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RESEARCH ARTICLE

PHENOTYPIC DETECTION OF B-LACTAMASE PRODUCING *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES.

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Abstract

More than 90% of *Staphylococcal Aureus* isolates produce penicillinase, regardless of the clinical setting. However, penicillin remains the treatment of choice for penicillin-susceptible *Staphylococcus aureus*. A number of phenotypic methods for the detection of β-lactamase production in *Staphylococcus* species have been investigated and compared to detection of the *blaZ gene* by PCR. All phenotypic methods had a sensitivity of less than 72%**, s**o phenotypic tests with high sensitivity should be adopted to detect β-lactamse production in *S. aureus.* This would guide the treatment of cases of serious infections requiring penicillin therapy. The present study aims to identify prevalence of *S. aureus* resistance to penicillin and to evaluate the nitrocefin reaction in detecting β-lactamse production in *S. aureus,* compared to detection of blaZ- *gene* presence by real time PCR in *S. aureus* isolates. The present study was conducted on one hundred clinical isolates of *S. aureus* that were collected from various clinical specimens. Antimicrobial susceptibility was made for cefoxitin and penicillin. Nitrocefin disks were used to detect β-lactamase production. Real time PCR was used to detect *blaZ gene*. Real time PCR results showed that 100% of *S. aureus* isolates were positive for *blaZ g*ene presence. Out of 100 staph aureus isolates 97 (97%) were resistant to penicillin by disk diffusion and gave positive reaction by nitrocefin test. Penicillin disk and nitrocefin test has a sensitivity of 97% and positive predictive value of 100% to detect positive β-lactamase producer *S. aureus* strains. In conclusion, the high sensitivity of phenotypic methods like penicillin susceptibility and nitrocefin reaction would facilitate more routine testing of the β-lactamse production in *S. aureus* clinical isolates.

Introduction:-

Staphylococci are most often associated with chronic infections and infect indwelling medical devices which had considerable impact on the role of *Staphylococci* in clinical medicine. The predominant species isolated in these infections are *Staphylococcus epidermidis* and *Staphylococcus aureus.* (**Donlan and Costerton, 2002**).

The mortality rate of patients with *Staphylococcus aureus* bacteremia in the pre-antibiotic era exceeded 80%, and over 70% developed metastatic infections. The introduction of penicillin in the early 1940s dramatically improved

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the prognosis of patients with *staphylococcal* infection. However, as early as 1942, penicillin-resistant *staphylococci* were recognized, first in hospitals and subsequently in the community**(Franklin, 2003)**.

Although rates of *Staphylococcus aureus* penicillin susceptibility are generally low, ranging from 5 to 20% (**Lowy, 1998)**. However, penicillin remains the treatment of choice for penicillin-susceptible *Staphylococcus aureus* based on in vitro data indicating increased susceptibility to penicillin **(Nissen et al., 2013).**

Two mechanisms confer penicillin resistance in staphylococci. The most important is production of β-lactamase, which inactivates penicillin by hydrolysis of its beta-lactam ring. The second is primarily associated with human isolates and confers resistance due to a penicillin-binding protein, PBP2a, encoded by mecA. (**Niemeyer et al., 1996).**

More than 90% of staphylococcal isolates now produce penicillinase, regardless of the clinical setting. The gene for β-lactamase is part of a transposable element located on a large plasmid, often with additional antimicrobial resistance genes (e.g., gentamicin and erythromycin). Spread of penicillin resistance primarily occurs by spread of resistant strains**(Nissen et al., 2013)**.

Staphylococcal resistance to penicillin is mediated by *blaZ gene*, the gene that encodes β-lactamase. This predominantly extracellular enzyme, synthesized when staphylococci are exposed to β-lactam antibiotics, hydrolyzes the β-lactam ring, rendering the β-lactam inactive. The *blaZ gene* is under the control of two adjacent regulatory genes, the antirepressor *blaR1* and the repressor *blaI*. Studies have demonstrated that the signaling pathway responsible for β-lactamase synthesis requires sequential cleavage of the regulatory proteins *BlaR1* and *BlaI*. Following exposure to β-lactams, *BlaR1*, a transmembrane sensor-transducer, cleaves itself**(Zhang et al., 2001)**.

