

Original Article

Nasal Colonization and Antimicrobial Susceptibility Pattern of *Staphylococcus* Species among Children in Lahore, Pakistan

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Introduction: *Staphylococcus* is a genus of pathogenic bacteria, which asymptotically colonizes the upper respiratory tract of the human. The incidence of invasive Staphylococcal infections and the disease burden are high among children in South Asia, including Pakistan. This study aims to determine the nasal colonization and antimicrobial susceptibility pattern of *Staphylococcus* species isolated from preschool children in Lahore, Pakistan. **Methods:** A community-based study was conducted in two camps named Shah Di Khui and Jeevan Haana in Lahore city. A total of 100 nasal samples, were collected from preschool children from lower-middle-class families during January to March 2018. Species identification was performed using the coagulase test, catalase test, and Gram staining. Also, a 370 bp fragment of the *tuf* gene was targeted using specific primers for the genus *Staphylococcus*. Antibiotic resistance pattern of the isolates was defined by an antibiotic susceptibility test using a series of antibiotic discs. **Results:** The results of this study indicated the presence of *Staphylococcus* species, mainly *Staphylococcus aureus* in more than 85% of the children. PCR amplification of *tuf* gene confirmed the identity of the *S. aureus* isolates from the nasal cultures. Many showed resistance to more than two broad-spectrum antibiotics. **Conclusion:** The prevalence of nasal colonization of *S. aureus* was more than 85% among preschool children. Most of the isolates were resistant to β -lactam antibiotics. *J Med Microbiol Infect Dis*, 2018, 6 (4): 91-98.

Keywords: *Staphylococcus*, Drug resistance, Invasive burden, MRSA.

INTRODUCTION

The human body contains numerous microorganisms, the so-called microbiota that outnumbers human cells. The human nasal passage is one of the primary habitats for microflora as well as pathogenic agents. The nasal passage of the human nostrils leads to the nasopharynx and the upper back part of the throat. The microbial communities inhabiting the nasal passages of humans colonize soon after the birth and distinctly changes over the lifespan of an individual with high inter-individual variations. During a human lifetime, the bacterial colonization of the human nasal cavities and its variations are affected by various factors, including the development of the immunity, hormonal changes, and age. Other affecting factors include environmental variations, e.g., temperature, humidity, pollution, and airborne microbes [1].

In children, a very complex community of bacteria inhabit the nasal passages. Identification of composition and dynamics of these bacteria can provide more insights into the basis of respiratory diseases [2]. Many works have demonstrated that the structure of pediatric bacterial microbiota of the nose is affected by acute respiratory tract infections (ARIs). Some studies have also reported that bacterial flora of the nasal cavities plays a vital role in regulating various immune responses in humans [3]. The interaction between bacterial species in nasal passages and the host include mutualism, commensalism, and pathogenic associations [4]. The most common species of nasal microflora comprise *Staphylococcus aureus*,

Staphylococcus epidermidis, *Streptococcus pneumoniae*, *Micrococcus luteus*, *Haemophilus influenzae*, *Proteus vulgaris*, *Proteus mirabilis*, and *Bacillus* sp. [2, 3].

The members of the genus *Staphylococcus* are among the common bacteria inhabiting the nasal passages of children and *S. aureus*, *S. epidermidis*, and *S. hominis* are among the most prevalent species. These Gram-positive bacteria are catalase positive, non-motile, and facultative anaerobes. They cause acute to severe infections, such as serious skin infections, pimples, boils, carditis, meningitis, septicemia, arthritis, endocarditis, abscess, osteomyelitis, central venous catheter-associated bacteremia, pneumonia, and ventilator-associated pneumonia. These bacteria produce the enzyme coagulase and are characterized as pathogenic or relatively pathogenic bacteria [5].

Pathogenic strains of the genus *Staphylococcus* such as *S. aureus* are usually coagulase-positive. The coagulase-negative strains, e.g., *S. epidermidis*, are mostly less invasive.

However, they are progressively considered pathogens

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as they may cause nosocomial infections [6-7].

