Studying the resistance of methicillin –resistant *staphylococcus aureus* against Different groups of antibiotics

Huda S. Alagely[1] Eman N. Ismail[1] Tagreed A. Kreem[2] Abeer A. Baqer[3] Ayad W. Alenawey[4] Muhammad Iqbal[5]

Affiliation: [1]Biotechnology Research Center / Al Nahrain University
[2]Medical and Pharmacentical Sciences / AIbn Sina University
[3]Dijlah University College / Iraq
[4]Babylon Agriculture Directorate
[5]Government College University / Faisalabad / Pakistan

***** Correspondence: **huda_alagely@yahoo.com**

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Abstract:

Back ground: MRSA developed resistance to β-lactam antibiotics through the acquisition of the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a), which has a significantly reduced affinity for β-lactam antibiotics, thereby conferring β- lactam resistance . The detection of *mecA* by the (PCR) is considered a gold-standard technique for methicillin resistance detection .

Objective: This study was aimed to isolation and identification of *S.aureus* by traditional and molecular methods and determine their susceptibility to different groups of antibiotics**.**

Materials and Methods: Two hundred and ten samples were collected from different sources of patients in different age groups and for the period from the beginning of January 2015 until the end of June 2015, from different Hospitals in Baghdad (Ibn Albalady, Al Yarmouk, Baghdad teaching hospital and AlKindy).

Results: Depending on the molecular methods (137) out of (210) isolates was methicillin –resistant *Staphylococcus aureus* MRSA. Antibiotic sensitivity test was conducted using 20 types of antibiotics include:- Amikacin, Amoxicillin, Ampicillin, Azithromycin, Cefoxitin, Ciprofloxacin, Clarerythromycin, Clindamycin, Chloramphenicol, Erythromycin, Gentamycin, Methicillin, Norfloxacin, Oxacillin, Penicillin, Rifampin, Teicoplanin, Tetracycline, Tobramycin and Vancomycin.

Conclusion: The results showed that the highest resistance was among beta-lactam group, where the ratio was 100% resistance to Ampicillin and Penicillin, and ratio of 91% to 86.66% and 86.66% resistance to Methicillin, Oxacillin and Amoxicillin respectively.

The lowest resistance was among a group of antagonists Aminoglycosides resistance and ratios was 37.77%, 31.11% and 35.55% to Amikacin, Tobramycin and Gentamycin, respectively, while the rest have different kinds of antibiotics effects.

Key words: *Staphylococcus aureus*, Antibiotics sensitivity, MRSA.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major infection prevention and control challenge globally. *S. aureus* is among the most important and commonly isolated human bacterial pathogens commonly known for causing mild to severe skin infections resulting in death if not treated promptly (1). Since its first appearance, methicillin resistance in *S. aureus* strains has become widespread in hospitals and intensive care units (ICUs).Methicillin resistance is almost exclusively caused by the production of an additional penicillin binding protein (PBP2a) encoded by the *mec*A gene, although other mechanisms have been described (2).Many drugs and antibiotics are become useless for MRSA and new antibiotics begins to use now a day .

Material and Methods

1- Clinical Isolates Two hundred and ten clinical samples collected from three hospitals Al-kindy, Al-Yarmouk and Ibn Al-balady hospitals. From the beginning of January 2015 till the end of May 2015 .

2- Isolation and identification of *S. aureus* by traditional methods -Culturing on selective media Direct smear examination by Gram's stain followed by culture on Mannitol salt agar at 37°C overnight incubation. The isolates were identified by characteristic colony morphology of Staphylococcus. Yellow-colored colonies were obtained on Mannitol salt agar, further confirmed by biochemical reactions using API Staph.,

3- Molecular identification of *S. aureus* -Bacterial Genomic DNA Extraction: - an overnight culture in brain heart infusion broth was collected by and Extraction of DNA from isolated bacteria, carried out by using genomic centrifugation DNA kit (Gene aid). The DNA was preserved with 50-100µl of TE solution in Eppendorf tubes at -20Cº**.**

1- Detection of *S. aureus nuc* gene by polymerase chain reaction (PCR)

Specific primer PCR used for detection of the *nuc* gene for conformation the *S. aureus*, according to (3). These primers synthesized by Cinna Gen Company Table (1).

