(22. 2)	14 (77.8)
Norfloxacin	12(66.6)	8(33,4)
Levofloxacin	14(77.7)	4(33,3)
Ofloxacin	12(66.6)	6(33,4)

The result revealed that MRSA isolates appeared completely resistant (100%) to Ceftazidime and Cefepime. The results showed that the resistance rate of MRSA isolate to Amikacin, Genetamicin and Kanamycin was 10\18(55.6%), 12\18 (66.6%)and 16 (88.9%)respectively. *S.aureus* isolates showed same levels of resistance of MRSA isolates to quinolone antibiotic such as Levofloxacin 14/18(77.7%), Norfloxacin 12/18 (66.6%) , Ofloxacin 12/18(66.6 %) ,Ciprofloxacin4/18(22.2%. According to antibiotic Susceptibility as shown on table (7) ; the isolates divided into the into two categories , the four of isolates were XDR(22%) which was resistance all 12 antibiotic that had been used in the study,but the fourteen isolates were MDR(88%) which had been resistance (six- ten) antibiotic from twelve .

The efflux system activity in the 18 MRSA isolates was assessed using the Ethidium Bromide Agar Cart Wheel technique (EtBrCW. In this study, we categorized these isolates into two distinct groups. The first group consisted of 14 isolates that exhibited fluorescence exclusively at the highest concentration of Ethidium Bromide (EtBr) tested (2 mg/ml), accounting for 77.7% of the total. This is depicted in Figure (B). The second group comprised 4 isolates, which showed fluorescence only at the lowest EtBr concentrations tested (0.25 mg/ml), representing 22.22% of the total. These isolates were designated as EtBrCW-negative and are illustrated in Figure (A). The remaining isolates, 4 in number, exhibited a combination of positive and negative results, suggesting an increased efflux activity. These isolates were designated as EtBrCW-positive .

A B

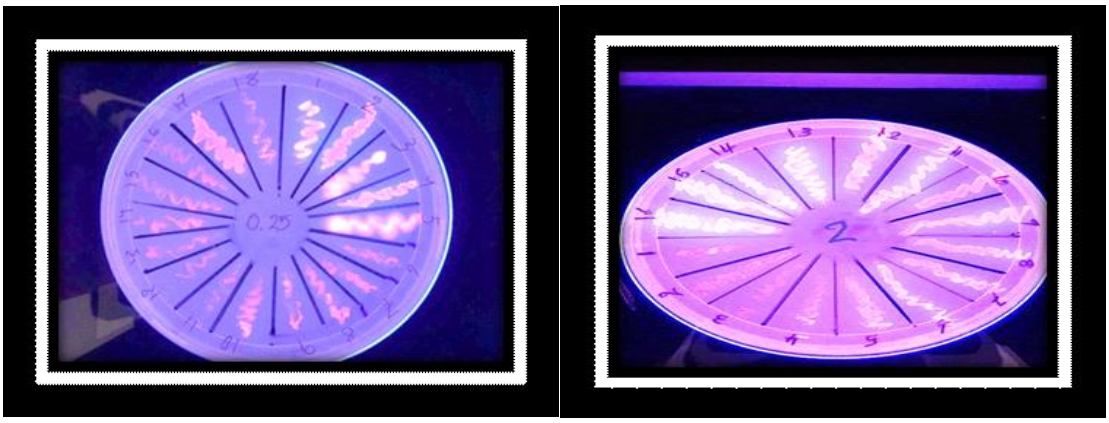

Figure 1: The EtBr-agar cartwheel method applied to *S.aureus* sultures were swabbed on TSA plates containing increasing concentrations of EtBr(A: 0.25mg/mL,No,1 ATCC25923 reference strain, SA2,3,4,5 EtBrCW-negative result, (B: 2mg/mL, No,1 ATCC25923 reference strain, SA 6-16 EtBrCW-positive result, following overnight incubation at 37ؚC for 18 hours, fluorescence was detected under UVlight .

The 18 isolates were evaluated for the existence of a model that encodes the MFS efflux pump using PCR. A unique PCR product of 677 bp, specific to the model gene, was observed in 16 isolates of MRSA. The incidence of mdeA in patients at Baquba Hospital was 88.8%. Out of the total isolates, 2 (12.2%) were mdeA-negative, as shown in Figure 2.