The bacteria *S. aureus* can pathogenically or non-pathogenically colonize humans [8]. It colonizes the nasal passages in about 33% of humans and has emerged as a significant antibiotic-resistant bacterium since 1960 [9]. One of the major underlying cause of antibiotic-resistance development is the inappropriate use of these medications. Apart from the inherent ability of microbes, agents, various factors including overuse and lack of access to appropriate combinations as well as failure to complete treatment regimens are involved in development of resistance. Moreover, technological advances and ease of travel contribute to the global spread of antibiotic resistance [1].

Resistance to almost all classes of antibiotics has been reported in different strains of *Staphylococcus* species. The DNA analysis of various strains of *S. aureus* has revealed the presence of virulence factors in these bacteria, explaining their pathogenic and invasive nature [10]. In addition to the presence of virulence factors and toxic genes, the occurrence of mutations in chromosomal and plasmid DNA is common and widespread in *Staphylococcus* species.

The antibiotics families macrolides, lincosamides, and streptogramins (introduced in 1952) kills the bacteria by targeting the bacterial 50S ribosomal subunit and inhibiting protein synthesis. However, shortly after their introduction for the treatment of staphylococcal infections, resistance, mainly among *S. aureus* strains, was observed. In this species, *ermA*, *ermB*, or *ermC* genes, located on plasmids or chromosomes, are responsible for ribosomal modification and making the antibiotics ineffective. Also, aminoglycoside resistance in *Staphylococcus* species is due to a chromosomal modification, leading to altered binding sites of this combination, *i.e.*, ribosomes [11]. Fluoroquinolones, initially introduced to treat Gram-negative infections, were also effective against some Gram-positive species including staphylococci through inhibition of bacterial DNA gyrase. Resistance against these combinations rapidly emerged as mutations developed in bacterial DNA gyrase gene. Resistance to β -lactam antibiotics is the most widespread antibacterial resistance in staphylococci, mainly *S. aureus* and *S. epidermidis*. The β -lactams are a family of broad-spectrum antibiotics including cephalosporins, penam-penicillin derivatives (methicillin, oxacillin, nafcillin, ampicillin), and carbapenems. [12]. The strains resistant to these antibiotics are classified as methicillin-resistant bacteria, *e.g.*, methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. epidermidis* (MRSE). The emergence of multi-antibiotic resistant strains, such as MRSA has made the treatment of the infections much more difficult [13].

As mentioned above, the environmental factors and climatic conditions play a major role in the development of the microbial flora. In Pakistan, climatic conditions show a high variation in different regions of the country, and in some parts of the country, the weather is hot and humid most time of the year. The variation of weather conditions contributes to the prevalence of different microbial flora, including *Staphylococcus* species. There is no regional study on the incidence and burden of invasive

staphylococcal species in children. There is also data on nasal colonization of *Staphylococcus* among pre-school children in Pakistan. This study aims to investigate the nasal colonization and antimicrobial susceptibility pattern of *Staphylococcus* species among children \leq five years of age in Lahore, Pakistan. The results of the study will be helpful in the diagnosis and eradication of *Staphylococcus*-induced pneumonia, the primary deadly disease in children of Pakistan.

MATERIAL AND METHODS

Collection of nasal samples. Two camps, Shah Di Khui (near Punjab University) and Jeevan Haana (near Barkat market) in Lahore Pakistan were selected. The nasal secretions were collected by sterilized swabs from healthy children \leq 5 years of age belonging to lower and middle-class families. The swabs were individually placed in the sterile tubes and transported to the laboratory.

The consent for the collection of specimens was obtained from childrens' parents. The study was approved by Higher Education of Pakistan S.No NRPU 4269.

Preparation of STGG transport medium. The skim milk-tryptone-glucose-glycerol (STGG) transport medium was prepared for inoculation and culture of nasal samples. The media was prepared by skim milk powder (2%), tryptone soy broth (TSB), (3%), glucose (0.5%), and 10% glycerol in distilled water. The media were sterilized and stored at 4°C until use.