Table (1): The sequence and concentration of forward and reverse primers of *nuc* gene.

Primers Type	Primers Sequence	Concentration in Pico moles	Product size
nuc geneForward	5-GCGATTGATGGTGATACGGTT--3	30262.27	300bp
nuc gene Reverse	5-AGCCAAGCCTTGACGAACTAAAGC-3	35265.50	300bp

PCR reaction was conducted in master mix tube with 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water (Table 2) .

Table (2): The mixture of conventional PCR working solution for detection of, *nuc* gene in *S. aureus*

Working solution	µl
Water **Forward primer** **Reverse primer** **DNA**	**13µl** **1µl** **1µl** **5µl**
Final volume	**20 µl**

Amplification was conducted using a master cycler Eppendorf programmed with 35 cycler for Initial denaturation 95°C for 3 min., Denaturation for 94°C 1min., Annealing 55°C 30 sec., Extension 72°C 1.5min and final Extension 72°C 3.5min. (Table 3).

Table (3): PCR program for *nuc* gene amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation **Denaturation** **Primmer annealing** **Primmer extension** **Final extension**	**94 °C** **94 °C** **55 °C** **72 °C** **72 °C**	**3min** **1min** **30sec.** **1.5min.** **3.5min**
Cycles number : 35 cycle		

2-Detection of *mec* A gene in *S. aureus* by specific primer:-

PCR used for detection of the *MecA* gene for conformation the identification of the *S. aureus* ,according to (4). These primers synthesized by Cinna Gen Company Table (4).

Table (4): The sequence and concentration of forward and reverse primers of *mecA* gene.

Primers Type	Primers Sequence	Product size
***mecA* Forward**	**5-AACAGGTGAATTATTAGCACTTGTAAG-3**	**170 bp**
***mecA* Reverse**	**5-ATTGCTGTTAATATTTTTTGAGTTGAA-3**	**170 bp**

PCR reaction was conducted in 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water (Table 5).

Table (5): The mixture of conventional PCR working solution for detection of, *mecA* gene in *S. aureus*.

Contained	µl
Water **Forward primer** **Reverse primer** **DNA**	**13 µl** **1 µl** **1 µl** **5 µl**
Final volume	**20 µl**

Amplification was conducted using a master cycler Eppendorf programmed with 35 cycler for Initial denaturation 95°C for 4 min., Denaturation for 94°C 30 sec., Annealing 55°C 1min., Extension 72°C 1min and final Extension 72°C 5min. (Table 6).

Table (6): PCR program for *Mec*A gene amplification by the conventional methods

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation **Denaturation** **Primmer annealing** **Primmer extension** **Final extension**	**94°C** **94°C** **55°C** **72°C** **72°C**	**4min.** **30sec.** **1min.** **1min** **5min.**

3- Antibiotic susceptibility test was performed by the modified Kirby-Bauer method to twenty different antibiotics. Antimicrobial Susceptibility testing by disk diffusion Method was performed by the modified Kirby-Bauer method (5), Mueller-Hinton Plates Mueller-Hinton agar was prepared according to the manufacturer's instructions, and then the medium was cooled to 45-50 °C and poured into the plates, allowed to set on a level surface to a depth of approximately 4mm. When the agar was solidified, the plates were stored at 4 °C until use . And the inoculate colonies from overnight culture of staphylococcal isolates were transferred to 5 ml tube of normal saline to obtain culture with 1.5×10 CFU/ml by adjusting to 0.5 McFarland standard .

The plates were inoculated by dipping a sterile swab into the inoculate; care must be taken to express excess broth from the swab prior to inoculation, by pressing and rotating the swab firmly against the side of the tube above the level of the fluid. The swab was rubbed over the surface of the medium three times rotating the plate through at an angle of 60 after each application. Finally the swab was passed around the edge of agar surface. The inoculate were left for a few minutes to dry at room temperature with the lid being closed. By using a sterile forceps, antibiotic discs were placed on the inoculated plate. Discs should be warmed to room temperature, and then dispensed on the agar surface; they should gently pressed down with sterile forceps.Reading the Results After incubation, the diameters of the complete zone of inhibition were noted and measured in millimeters. The diameter of inhibition zone for individual antimicrobial agent was translated in terms of sensitive, intermediate and resistant categories by comparison with the standard inhibition zone (Table 7) .