A number of phenotypic methods for the detection of β-lactamase production in *Staphylococcus* species have been investigated then compared to detection of the *blaZ gene* by PCR. All phenotypic methods had a sensitivity of less than 72%**.** Various PCR methods have been described for the detection of *blaZ gene* in *Staphylococcus species*. The Clinical and Laboratory Standards Institute (CLSI) recommended that *blaZ gene* detection to be considered for penicillin sensitive *S. aureus* (PSSA) isolates from cases of serious infection requiring penicillin therapy (**Pereira et al., 2014**).

The advantages of the real-time PCR over conventional PCR include a faster turnaround time, less specimen handling with subsequent reduced workload and risk of specimen contamination, lower cost, and equivalent sensitivity (100%) and specificity (100%). These advantages would facilitate more routine testing of the *blaZ* gene**(Nissen et al., 2013).**

The present study aims to identify prevalence of *S. aureus* resistance to penicillin and to evaluate the nitrocefin reaction in detecting β-lactamse production in *S. aureus* isolates. This would be compared to detection of blaZ- *gene* presence by real time PCR in *S. aureus* isolates obtained from various clinical samples.

Materials and Methods:-

One hundred clinical isolates of *S. aureus* were collected from various clinical specimens, submitted for routine culture and susceptibility testing to the Main Microbiology Laboratory, Ain Shams University Hospitals, Cairo, Egypt. All isolates were identified using conventional methods such as Gram stain and catalase. Then, the isolates were identified to the species level by slide and tube coagulase test, subculture on DNAse agar, and mannitol salt agar.

Antimicrobial Susceptibility and phenotypic testing:-

Antimicrobial Susceptibility testing was done using cefoxitin disc 30ug and penicillin disc 10ug using Kirby-Bauer disc diffusion according to CLSI guidelines. Zone edge assessment of penicillin disc was made on all isolates and recorded as being either a sharp "cliff" edge suggestive of β-lactamase production or a tapered "beach" edge suggestive of absence of β-lactamase production.

Detection of *Staphylococcal*β-lactamase is enhanced by testing growth from a medium containing sub-inhibitory concentrations of a β-lactam antibiotic. Colonies of *S. aureus* located at the penicillin zone edge were tested with

nitrocefin-impregnated discs for β-lactamase production. Positive reaction is detected by development of a red color in the area of the disc where the culture was applied. No color change is a negative reaction. For most bacterial strains a positive results will develop within 5 minutes. However, positive reactions for some *staphylococci* may take up to 60 minutes to develop **(MacFaddin, 2000)**

Real time PCR:-
Real time Polymerase chain reaction (PCR) was performed for detection of *blaZ* genein the *S. aureus* isolates (Roter gene 5plex). Bacterial DNA was extracted using DNA purification kit (QIA ampDNA Mini Kit supplied by Qiagen, USA). The reaction mixture included Maxima® SYBR Green qPCR Master Mix (2X) Sigma, Germany), Oligonucleotide primers; ***blaz gene*:**

Forward primer:5'ACTTCAACACCTGCTGCTTTC 3'
Reverse primer: 5'TGACCACTTTTATCAGCAACC 3' **(*Olsen et al., 2006).***

The PCR conditions were with initial denaturation step at 95°C for 10 min and 40 cycles of amplification consisting of: denaturation at 95°C for 15s., annealing at 60°C for 30s, extension at 72°C for 30s. Detection of the PCR amplified product was done by SYBR Green (Sigma, Germany) ***(Muldrew, 2009).***

Statistical analysis:-
Categorical variables were expressed as number (%).The diagnostic test evaluation; sensitivity, the positive predictive values and MC NEMAR testfor detection of statistical difference between two tests were calculated for determining the diagnostic validity of the test. Cohen's Kappa coefficient (k) for agreement between thetwo tests were calculated. Kappa's over 0.75 is excellent, 0.40 to 0.75 is fair to good, and below 0.40 is poor. All the analyses were performed with commercially available software (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, USA, 2001).