Inoculation of STGG with nasopharyngeal swabs. The nasal samples were inoculated into vials containing 3 ml of STGG medium followed by overnight incubation in a 120 rpm shaking incubator at 37°C. The cultures were checked after 18 hours for bacterial growth.

Culture. Blood agar medium was prepared by dissolving blood agar base in distilled water followed by autoclaving. Once, the culture media cooled to 50 °C, 5% chicken blood was added to them. Amounts of 20 ml of the blood agar were dispensed into Petri dishes and allowed to cool and solidify, and then, sealed with parafilm and stored at 4°C. The plates were streaked by previously cultured nasal samples using the standard streaking technique, and sealed with parafilm and incubated at 37°C overnight. The following day, the plates were examined for bacterial growth.

Identification of *Staphylococcus* species. The *Staphylococcus* colonies appeared as round, creamy white colonies. The plates were also checked for beta hemolysis, which is the result of alpha-toxin production by *Staphylococcus* species. Further identification of the bacteria was performed by using standard Gram staining technique and coagulase, catalase, oxidase, and indole tests. For the catalase test, a bacterial colony was placed on a slide and mixed with a drop of distilled water to form a white suspension and then a drop of 3% hydrogen peroxide solution was added to it. The rapid evolution of O₂ along with bubble formation indicated a positive result.

For coagulase test, a bacteria colony was emulsified in a drop of water on a clean glass slide, and then some

undiluted plasma was picked with a sterilized loop and mixed with the milky solution of the bacterial colony. The clumping of cocci, visible to naked eye within 10 seconds, revealed coagulase positive isolates. For indole assay, the bacteria were grown in tryptic soy broth in 10-ml culture tubes for 24 hours. The following day, few drops of Kovac's reagent (isoamyl alcohol, para-dimethylaminobenzaldehyde (DMAB), and concentrated HCl), were added into the tube. Formation of a red ring at the interface indicated a positive indole result.

Oxidase test was performed using filter papers soaked with tetramethyl-p-phenylenediamine dihydrochloride substrate. The filter paper was moistened with sterile water, and a bacteria was smeared on the filter paper. Color change to purple or deep blue within 10-30 seconds indicated a positive oxidase result [14].

Identification of *S. aureus* by PCR. The identity of *Staphylococcus* spp. was confirmed by amplification of the *tuf* gene as described by others [15].

Table 1. The primers used in this study

Primer	Name	Sequence	The binding region on the gene	Size of PCR product
Forward primer	TStaG422	5'-GGC CGT GTT GAA CGT GGT CAA ATC A-3'	422-446*	
Reverse primer	TStag765	5'-TIA CCA TTT CAG TAC CTT CTG GTA A-3'	765-792*	370 bp

*The nucleotide positions are given with reference to the *tuf* gene sequence of *E. coli* (Accession no: J01690)

PCR amplification was performed in a 25 μ l reactions containing 5 μ l of microbial culture, 0.5 μ M of forward and reverse primers (Table 1), and 12.5 μ l of GoTaq Green Master Mix, (Promega, Madison, WI, USA), and nuclease-free water.

Antibiotic susceptibility assay. Bacteria susceptibility assays were performed by disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) reference methods [16]. For examination of the susceptibility pattern of the isolates, antimicrobial testing was performed by using standardized Kirby-Bauer disc diffusion test [17, 18] on 10 mm Mueller-Hinton agar plates (pH 7.2-7.4). The bacteria density of isolates in suspensions was adjusted to 0.5 McFarland standard. Amounts of 2 ml saline solution were prepared for each sample, and colonies from blood agar plates were suspended in salt solution. The prepared suspensions were then streaked 2-3 times onto Mueller-Hinton agar plates. The antibiotic susceptibility testing (including MRSA screening with cefoxitin as the recommended by NCCLS) [16], was performed using the antibiotic discs, cefoxitin (30 μ g), trimethoprim/

sulfamethoxazole (25 μ g), ciprofloxacin (5 μ g), erythromycin (15 μ g), amikacin (30 μ g), penicillin (10 units), novobiocin (30 μ g), and vancomycin (30 μ g) (Thermo Fisher Scientific, Hampshire, England). The plates were incubated at 37°C for 18-24 h and then were examined for bacterial growth. The clear zone of inhibition around each disk was measured by calipers.

