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Original Research Article

PHENOTYPIC DETERMINATION OF INDUCIBLE CLINDAMYCIN RESISTANT AND METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* FROM CLINICAL ISOLATES OF KHYBER TEACHING HOSPITAL, PESHAWAR

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Abstract *Methicillin Resistant Staphylococcus aureus (MRSA) is a major pathogen involved in nosocomial infections and to some extent, in community acquired infections. Among Macrolide Lincosamine Streptogramin B (MLSB) class of drugs, Clindamycin was vigorously preferred for treating staphylococcal infections in the past few decades but, some genetic factors i.e. erm and msrA genes contribute in developing Inducible Clindamycin Resistance (iCR). Sensitivity tests performed on a routine basis cannot detect inducible resistance and may result in the failure of Clindamycin to be used as an effective medication. This study aimed to detect the phenotype of MRSA and iCR S. aureus from the clinical samples of Khyber Teaching Hospital, Peshawar. A total of 204 samples were collected randomly from each gender, 130 (63.72%) samples were isolated as S. aureus, while 74 (36.27%) were other bacterial species. Double disk diffusion (D-test) was performed to detect iCR phenotype, and 80 (61.5%) isolates showed iCR, while 50 (38.4%) were negative in this regard. MRSA phenotype was determined by strains conferring resistance to Cefoxitin antibiotic, which resulted in 84 (64.6%) isolates of MRSA and 46 (35.3%) of Methicillin Sensitive Staphylococcus aureus (MSSA). Antibiogram analysis showed efficient antimicrobial activity by Tigecycline 129 (99.2%), Fusidic Acid 126 (96.9%), and Doxycycline 124 (95.3%), while the highest resistance pattern was recorded against Ciprofloxacin 31(23.8%) and Clindamycin 28(21.5%). Our study concludes that misuse of antibiotics should be avoided to inhibit the spread of MRSA, and implementation of D-test regularly in hospitals is crucial varieties.*

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Keywords: Methicillin Resistant *Staphylococcus aureus* (MRSA); Inducible Clindamycin Resistance (iCR); Macrolide Lincosamine Streptogramin B (MLSB); Double disk diffusion (D-test)

Introduction

Staphylococcus aureus is a Gram positive bacteria and an etiological agent of a variety of severe infections including endocarditis, pneumonia, septicemia and skin-associated disorders. *S. aureus* and Coagulase Negative Staphylococci (CoNS) are the causative agents of a wide range of hospital and community acquired infections, worldwide. The emerging Methicillin resistance caused by staphylococci is the major health issue to be effectively treated (Fokas et al., 2005; Lall and Sahni, 2014). Resistance to Methicillin occurs due to modification in PBP-2a, which is a penicillin binding protein that results in resistance to entire ß lactam antibiotics (Ghosh and Banerjee, 2016).The infections caused by staphylococci are routinely treated with MLSB antibiotics. Among MLSB antibiotics, Clindamycin was the drug of choice for treating skin and soft tissue disorders, including MRSA and MSSA infections. But on the other hand, the emergence of iCR species of *S. aureus* reduced the efficacy of Clindamycin (Patel et al., 2006). Resistance to MLSB antibiotics developed by *S. aureus* is because of three important factors i.e. transformation of the target site, enzymes that disable MLSB drugs, and efflux pumps against macrolides. The lack of inducible MLSB detection fails Clindamycin therapy (Goldman and Capobianco, 1990). Erythromycin, the inducer of Clindamycin resistance diffuses in the agar and confers resistance by forming a flattened D-shape zone of inhibition around Clindamycin disk. So,

MLSB resistance could be determined by placing Erythromycin and Clindamycin antibiotic disks at a distance of approximately 10 to 15 mm or up to 20 mm apart (Fiebelkorn et al., 2003). The MLSB antibiotics function's as protein synthesis inhibitors in Gram positive bacteria. Their mechanism of action is to bind the 23S rRNA on a large ribosomal subunit. Erythromycin and Clindamycin (MLSB antibiotics) also carry out similar mechanism by inhibiting the 50S ribosomal subunit of the pathogen. MLSB resistance can be constitutive or inducible. In the case of inducible resistance, Erythromycin is the inducer of methylase secretion, resulting in resistance to Clindamycin (Kilany, 2016).