Results and Discussion:-
Clinical isolates:-
Out of the one hundred *S. aureus* isolates: 43 (43%) are from Pus specimens, 28 (28%) are from wound swabs, 26 (26%) are from blood, and 3 (3%) are from urine. Out of the one hundred *S. aureus* isolates 60% of isolates were from males.

Real time Polymerase chain reaction:-
Real time PCR results showed 100% of *S. aureus* isolates were positive for *blaZ* gene presence.

Phenotypic testing:-
Out of the one hundred *S. aureus* isolates 97 (97%) were resistant to penicillin by disk diffusion, Penicillin disk diffusion has a sensitivity of 97% and a positive predictive value of 100%to detect positive β-lactamase producer strains, if compared to real time PCR. Out of 97 *S. aureus* isolates resistant to penicillin there were 84 isolates showing no penicillin zone and 13 isolates had a penicillin zone edge characterized by a sharp zone edge ("cliff") = β-lactamase positive.

Out of 100 staph aureus isolates 3 (3%) were susceptible to penicillin by disk diffusion and showed penicillin zone edge characterized as Fuzzy zone edge ("beach") = β-lactamase negative.

By MC NEMAR test, there was a non-significant difference between Penicillin susceptibility and *blaZ*gene PCR positivity as regard detection of β-lactamase producing *S. aureus* isolates as penicillin resistance was detected in 97% of *S. aureus*isolates compared to 100% of *S. aureus* isolates positive for*blaZ*gene by real time PCR.

Out of the one hundred *S. aureus* isolates 97 (97%) gave Nitrocefin positive reaction which represented 97 (97%) of *blaZ*gene PCR positive *S. aureus* isolates. Nitrocefin had a sensitivity of 97%, and a positive predictive value of 100% with 85% of isolates showing strong positive reaction by nitrocefin test.

By MC NEMAR test, there was no significant difference between Nitrocefin test and *blaZ*gene PCR test as regard detection of β-lactamase producing *S. aureus* isolates as Nitrocefin test was positive in 97% of *S. aureus*isolates

compared to 100% of S. *aureus* isolates positive for blaZgene byreal time PCR .

The same ninety seven S. *aureus* isolates that were resistant to penicillin by disk diffusion were positive for β-lactamase production by Nitrocefintest indicating positive β-lactamase production by *S. aureus*. The three *S. aureus* isolates that were sensitive to penicillin were Nitrocefin test negative indicating negative β-lactamase production by *S. aureus*. By MC NEMAR test, there was no significant difference between Nitrocefin test and penicillin test as regard detection of β-lactamase production by *S. aureus.*

There was a highly significant excellent agreement between penicillin disk diffusion technique and nitrocefin test results (Kappastatistics =0.852), as 100 % of positive nitrocefin*S. aureus* isolates were penicillin resistant and 100% of negative nitrocefin*S. aureus* isolates were penicillin sensitive with Fuzzy zone edge ("beach").

Out of the one hundred *S. aureus* isolates 89 (89%) of *S. aureus* isolates were resistant for methicillin by using Cefoxitin disk diffusion technique (MRSA), whereas 11isolates (11%) were Methicillin sensitive isolates (MSSA). There was a moderate agreement between Methicillin susceptibility and β-lactamase production by *S. aureus* isolates (Kappa=0.400).