Table (7): Antibiotics and their Zone diameter interpretation standards (6)

Id	Antimicrobial agent	Disc potency (µg /Disc)	Resistant	Intermediate	Sensitive
1	Amikacin	30	≤ 14	15-16	≥17
2	Amoxicillin	25	≤19	-	≥20
3	Ampicillin	10	≤28	-	≥29
4	Azithromycin	15	≤13	14-17	≥18
5	Cefoxitin	30	≤14	15-17	≥18
6	Ciprofloxacin	10	≤10	11-15	≥16
7	Clarerythromycin	10	≤10	11-13	≥14
8	Clindamycin	10	≤14	15-20	≥21
9	Chloramphenicol	30	≤12	13-17	≥18
10	Erythromycin	15	≤13	14-22	≥23
11	Gentamycin	10	≤10	11-13	≥14
12	Methicillin	10	≤19	10-13	≥16
13	Norfloxacin	10	≤12	13-16	≥17
14	Oxacillin	1	≤10	11-12	≥13
15	Penicillin G	10	≤ 28	-	≥ 29
16	Rifampin	15	≤16	17-19	≥20
17	Teicoplanin	30	≤10	11-13	≥14
18	Tetracycline	10	≤ 14	15-18	≥ 19
19	Tobramycin	10	≤12	13-14	≥16
20	Vancomycin	30	-	-	≥15

Results

Clinical Samples -Identification of *S. aureus* by traditional methods a total number of 210 clinical samples were collected from different three hospitals in Baghdad city Al-kindy, Al-Yarmouk and Ibn Al-balady hospitals. The specimens included nasal swab, wound swab, burn swab, abscess and pus, sputum, ear swab, urine and blood culture. (150) isolates identified as Staphylococci on a mannitol salt agar depending on yellow color of the colonies, the media considered a selective and differential growth medium which is used for encouraging the growth of Staphylococci and inhibit others by containing high concentration of NaCl and phenol red as an indicator figure(1) (7).150 isolates gave a positive result and were identified as *S. aureus* due to the production of catalase enzyme which is distinguished them from Streptococcus spp. (5; 8). Finally, the API Staph. System was used for accurate identification of the isolates at generic and species level. The test was applied on all isolates, which previously identified by conventional biochemical tests the results gained from API Staph system figure(2).

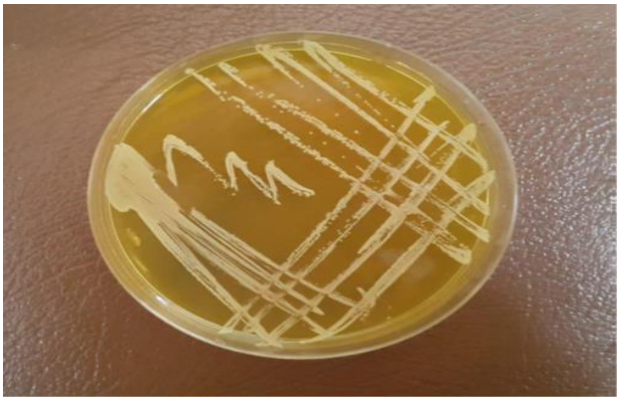

Figure (1): Colonies of *S. aureus* growth on mannitol salt agar in 37 °C for 24 h.

Figure (2): API Staph system for *S. aureus*

Identification by Molecular methods

All isolates were submitted to conventional PCR for further identification on molecular level by using specific primers for detection *nuc* gene, from (150) *S. aureus* isolates recognizing depending on traditional methods (143) isolates were positive for *nuc* gene with product size 300pb figure (3) .The *nuc* primer set recognized all tested isolates belonged to *S. aureus*, but not other bacteria tested Published data indicate that treatment with antibiotics does not interfere with the detection of the *nuc* gene as long as minimum quantities of the target DNA sequences are still present in the clinical specimens.