The target site modification developed by staphylococcal species is one reason for resistance against MLSB drugs. This modification is induced by *erm* genes. Four types of *erm* genes carry out MLSB resistance i.e. *ermA, ermB, ermC* and *ermF* but MLSB resistance is mainly caused by *ermA* and *ermC*. The inducible-resistant strains produce an inactive mRNA incapable of encoding methylase because mRNA requires an inducer (macrolide) to be activated. So, the strains having an inducible *erm* gene became resistant to the inducing agent and susceptible to non-inducing MLSB drugs. The constitutive strains produce active mRNA without the presence of an inducing agent. Hence, cross-resistance to MLSB drugs is attained in constitutive phenotype. Increasing resistance to methicillin developed by *S. aureus* has resulted in limited treatment options for staphylococcal infections. In addition, the efflux pumps in staphylococci are encoded by *msrA* gene responsible for pumping out macrolides and streptogramin B antibiotics but not lincosamides. That's why these strains are Erythromycin resistant and Clindamycin susceptible exhibiting Macrolide Streptogramin (MS) phenotype differs from inducible, having no D-zone. Clindamycin can be safely administered in this case (Adhikari et al., 2017; Aktas et al., 2007). Almost one-third of healthy individuals are expected to be the carriers of S. aureus,which may lead to severe staphylococcal infections in the case of immunocompromised patients. The strains conferring resistance to macrolide (Erythromycin) and susceptibility to lincosamide (Clindamycin) need to be carefully identified. The inducible MLSB strains are often incorrectly considered Clindamycin sensitive. Polymerase Chain Reaction (PCR) would facilitate identifying of resistant genes responsible for causing MLSB resistance (Elkammoshi et al., 2016). Our study was designed to determine the prevalence of iCR and methicillin resistant strains of *S. aureus* among clinical isolates collected from Khyber Teaching Hospital, Peshawar.

Materials and Methods

Collection and processing of samples

A total of 204 samples were collected randomly from Khyber Teaching Hospital (KTH), Peshawar from February, 2019 to July, 2019 from patients of both genders and all age groups. The clinical samples were collected from different sources i.e. blood, pus, wound and Cerebrospinal Fluid (CSF). The samples were cultured on blood agar and sub-cultured on Mannitol Salt Agar (MSA) plates and incubated at 37°C for 24 hours. Different biochemical tests (Catalase and Coagulase) were carried out to identify Gram Positive Cocci (GPC's) (Gharib et al., 2013).

Kirby Bauer disc diffusion method

Antibiotic susceptibility test was performed on Muller Hinton Agar (MHA) plates by following the protocol of "Kirby Bauer disc diffusion method" as followed by Yadav (Yadav et al.) and as per Clinical and Laboratory Standard Institute (CLSI) 2019 guidelines. Different antibiotics were used in the study which includes; Clindamycin (CLI), Erythromycin (ERY), Cefoxitin (FOX), Ciprofloxacin (CIP), Vancomycin (VA), Augmentin (AUG), Doxycycline (DXT), Fusidic acid (FC), Linezolid (LZD), Tigecycline (TGC), Cefaclor (CEC), Cephradine (CRD), Cefotaxime (CTX) and Co-trimoxazole (SXT).