RESULTS

Microflora. The cultures revealed different species of pathogenic microorganisms, including *S. aureus*, *Bacillus* spp., *Proteus mirabilis*, *Staphylococcus epidermidis*, *Acinetobacter* spp., *Haemophilus* spp., *Proteus vulgaris*, and *Macrococcus* species. The presence of *Staphylococcus* species was confirmed by different biochemical tests, including catalase, coagulase, oxidase, and indole tests as well as Gram staining. The species *S. aureus* was the most prevalent bacteria in the nasal samples with a prevalent rate of over 85%. Other species of bacteria and yeast cells were also identified in the nasal cavities of the children (Fig. 1).

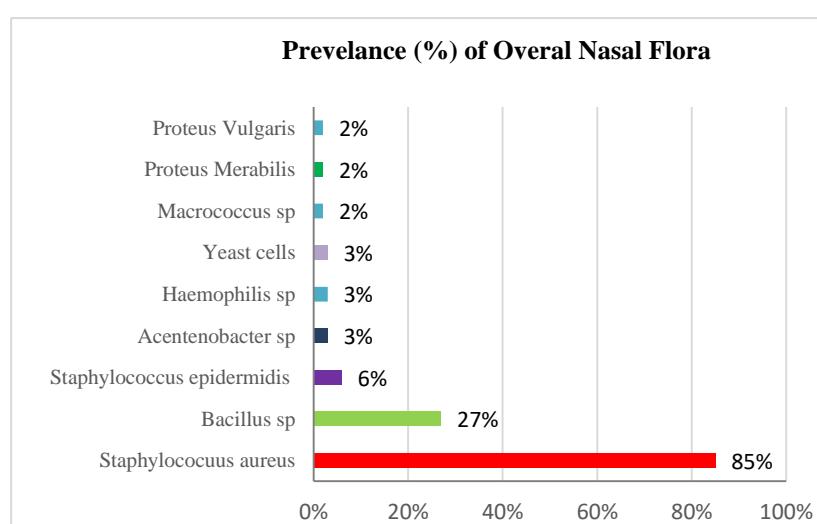


Fig. 1. Prevalence of pathogenic microbes in the nasal samples of children in Lahore, Pakistan

PCR Assay for *S. aureus*. The species *S. aureus* was identified by PCR amplification of a 370 bp fragment of the *tuf* gene specific to this species (Fig. 2).

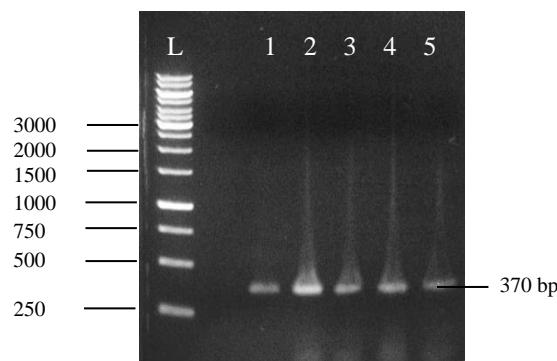


Fig. 2. PCR amplification of a 370 bp fragment of *tuf* gene specific to *S. aureus*. Lane L, DNA ladder; lanes 1-5 positive samples

Susceptibility pattern of *Staphylococcus* species. Different combinations of drugs were used to identify resistance and susceptibility pattern of the Staphylococcal species. The isolates exhibited the highest resistance rates to penicillin, erythromycin, trimethoprim/sulfamethoxazole, and ciprofloxacin (Table 2).