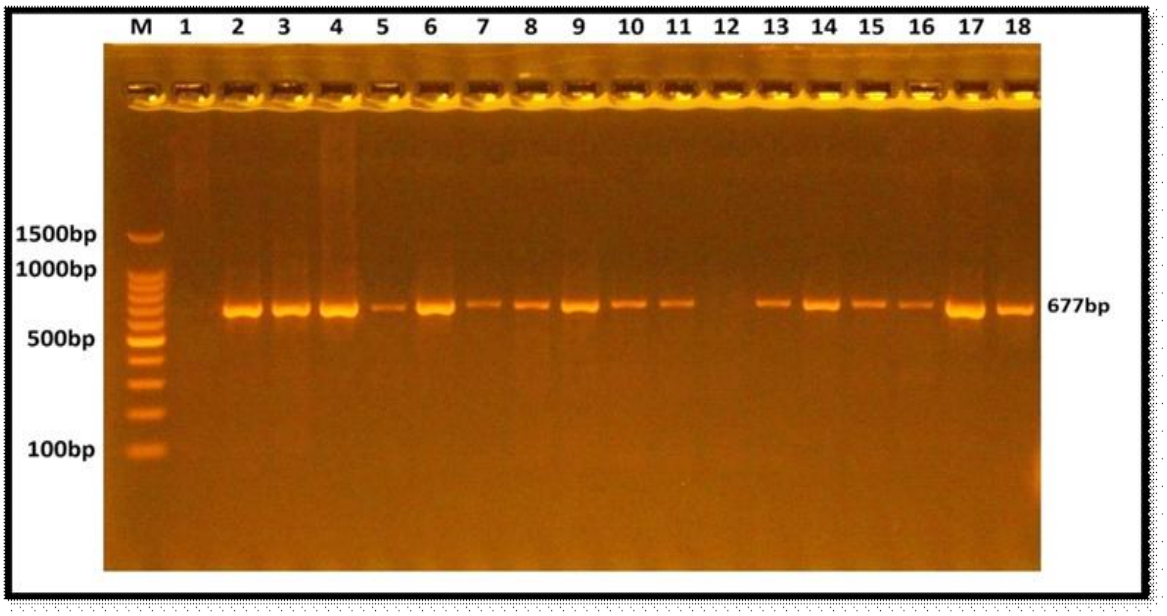

Figure 2: PCR amplification of the mdeA gene from S. aureus, with the amplicon size 677bp. DNA amplification products were electrophoresed in 2% agarose gel. Electrophoresis took 1.5 hours at 70 volt. Lanes 1-18 represent the amplified PCR products (SA1,12) negative amplification of mdeA and (SA2,3, 4,5,6,7,8,9,10, 11.13,14,15,16,17,18) positive amplification.

4. DISCUSSION

S. aureus (an opportunistic human bacterium) causes a wide spectrum of clinical community and nosocomial infections .Infections caused by multidrug-resistant (MDR) microorganisms are correlated with high mortality compared to those caused by susceptible bacteria and carry important economic burdens [22]. The eighteenth clinical isolates were identified as *Staphylococcus aureus* using routine biochemical testing. Several strains were shown to be makers of β-hemolysin, and this trait is considered a presumptive indication of the pathogenicity of staphylococci [23]. This particular outcome is classified as β-hemolysis by the classification of [13, 17]. The result revealed that MRSA isolates appeared completely resistant (100%) to Ceftazidime and Cefepime. These findings were close to local studies related to MRSA isolates by Kareem et al.,(2015) [24], who found that (100%) of MRSA isolates in Baghdad hospitals resisted Oxacillin, Ceftazidime and Cefepimeand the results were almost similar [25]. The results of this study agreed with the study conducted by Abdullahi and Iregbu, (2018)[26] in the National Hospital, Abuja, Nigeria who revealed that (90.7%) MRSA isolates were susceptible to Imipenem.