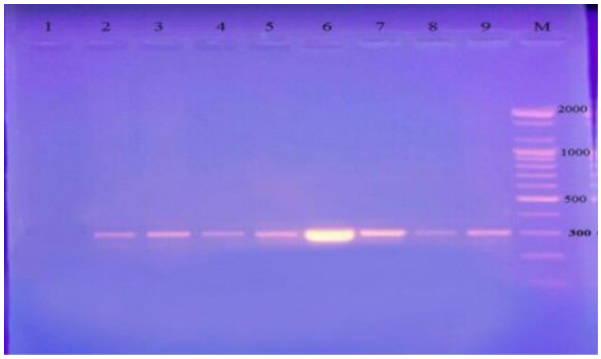

Figure (3): Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *nuc* gene (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular Wight marker (100 bp ladder); Lanes (2-9) positive amplification of 300 bp for *nuc* gene

The isolates that were previously identified by morphological, biochemical characteristics and molecular level by *nuc* gene primers as *S. aureus* were tested for antibiotic susceptibility using Methicillin antibiotic discs (5µg/disc) by applying the antibiotic disc diffusion method .The results of this study confirmed that out of (143) tested *S. aureus* isolates that were (137) isolated exhibited a high level of resistance to Methicillin, the target antibiotic, which is reflected MRSA. This result is agreed with the outcome obtained by (9) in Saudi Arabia .

Rapid and accurate detection of methicillin resistance in *S. aureus* is essential for the use of appropriate antimicrobial therapy and for the control of nosocomial spread of MRSA strains. Thus evaluation the efficiency of the disk diffusion method is important. All the positive isolates (137) that were characterized as MRSA by the (methicillin disc test) were subjected to PCR to detect the presence of *mecA* gene, all of them gave positive results with 170pb PCR product figure(4). The acquisition of *mecA* gene is considered to be the first genetic requisite for methicillin resistance in Staphylococci (10 , 11 ,12).

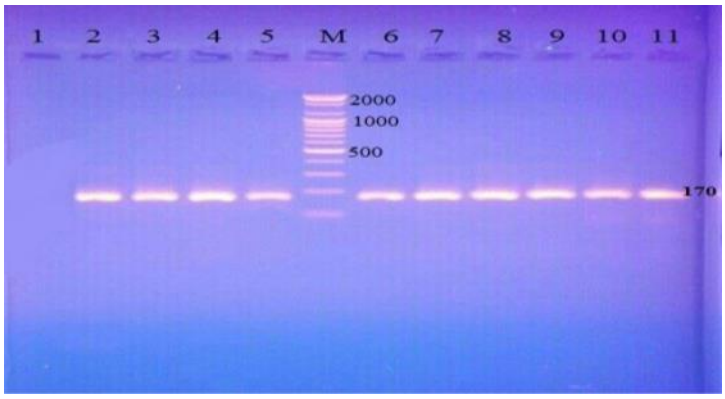

Figure (4):- Agarose gel electrophoresis of PCR amplification products of *S.aureus, mecA* gene (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular Wight marker (100 bp ladder); Lanes (2-11) positive amplification of 170 bp for *mecA* gene.

Antibiotic Sensitivity Profile of the MRSA isolates

Susceptibility of *S. aureus* isolates was detected against 20 types of antibiotics, which are differ in their action (figure 5), table (8).

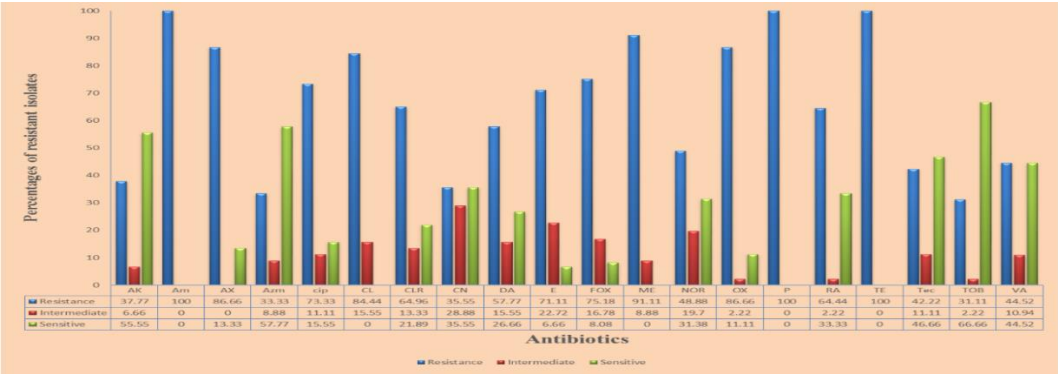

Figure (5):- Susceptibility of MRSA *S. aureus* isolates to 20 different antibiotics