Sensitivity analysis of *Staphylococcus* species to antibiotics. In our study, most isolates were found to be resistant to more than 2 antibiotics. The bacteria species in the nasal samples showed the highest resistance to penicillin (98.90%) followed by erythromycin (81.32%), trimethoprim/sulfamethoxazole (79.12%), amikacin

(41.6%), ciprofloxacin (38.46%), cefoxitin (10.99%), and Novobiocin (3.30%). All the species of microbes were found to be sensitive to vancomycin with 0% resistance (Fig. 3). The number of resistant and susceptible isolates of *S. aureus* and *S. epidermidis* to the tested antibiotics are shown in the Figure 4.

DISCUSSION

Nasal colonization of *Staphylococcus* species is a risk factor for the development of lung diseases. Children are considered as the persistent carriers of nasal pathogens with the highest rate of the carriage. The role of nasal carriage in *S. aureus* infections has been extensively studied and reported worldwide [19]. The nasal carriage and prevalence of *S. aureus* vary in children with socioeconomic status, general health, and disease conditions [20]. In Japan, 17.5% of nasopharyngeal colonization of respiratory bacterial pathogens was observed among children attending day-care centers [21]. In India, nasal carriage of *S. aureus* among healthy preschool children of Ujjain was 6.3%, from of which 16.3% were methicillin-resistant (MRSA) isolates [22]. In Ghana, 22.1% of children showed colonization with *S. aureus*, with the highest carriage rates during the rainy seasons [23]. In Brazil, the highest colonization rates with *S. aureus* was reported among children (48%) with 6.2% identified as MRSA [23, 24]. The identity of *Staphylococcus* species in our study was confirmed by the PCR amplification of *tuf* gene, a 370 bp sequence specific to *Staphylococcus* genome (Fig. 2). The *tuf* gene encodes elongation factor tu (EF-tu), which is required for the peptide chain formation [15].