Discussion:-

The recent increase in the antibiotic resistant bacteria such as *S. aureus* was largely because of the irrational usage of antibiotics in both community and hospitals in third world countries. Statistical studies are very important to monitor the resistant strains in the world**(kasse et al., 2008)**. The current study aims to identify prevalence of *S. aureus* resistance to penicillin and to relate this to *blaZ gene* presence, and to evaluate nitrocefin as a sensitive method to detect β-lactamase production by *S. aureus* isolates. This would help in modifying antibiotic regimens, whether to use penicillin as a treatment or not, because penicillin is known to be the drug of choice to treat infections by *S. aureus* that do not secret penicillinase.

The current study was conducted on one hundred clinical isolates of *S. aureus* that were collected from various clinical specimens, submitted for routine culture and susceptibility testing to the Main Microbiology Laboratory, Ain Shams University Hospitals, Cairo, Egypt. All *S. aureus* isolates were identified to species level using conventional methods and biochemical testing then antimicrobial susceptibility testing was done using cefoxitin 30ug and penicillin 10ug disks. Detection of β-lactamase production was done by nitrocefin disks. Real time PCR was done to detect *blaZ*gene.

In the current study, out of the one hundred *S. aureus* isolates 97 (97%) were resistant to penicillin by disk diffusion, Penicillin disk diffusion has a sensitivity of 97% anda positive predictive value of 100% to detect positive β-lactamase producer strains, if compared to real time PCR.Similarly, **Egyir et al., 2014** made a study on 308 *S. aureus* isolates, penicillin-resistant isolates were (97%), Other African studies have reported similar levels of resistance to penicillin (86%–93%) (**Egyir et al., 2014)**. Also**YANG et al., 2015** foundthat94.6% of *S. aureus* isolates in their study were resistant to penicillin. **Lidiane et al., 2012** made a study on 100 *coagulase-negative Staphylococcus*, they found that penicillin resistance was 79%. This worldwide increase in penicillin resistance between *S. aureus* isolates is due to increased use of penicillin as a treatment of choice for many *S. aureus* infections which induced production of penicillinase (**Lynette et al., 2014**).

In the current study, Out of the one hundred *S. aureus* isolates 97 (97%) gave Nitrocefin positive reaction which represented 97 (97%) of *blaZ*gene PCR positive *S. aureus* isolates. Nitrocefin had a sensitivity of 97%, and positive predictive value of 100% with 85% of them showing strong positive reaction by nitrocefin test.Similarly, in a study made by **Pitka et al., 2007,** they used a total of 175 staphylococcal isolates to compare between PCR as a reference method, Cefinase and nitrocefine based tests for detection of production of β-lactamase. They found that sensitivity of nitrocefin is 90% and specificity 100%. However, the numbers of staphylococci with only a very weak color reaction were very high may be due to different β-lactamase enzymes that exhibit differences in substrate specificities, which may explain the partial color reactions (**Pitka et al., 2007)**. Also **Lidiane et al., 2012** made a study on 100 *coagulase-negative Staphylococcus*, they detected *blaZ* gene in only 16% of samples which were consistent with nitrocefin results for detection of β-lactamase. They concluded that nitrocefin-based assays can be recommended for routine clinical use for detection of β-lactamase. A nitrocefin positive result should be interpreted as β-lactamse producing strain which confer resistance to penicillin and cephalosporin activity.

In the current study, there was a highly significant excellent agreement between penicillin disk diffusion technique and nitrocefin test results, as 100 % of positive nitrocefin*S. aureus* isolates were penicillin resistant and 100% of negative nitrocefin*S. aureus* isolates were penicillin sensitive with Fuzzy zone edge ("beach").Similarly, **Lynette and colleagues 2014,** made a study on one hundred *S. aureus* bloodstream isolates (BSI) in Path West Laboratory Medicine WA Queen Elizabeth II Medical Centre Department of Microbiology. Of these 100 *S. aureus* isolates, 50 isolates were susceptible to penicillin and 50 were resistant to penicillin and tested these resistant strains by nitrocefin as well. Theyfound the same good agreement between penicillin and nitrocefin disk.