Double Disk Diffusion (D-test)

Inducible Clindamycin Resistance (iCR) in *S. aureus* was detected by following the protocol of Double disk diffusion test (D-test). The two antibiotic disks i.e. Erythromycin (15µg) and Clindamycin (2µg) were placed 15 to 20mm apart on a MHA plates as shown in figure 1. The plates were incubated at 37°C for 24 hours (Prabhu et al., 2011). The isolates exhibited three different MLSB phenotypes;

Inducible MLSB resistant or iCR phenotype

The isolates which showed diffusion of Erythromycin in the agar (zone of inhibition ≤13 mm) and sensitivity to Clindamycin with a flattened zone of D-shape around it (zone of inhibition ≥ 21mm) were regarded as inducible MLSB resistant or iCR isolates.

Constitutive MLSB resistant phenotype

The isolates that exhibited resistance to both Erythromycin and Clindamycin with a zone of inhibition ≤13 mm around both antibiotics were considered constitutive MLSB-resistant isolates.

MS phenotype

The isolates which were resistant to Erythromycin (zone of inhibition ≤13 mm) and sensitive to Clindamycin (circular zone of inhibition ≥21 mm) exhibited MS phenotype.

Detection of Methicillin Resistant *Staphylococcus aureus*

Methicillin resistance in *S. aureus* was detected by using Cefoxitin (30µg) antibiotic disk on MHA plates, as followed by (Kale and Patil, 2019) and as shown in figure 1. The strains which exhibited a zone of inhibition less than 19mm around Cefoxitin were regarded as MRSA.

Figure 1 D-test positive and MRSA,*MRSA: Methicillin Resistant *Staphylococcus aureus*

Results

Gender wise analysis of positive isolates

Out of 204 collected samples, 130 (63.72%) were positive for *S. aureus*, while 74 (36.27%) were negative (Gram negative bacteria). Among 130 positive samples, 61(46.9%) were male, and 69 (53.1%) were female patients.

Frequency distribution of positive isolates

Out of 130 positive isolates, *S. aureus* was isolated in majority of pus samples 62 (47.69%), followed by wound 39 (30.0%), blood 21 (16.15%), and CSF 8 (6.15%), respectively.

Antibiotic Sensitivity Pattern

The sensitivity pattern recorded against different isolates revealed the highest sensitivity against Tigecycline 129 (99.2%), followed by Fusidic Acid 126 (96.9%) and Doxycycline 124 (95.3%). A variation in sensitivity pattern was revealed by the rest of the antibiotics i.e. Erythromycin 50 (38.4%), Clindamycin 28 (21.5%), Cefoxitin 46 (35.3%), Ciprofloxacin 31(23.8%), Vancomycin 44 (33.8%), Augmentin 55 (42.3%), Linezolid 119 (91.5%), Cefaclor 120 (92.3%), Cephradine 118 (90.7%), Cefotaxime 112 (86.1%) and Co-trimoxazole 32 (24.6%).

Phenotypic detection of clinical isolates

D-test was carried out to identify iCR strains of *S. aureus*. The results showed that out of 130 isolated strains of *S. aureus*, 80 (61.5%) isolates showed a positive D-test while 50 (38.4%) were negative as shown in Table 1 and Fig.2. Along with it, 22 (16.92%) isolates showed constitutive MLSB and 28 (21.53%) showed MS phenotype as shown in Table 2 and Fig.3. the use of Cefoxitin antibiotic disk specified MRSA strains, the results of which revealed that; 84 (64.6%) isolates were found resistant to Cefoxitin (MRSA) and 46 (35.3%) were sensitive to Cefoxitin or Methicillin susceptible strains (MSSA) as shown in Table 3 and Fig.2. Phenotypic results also revealed that 80 (61.5%) isolates were iCR and MRSA as well.