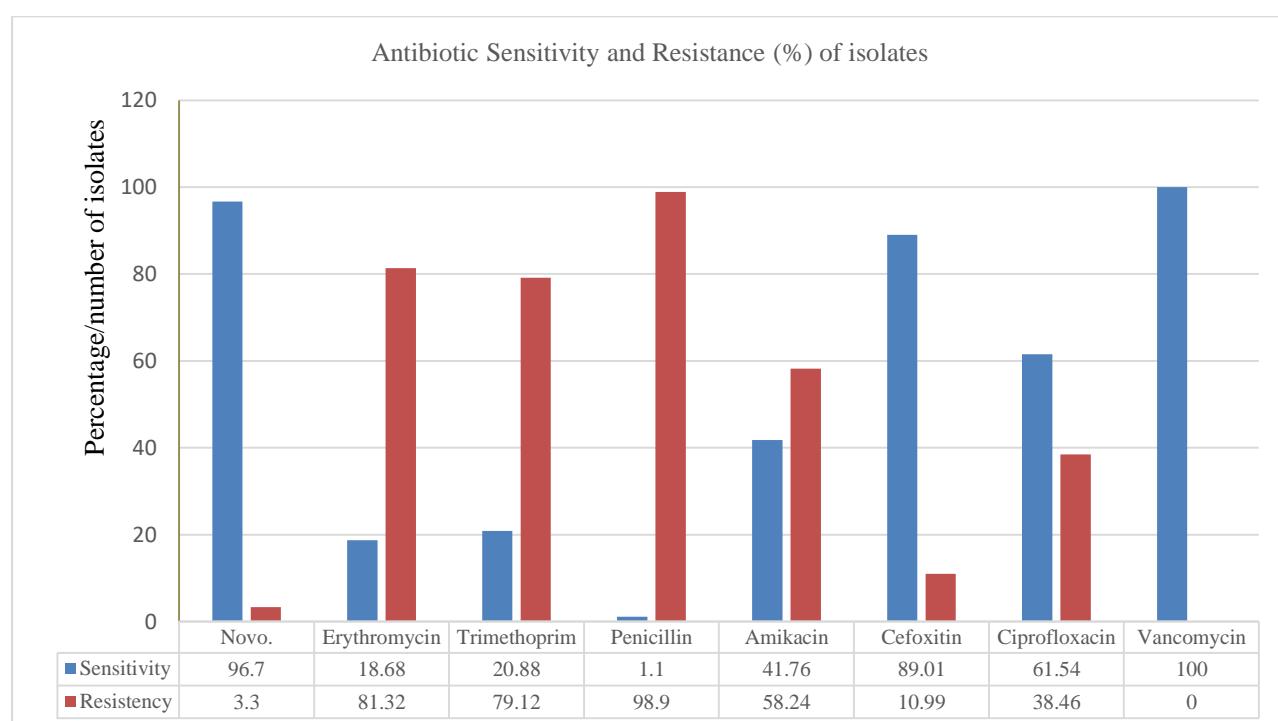


Fig. 3. Susceptibility pattern of *Staphylococcus* species in children under 5 years

Table 2. Susceptibility of *S. aureus* and *S. epidermidis* to the tested antibiotics

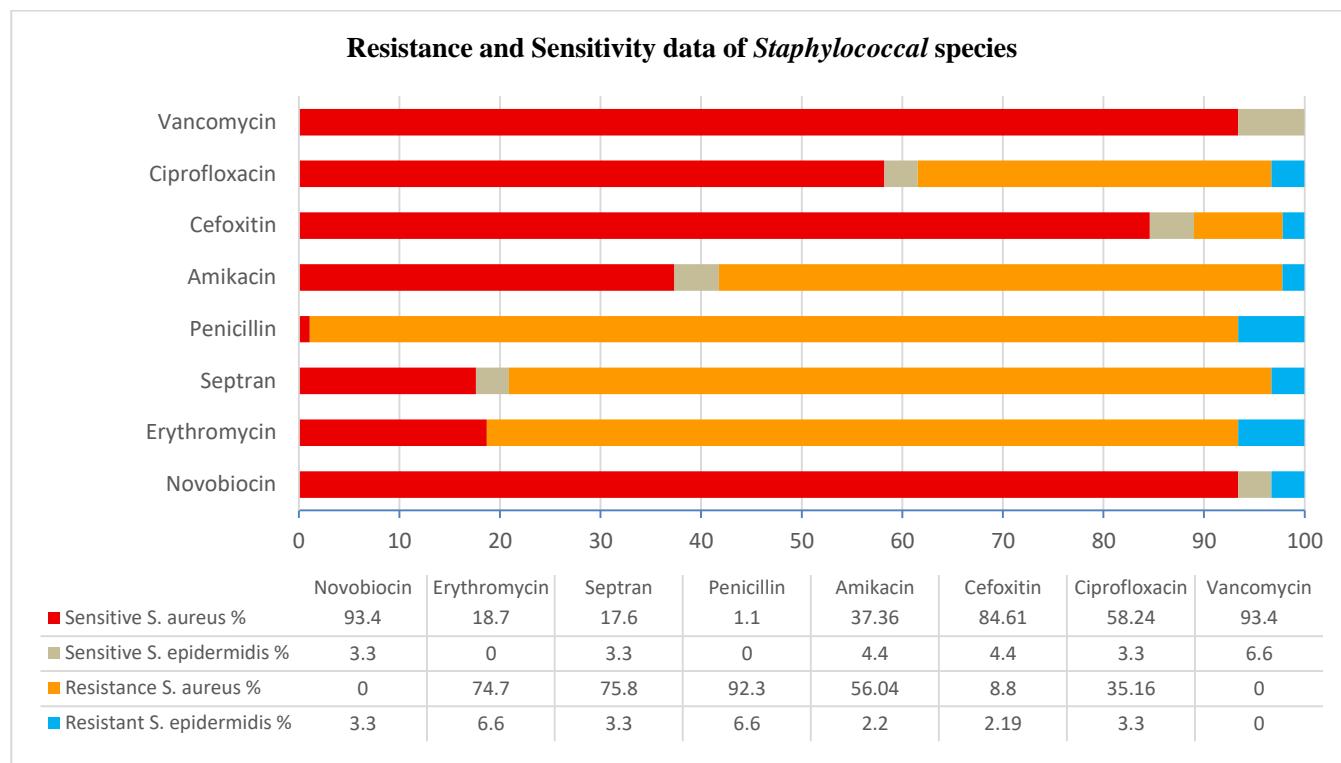


Fig. 4. Resistance and sensitivity data of *Staphylococcus* species to the tested antibiotics