Table (8): Susceptibility of MRSA *S. aureus* isolates to 20 different antibiotics

Antibiotics	Resistance		Intermediate		Sensitive	
	NO.	%	NO.	%	NO.	%
AK	52	37.77	9	6.66	76	55.55
Am	137	100	0	0	0	0
AX	119	86.66	0	0	18	13.33
Azm	46	33.33	12	8.88	79	57.77
cip	101	73.33	15	11.11	21	15.55
CL	116	84.44	21	15.55	0	0
CLR	89	64.96	18	13.33	30	21.89
CN	49	35.55	39	28.88	49	35.55
DA	79	57.77	21	15.55	37	26.66
E	98	71.11	30	22.72	9	6.66
FOX	103	75.18	23	16.78	11	8.02
ME	125	91.11	12	8.88	0	0
NOR	67	48.88	27	19.7	43	31.38
OX	119	86.66	3	2.22	15	11.11
P	137	100	0	0	0	0
RA	88	64.44	3	2.22	46	33.33
TE	137	100	0	0	0	0
Tec	58	42.22	15	11.11	64	46.66
TOB	43	31.11	3	2.22	91	66.66
VA	61	44.52	15	10.94	61	44.52

Amikacin AK , amoxicillin AX , ampicillin AM , azithromycin AZM , cefoxitin FOX , ciprofloxacin CIP , clarerythromycin CLR , clindamycin DA , clorophenicol CL , erythromycin E , Methicillin ME , Norfloxacin NOR , Oxacillin OX , Penicillin GP , Rifampin RA , Teicoplanin TEC , Tetracycline TE , Tobromycine TOB , Vancomycine VA , Gentamycin CN.

Discussion

The results demonstrated that out of 137 tested *S. aureus* isolates (MRSA), 125 isolate (91.11%) were resistant to methicillin while the rest (12 isolate) (8.88%) were intermediate .The disc diffusion method currently recommended by the Clinical Laboratory Standard Institute (CLSI) in (2011) for phenotypic detection of methicillin resistance in all staphylococci (methicillin is a narrow-spectrum β-lactam antibiotic of the penicillin class). Like other beta-lactam antibiotics, methicillin acts by inhibiting the synthesis of bacterial cell walls .It inhibits cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell wall of Gram-positive bacteria (13). Also the results of this study showed that 119 (86.66%) isolates were resistant to oxacillin (ORSA) table(9), 3(2.22%) were intermediate and 15 isolates (11.11%) were sensitive. Oxacillin has been the agent recommended by the CLSI for phenotypic tests to predict resistance to penicillinase-stable penicillin's (PSPs) due to its stability and superior sensitivity over other PSPs susceptibility tests. The majority of ORSA isolates were found to be resistant to all β-lactam antibiotics used (14).

Furthermore, 100% of the isolates showed resistant to penicillin which is close to the percentage obtained by the oxacillin as the two antibiotics belonged to the same class of antibiotic that kill the bacteria by the inhibition of the cell wall synthesis. This may be due to the irrational use of this antibiotic. Such results are in agreement

with the study of(15) who reported that 100% resistance to penicillin and oxacillin by *S. aureus* isolates from clinical hospitals in Cairo and to those results obtained in Saudi Arabia by(16). They partially agreed with the data published by Khan *et al*. (2007) who recorded that 40% are resistance to the penicillin among their tested isolates. On the other hand, vancomycin used in this study and it was found that Out of 137 MRSA isolates 61 (44.52%) isolates were resistant to vancomycin and of the same percentage were sensitive to it .A maximum percentage of resistance 100% was observed by the MRSA S. aureus isolates against, Ampicillin, Tetracycline, Penicillin; 91.11% for Methicillin, 86.66% for Oxacillin and 86.66% for Amoxicillin. This may be due to their belonging to the same group (β-lactam group) and to their similarity in action on the cell. They act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity.