Table 1 Phenotypic Detection of Inducible Clindamycin Resistant *S. aureus*

Phenotype	D test Positive	D test Negative
iCR *S. aureus*	80 (61.5%)	50 (38.4%)

*iCR: Inducible Clindamycin Resistance

Table 2 Phenotypic Detection of Clinical isolates

Phenotype	Samples Frequency	Samples Percentage
Inducible MLSB	80	61.53%
Constitutive MLSB	22	16.92%
MS Phenotype	28	21.53%
MRSA	84	64.61%
MSSA	46	35.38%

*MLSB: Macrolide-lincosamide-streptogramin B; MS Phenotype: Macrolide Streptogramin phenotype

Table 3 Phenotypic Detection of Methicillin Resistant *S. aureus*

Phenotype	MRSA	MSSA
MRSA	84 (64.6%)	46 (35.3%)

*MRSA: Methicillin Resistant *S. aureus*; MSSA: Methicillin Sensitive *S. aureus*

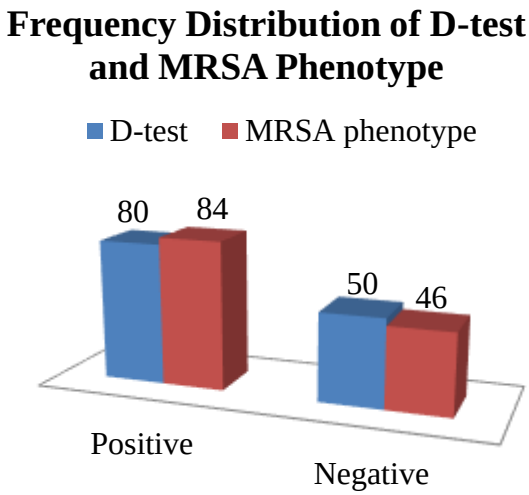

Figure 2 Phenotypic Detection of iCR and MRSA isolates

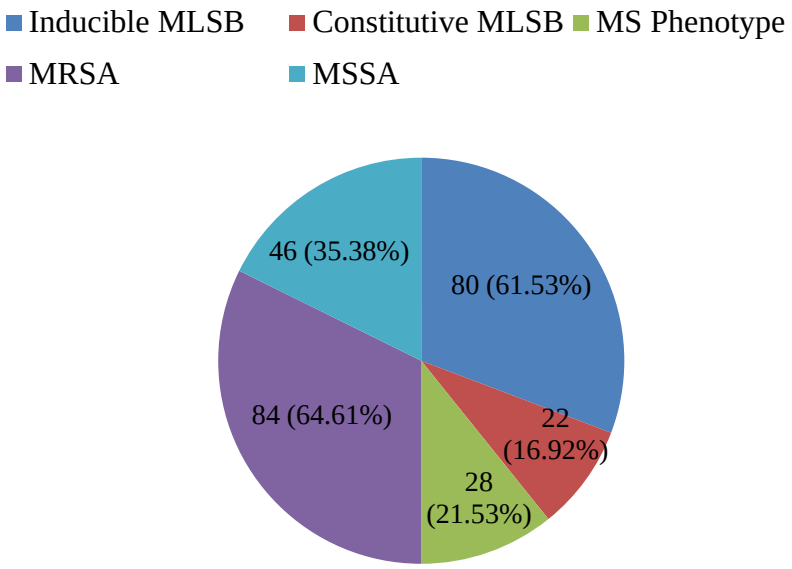

Figure 3 Phenotypic Detection of Clinical isolates

Discussion

Methicillin resistance is a global health issue since the past few decades affecting nosocomial and community settings. Penicillin's particularly Methicillin and Oxacillin were greatly considered for use previously, but due to emerging resistance to these antibiotics, has made us aware of treating this problem effectively. Similarly, MLSB family of antibiotics was the major option to cure staphylococcal infections. Among MLSB antibiotics, Clindamycin was mostly preferred to treat skin and soft tissue infections (SSTI's). Later on, this antibiotic became ineffective due to the presence of *erm* mutants in different strains of *S. aureus*. In this study, collection and processing of a total of 204 samples were carried out. Of 204 collected samples, 130 (63.72%) were *S. aureus* while 74 (36.27%) were Gram negative bacteria. Among 130 positive samples, 61(46.9%) were male, and 69 (53.1%) were female patients. In positive isolates, pus infections were the most prominent in all patients. The culture and sensitivity results indicates that *S. aureus* was isolated in the majority of pus samples 62 (47.69%), followed by wound 39 (30.0%), blood 21 (16.15%) and CSF 8 (6.15%), respectively. Staphylococcal strains become more resistant if they develop resistance to MLSB drugs and Penicillin's (Methicillin and Oxacillin). Our study revealed those strains of *S. aureus* which were inducible MLSB resistant and Methicillin resistant. Similar study was performed by Lall & Sahni (Lall and Sahni, 2014) to determine the frequency of iCR in *S. aureus* among clinical samples.in which 305 *S. aureus* isolates were collected and among them 140 (45.9%) were MRSA, and in entire 140 MRSA isolates, 52 (37.1%) were iCR as well.