Today, a large number of antibacterial combinations are available. Some combinations show similar behaviors to each other when assayed *in vitro*. Therefore, one drug can be tested to represent other similar or closely related compounds. In the present study, *Staphylococcal* species were tested against various antibiotics including cell wall synthesis inhibiting compounds and protein synthesis disrupting combinations, like erythromycin, trimethoprim-sulfamethoxazole, penicillin, ciprofloxacin, cefoxitin, vancomycin and two non-specific drugs, novobiocin, and amikacin. Today, methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) have emerged as a critical threat in both hospitals and communities [25]. Therefore, the samples were also tested to identify MRSA and MRSE. The compounds oxacillin or cefoxitin can be used as an alternative for all other β -lactams such as cephamycins), and susceptibility testing to these two drugs can predict resistance to all class of β -lactam antibiotics [26]. In a study, the cefoxitin disk diffusion test showed to be preferable to the oxacillin disk diffusion method for routine screening of MRSA. [27]. The bacteria species in the nasal samples showed the highest resistance to penicillin ($\approx 98.90\%$) followed by erythromycin (81.32%). Previous studies have also reported 100% resistance of *Staphylococcus* species to penicillin and high rates of resistance to erythromycin [28]. The highest resistance rate to a single drug was observed for penicillin, followed by trimethoprim/sulfamethoxazole, erythromycin, ciprofloxacin, and cefoxitin. In the two-drug combinations, the highest resistance was observed to penicillin and trimethoprim/sulfamethoxazole (71 isolates), followed by penicillin and erythromycin (66 isolates),

erythromycin and Trimethoprim/Sulfamethoxazole (52 isolates), penicillin and ciprofloxacin (29 isolates), ciprofloxacin and trimethoprim/sulfamethoxazole (25 isolates). In the three-drug combinations, the highest resistance was observed to the combination of penicillin, erythromycin, and trimethoprim/ sulfamethoxazole (61 isolates), followed by penicillin, ciprofloxacin, and trimethoprim/sulfamethoxazole (27 isolates), trimethoprim/sulfamethoxazole, erythromycin, and ciprofloxacin (25 isolates), and penicillin, erythromycin, and ciprofloxacin (25 isolates). In the four drugs combinations, the highest resistance was observed to erythromycin, penicillin, trimethoprim/sulfamethoxazole, and ciprofloxacin (23 isolates). Whereas, in the combination of five drugs, only 3 isolates were found to be resistant to the combination of trimethoprim/sulfamethoxazole, penicillin, erythromycin, cefoxitin, and ciprofloxacin. All other combinations of five drugs were efficient against all *Staphylococcal* isolates (Table 2).

Resistance and sensitivity data of *S. aureus* and *S. epidermidis* indicated high resistance to penicillin in both species. High resistance to trimethoprim/sulfamethoxazole and erythromycin was also observed. The susceptibility to cefoxitin and ciprofloxacin among the *S. aureus* and *S. epidermidis* isolates was higher compared to penicillin, erythromycin, and trimethoprim. All species of *Staphylococcus* were found to be sensitive to vancomycin (Fig. 4).

Staphylococcal species, especially *S. aureus* are very prevalent in our community and are the primary cause of

nosocomial and respiratory tract infections in children. In the current study, most of the isolates were identified to be *S. aureus*, and a smaller percentage were *S. epidermidis*. All the isolates were resistant to a minimum of 2 and a maximum of 5 antibacterial drugs. The MRSA was identified in 10% of the *S. aureus* isolates, whereas 33% isolates were MRSE. Our results provide a better understanding of the epidemiology and determinants of Staphylococcal nasal colonization and can help public health authorities in Pakistan to adopt proper control and prevention measures.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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