The highest level of MRSA *S. aureus* sensitivity to aminoglycosides representing by tobramycin (66.66%); amikacin (55.55%) and gentamycin (35.55%) antibiotics were found .Their resistance percentage agrees with the results obtained by (17) indicated that despite aminoglycosides resistance among clinical MRSA isolates, they are widespread but gentamycin remains active against most MRSA strains. The isolates were resistant to Ciprofloxacillin in a percentage of 73.33%, and 48.88% to norflaxacin from the fluoroquinolone used. First and second generation fluoroquinolones selectively inhibit the topoisomerase II ligase domain, leaving the two nuclease domains intact. This modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation via the nucleases activity of the intact enzyme domains. Among the aminoglycoside antibiotics used, erythromycins that act by inhibition of protein synthesis showed activity against the tested MRSA isolates in such a way that 71.11% were resistance and 6.66% of them were sensitive. For tetracycline, the percentage of the resistance was 100% , which was not agree with that of(18) who recorded resistance of 24% among *S. aureus* strains isolated from hospitalized patients. While the percentage of resistance to teicoplanin antibiotic was 42.22% and the percentage of sensitive isolates was 46.66%.

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دراسة مقاومة المكورات العنقودية الذهبية المقاومة للميثيسيلين لمجموعات مختلفة من المضادات الحيوية

هدى سلمان العجيلي[1] ايمان نعمان اسماعيل[1] تغريد عبد الرحمن كريم[2] عبير امين باقر[3] اياد وعد العناوي[4] محمد اقبال[5]

1 جامعة النهرين / مركز بحوث التقنيات الاحيائية
2 جامعة ابن سينا للعلوم الطبية والصيدلانية
3 كلية دجلة الجامعة / العراق
4 مديرية زراعه بابل
5 جامعة الكلية الحكومية / فيصل اباد / باكستان
*Correspondence: **huda_alagely@yahoo.com**

الخلاصة:
الخلفية: طورت MRSA مقاومة للمضادات الحيوية β-lactam من خلال اكتساب الجين mecA الذي يشفر البروتين المرتبط بالبنسلين 2 a (PBP2a)، والذي يقلل بشكل كبير من الفة المضادات الحيوية β-lactam ، وبالتالي يمنح مقاومة .β- lactam يعتبر اكتشاف mecA بواسطة (PCR) تقنية قياسية ذهبية للكشف عن مقاومة الميثيسيلين.
الهدف: هدفت هذه الدراسة إلى عزل وتشخيص بكتريا العنقودية الذهبية بالطرق التقليدية والجزيئية وتحديد مدى تعرضها لمجموعات مختلفة من المضادات الحيوية.
المواد والطرق: تم جمع مائتين وعشرة عينة من مصادر مختلفة للمرضى في مختلف الفئات العمرية وذلك للفترة من بداية كانون الثاني 2015 حتى نهاية حزيران 2015 ، من مستشفيات مختلفة في بغداد (ابن البلدي ، اليرموك ، بغداد. مستشفى الكندي التعليمي).
النتائج: بالاعتماد على الطرق الجزيئية (137) عزلة من أصل (210) عزلة كانت Staphylococcus aureus MRSA المقاومة للميثيسيلين. تم إجراء اختبار الحساسية للمضادات الحيوية باستخدام 20 نوعًا من المضادات الحيوية تشمل: - أميكاسين ، أموكسيسيلين ، أمبيسيلين ، أزيثروميسين ، سيفوكسيتين ، سيبروفلوكساسين ، كلاريثروميسين ، كليندامايسين ، كلورامفينيكول ، إريثروميسين ، جنتامايسين ، ميثيسيلين ، أوكسيلين ، أوكسيلين ، أوكسيلين. والفانكومايسين.
الاستنتاجات: أظهرت النتائج أن أعلى مقاومة كانت بين مجموعة بيتا لاكتام حيث كانت النسبة 100٪ مقاومة للأمبيسيلين والبنسلين ونسبة 91٪ إلى 86.66٪ و 86.66٪ مقاومة للميثيسيلين والأوكساسيلين والأموكسيسيلين على التوالي.
كانت أقل مقاومة بين مجموعة مضادات الأمينوغليكوزيدات وكانت النسب 37.77٪ و 31.11٪ و 35.55٪ لأميكاسين وتوبراميسين وجنتامايسين على التوالي ، في حين أن البقية لها تأثيرات مختلفة من المضادات الحيوية.

الكلمات المفتاحية: المكورات العنقودية الذهبية ، حساسية المضادات الحيوية ، MRSA.