The result of the present study indicated clearly the evaluation of aminoglycoside resistance in MRSA strains. These antibiotics are widely used in the treatment of staphylococcal infections. As a result of this wide spread use, the level of resistance of *S.aureus* was high in this study. The results showed that the resistance rate of MRSA isolate to Amikacin, Genetamicin and Kanamycin was 10\18(55.6%), 12\18 (66.6%)and 16 (88.9%)respectively. According to a local study performed in a hospital in Al-Najaf, Iraq Al-Khafaji, (2018) showed that the resistance rate of *S.aureus* to Gentamycin and Kanamycin was (10%) and (40%) respectively. Nezhad et al .,(2017) in Iran showed that the resistance rate of MRSA strains to Amikacin[27]. *S.aureus* isolates showed same levels of resistance of MRSA isolates to quinolone antibiotic such as Levofloxacin 14/18(77.7%), Norfloxacin 12/18 (66.6%) , Ofloxacin 12/18(66.6 %) ,Ciprofloxacin4/18(22.2%. The finding that almost supports a previous local study by Al-hamedawy and Mahmoud, (2019)[34] who demonstrated the resistance of *S.aureus* to Quinolone among Iraqi Patients in Baghdad hospitals was by Ciprofloxacin and Norfloxacin (50%).The results of sensitivity in this study were also approximately similar to another local study done by [28] who monitored in their study in Baghdad hospitals that the resistance rate of this bacteria to Levofloxacin, Norfloxacin and Ciprofloxacin was (37.19 %). in South Africa showed that the resistance rate of *S. aureus* to levofloxacin and Ciprofloxacin was (14.3%) and (34.3%) respectively[29]. And the result agreement (Hamza et al 2023)[30] to From a total of 78 S. aureus isolates, 18 (23.77%) and 19 (24.35%) isolates of *S. aureus* bacteria were sensitive and intermediate to quinolone compounds, respectively, whereas 41 (52.56%) isolates showed high-level quinolone resistance[30]. The majority of the antibacterial drugs that were used were considered as candidates for the staphylococcal efflux pump [31]. Some data suggests that efflux pumps can serve as a primary defensive mechanism for cells, preventing medicines from reaching deadly quantities within the cell [32]. The wide range of fluoroquinolone medicines, particularly Ciprofloxacin, has enabled the successful treatment of infections caused by *S.aureus* strains. However, these bacteria rapidly acquire resistance to these antimicrobial medicines[33] .

The efflux system activity in the 18 MRSA isolates was assessed using the Ethidium Bromide Agar Cart Wheel technique (EtBrCW). This method offers a realistic approach to assessing heightened efflux activity in clinical isolates across different bacterial species [14]. The 18 chosen isolates displayed resistance to fluoroquinolones, a group of antibiotics with a high fluorescence level. This fluorescence allows for easy monitoring of their presence and buildup in bacterial cells, eliminating the need for any external probe. As a result, these antibiotics are considered effective targets for efflux pumps[18].The isolates were deemed negative likely due to the absence of phenotypically active efflux pumps that facilitate the expulsion of EtBr. The data demonstrated a significant increase in fluorescence in strains that had an overexpression of efflux pumps compared to the reference strains [19].

The presence or upregulation of efflux pumps leads to decreased drug accessibility for inhibiting the specific target, as the drugs are continuously pumped out. This results in a lower concentration of antimicrobial agents reaching the target, which can increase the rate of new mutations. Consequently, this process generates novel resistant mutants, showcasing a unique resistance mechanism [20]. The primary drawback of this method is the toxicity of the dye used, ethidium bromide (EtBr), which necessitates the implementation of safety precautions during the tests and proper waste treatment for plates and effluents. In addition, similar to other dyes, EtBr does not possess antibiotic properties, making it challenging to establish a direct relationship between its internal concentration, antibiotic activity, and therapeutic significance [21]..The results of this study were almost similar to the local studies performed during different years such as [26] and [27] in Baghdad city which revealed that the positive results for this method of *S.aureus* isolates were (52.9%) and (64.28%) respectively. Our result was in agreement with those obtained also in Baghdad city by Saber et al., who observed that the positive result was (24.49%) by the EtBr-agar cartwheel method [19].

The 18 isolates were evaluated for the existence of a model that encodes the MFS efflux pump using PCR. A unique PCR product of 677 bp. There is only one report on the prevalence of mdeA from Iraq by [27] who showed that the prevalence rate of mdeA gene in (14) (64.2%) MRSA isolates. According to an Iranian study by [30], the prevalence of mdeA in S. aureus was (61.7%), while a Chinese study by Li et al., recorded (94.3%) prevalence rate [31]. Fluorescent antimicrobial drugs, namely ethidium bromide and acriflavine, are commonly employed as substrates for mdeA in the fluorometric assessment of multidrug efflux pump activity. Regarding mdeA, it appears that fluorescent acriflavine was a significantly superior substrate for mdeA. Additionally, it is involved in inherent resistance to numerous antimicrobial drugs, such as Ciprofloxacin [32].

5. CONCLUSION

According to resulting of present study, they have The quinolone antibiotics, such as Ciprofloxacin, are the most efficient in preventing the growth of *Staphylococcus aureus*, which is highly resistant to these antibiotics. In addition to its capacity to create the *mdeA* gene in the isolates, it also causes a rise in the extent to which bacteria are resistant to antibiotics that are isolated from our local area. Instead of depending on bioinformatics to identify substrates for membrane protein transporters, genomics technologies are generating large volumes of data on the genes of efflux transporters. However, the substrates for these transporters need be appropriately identified

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