In the current study real time PCR results showed 100% of *S. aureus* isolates were positive for *blaZ*gene presence. Out of these 100 isolates only 97 (97%) of isolates were resistant to penicillin and nitrocefin positive. The remaining 3 isolates were sensitive to penicillin and nitrocefin negative, these discrepant phenotypic and genotypic results is related to expression of the *blaZ* gene which may be affected by mutations in DNA encoding promoter or repressor regions. Similarly, **Lynette and colleagues 2014,** tested 50 penicillin resistant *S. aureus* BSI isolates by both the conventional PCR and real-time PCR. They found that the 50 penicillin resistant *S. aureus* had the *blaZ* gene. They tested other 50 phenotypically penicillin-susceptible *S. aureus* isolates, 48 isolates did not have *blaZ* gene by PCR. This phenotypic and genotypic results were consistent with the absence of β-lactamase. The remaining 2 penicillin-susceptible *S. aureus* isolates had *blaZ* gene, this discrepant results were related to gene expression effects. In another study made by **WANG et al., 2015** they tested 37 penicillin-resistant *S. aureus* strains from bovine mastitis by simplex PCR, 94.6% of *S. aureus* isolates were resistant to penicillin, and were shown to have the expected penicillin resistance *blaZ* gene.

On the other hand **Lidiane et al., 2012** made a study on 100 *coagulase-negative Staphylococcus*, they found that penicillin resistance was 79% but detected *blaZ* gene in only 16% of samples. Resistance to penicillin can be attributed to other mechanisms such penicillin-binding protein, PBP2a, encoded by mecA and biofilm formation which may play a major role in the resistance mechanisms (**Lidiane et al., 2012)**.

In the current study, out of 100 *S. aureus* isolates 89 (89%) of *S. aureus* isolates were resistant for Methicillin by using Cefoxitin disk diffusion technique (MRSA), whereas 11(11%) were Methicillin sensitive isolates (MSSA). There was a moderate agreement between Methicillin susceptibility and β-lactamase production by *S. aureus* isolates. In a study made by **Arêde et al., 2013,** they explained this agreement as they stated that in response to β-lactam chemotherapy, *S. aureus* has acquired two resistance determinants: *blaZ*, coding for β-lactamase, which confers resistance to penicillins only, and *mecA*, coding for an extra cell wall cross-linking enzyme with reduced affinity for virtually all other β-lactams. The transcriptional control of both resistance determinants is regulated by homologous repressors (*BlaI and MecI*, respectively) and sensor inducers (*BlaR1 and MecR1*, respectively).There is a cross-talk between the two regulatory systems, and it has been demonstrated that *bla* regulators stabilize the *mecA*acquisition.These observations point to important roles of the *bla* locus for the expression of the methicillin-resistant *S. aureus* (MRSA) phenotype. It has been recognized that the β-lactamase locus (*bla*) plays an important role in MRSA evolution, namely, by facilitating the acquisition and stabilization of the *mecA* gene (**Arêde et al., 2013)**.

The advantages of the real-time PCR over conventional PCR include a faster turnaround time, less specimen handling with subsequent reduced workload and lower risk of specimen contamination, lower cost, and an equivalent sensitivity (100%) and specificity (100%). The Clinical and Laboratory Standards Institute (CLSI) recommended that *blaZ gene* detection should be considered for PSSA isolates from cases of serious infection requiring penicillin therapy (**Pereira et al., 2014**). But in limited resource laboratories, purchasing the real time PCR may represent a financial challenge. Penicillin susceptibility and Nitrocefin test has a sensitivity of 97% to detect positive β-lactamase producer *S. aureus* strains. In conclusion, the high sensitivity of phenotypic methods like penicillin susceptibility and Nitrocefin reaction would facilitate more routine testing of the β-lactamse production in *S. aureus* clinical isolates.

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