Sensitivity pattern recorded against different isolates revealed the highest sensitivity against Tigecycline 129 (99.2%), followed by Fusidic Acid 126 (96.9%) and Doxycycline 124 (95.3%). A variation in sensitivity pattern was revealed by the rest of antibiotics i.e. Erythromycin 50 (38.4%), Clindamycin 28 (21.5%), Cefoxitin 46 (35.3%), Ciprofloxacin 31(23.8%), Vancomycin 44 (33.8%), Augmentin 55 (42.3%), Linezolid 119 (91.5%), Cefaclor 120 (92.3%), Cephradine 118 (90.7%), Cefotaxime 112 (86.1%) and Co-trimoxazole 32 (24.6%). Our antibiogram analysis is similar to the study of Kaleem (Kaleem et al., 2010) in which the pathogens showed 91% sensitivity to Tigecycline, 65% to Fusidic Acid, 41% to Doxycycline and 100% to Linezolid. The lowest sensitivity pattern was recorded against macrolide (Clindamycin) i.e. 22%, which correlates with our findings of 21.5% sensitivity to Clindamycin. Our study revealed that Tigecycline, Fusidic Acid and Doxycycline were highly effective with a sensitivity level of 99.2%, 96.9 and 95.3%. Clindamycin and Ciprofloxacin showed low efficacy with a sensitivity level of 21.5% and 23.8%, respectively. The current study was executed to detect the phenotype of iCR and MRSA from clinical samples of Khyber Teaching Hospital, Peshawar. D-test was carried out to identify iCR strains of *S. aureus*. The results showed that among 130 *S. aureus* isolates, 80 (61.5%) showed a positive D-test while 50 (38.4%) were negative. The D-test results of our study correlates with a study of Marais (Marais et al., 2009) in which among 248 collected MRSA isolates, 62.5% were D-test positive and 37.5% of the isolates were negative in this regard. Our study also detected MRSA isolates using Cefoxitin on MHA, revealing 84 (64.6%) MRSA and 46 (35.3%) MSSA isolates. A similar study was conducted by Frazee (Frazee et al., 2005) in which out of 119 isolates of *S. aureus* 89 (75%) isolates developed

MRSA phenotype while the rest of 39 (25.21%) isolates were MSSA.

Conclusion

The effective therapy to treat Methicillin resistance in *S. aureus* and CoNS has become problematic. So, routine monitoring of nosocomial infections and culture sensitivity tests for the resistant strains should be done to inhibit their proliferation in hospitals. Safety measures such as maintaining good hygiene by the hospital staff and preventive measures to control infection daily are mandatory to inhibit MRSA spread in hospitals. Routine antibiotic sensitivity fails to recognize inducible MLSB resistance; therefore, D-test, a simple and inexpensive method, must be implemented daily in each hospital to identify iCR strains of *S. aureus* and to combat the failure of Clindamycin therapy as well.

Declarations

Conflict of interest

The authors have no conflict of interest.

Data Availability statement

All data generated or analyzed during the study are included in the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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