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Incidence of multidrug resistant coagulase negative *Staphylococci* in clinical samples of Tertiary care hospitals of Khairpur and Sukkur cities of Pakistan

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Key words: Coagulase-negative *staphylococci,* Multidrug resistance, Antimicrobial sensitivity profiling, Clinical samples, Tertiary care hospitals

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Abstract

Coagulase-negative staphylococci (CoNS) adopting multidrug resistance have been increasingly becoming a threat to the patients in tertiary care hospitals worldwide. The aim of present study was to find out the incidence of multidrug-resistant CoNS in various samples of clinical origin. A total of 280 clinical samples of different origin were collected from tertiary care hospitals of Khairpur and Sukkur cities of Pakistan. Coagulase-negative staphylococci (CoNS) were isolated and identified using routine microbiological techniques and molecular characterization using 16S rRNA sequence-based homology. The antimicrobial sensitivity was determined using Kirby-Bauer's disc-diffusion assay and penicllin zone-edge test. Overall, 44 (21.15%) samples were found positive for Gram positive staphylococci, i.e. 35(80%) coagulase-positive and 9(20%) coagulase-negative. The highest percent prevalence of CoNS was found in urine samples 2/6(33%) and ear swab 1/3(33%) followed by throat swab 1/4(25%) and pus 5/27(19.1%). The results of antibiotic sensitivity profiling revealed complete resistance of the pus isolate CoNS5 to all the antibiotics tested. The phylogenetic correlation of amplified 16S rRNA gene sequence of CoNS5 isolate shared 98% similarity with *Staphylococcus haemolyticus* ATCC 29970 (GenBank accession no. D83367). Prevalence of extended multidrug-resistant pathogen *S. haemolyticus*in clinical specimenscalls for timely control measures against next superbug after methicillin-resistant *S. aureus* (MRSA).

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Introduction

The spread and development of antimicrobial resistance to contemporary medicine by limiting the available counteractive options in Pakistan as it is in whole globe (Khan, *et al.,* 2014). Coagulase-negative staphylococci (CoNS) infections among infants with very low birth weight are most common causes of death (Stoll, *et al.,* 2002). CoNS are major nosocomial pathogens and among all CoNS, *S. hemolyticus* (14% - 32%) is second only to *S. epidermidis* (58% - 76%) in its frequency of isolation from human clinical samples (Vignaroli, *et al.,* 2006; Gheibi, *et al.,* 2008; Fredheim, *et al.,* 2009; Dimitriou, *et al.,* 2011 and Brzychczy-Wloch M, *et al.,* 2013). Recently reports have accepted *S. haemolyticus* as the cause of very dangerous hospital-acquired opportunistic infections, i.e. meningitis, prosthetic joint infections, skin and skin structure infections, endocarditis and bacterimia (Fredheim, *et al.*, 2009 and Kim JW, *et al.,* 2012).Infections caused by hospital pathogen is adjusted with empirical treatment based on information on persisting antibiotic resistance profile of the bacteria (Brzychczy-Wloch, *et al.,* 2013). An elevated percentage of *S. haemolyticus* and *S. epidermidis* isolates impending from clinical samples of male and female, are resistant to good number of antibiotics: methicillin (86% – 100%), gentamicin (80% – 100%), erythromycin (65% – 100%), oxacillin (92% – 100%), or clindamycin (80% – 100%) (Qu, *et al.,* 2010).

Antibiotic resistance is the quality of microbes to endure the inhibitory as well as cidal effects of antibiotics which solely illustrates the adaptability of microbes in their efforts for existence (Akelere, *et al.,* 2013). The number of genes are frequently involved in multidrug resistance (MDR) to normally available and used antimicrobial agents including extended-spectrum β-lactamases (ESBLs) encoding genes, exogenously acquired 16S rRNA methyl transferase (16S-RMTase) genes, plasmid-mediated quinolone resistance (PMQR) genes, carbapenem-hydrolyzing β-lactamases (CHβLs) genes, and methicillin-resistance *mec*A gene related with *S. haemolyticus* or *S. epidermidis* (Strahilevitz, *et al.,* 2009; Pereira, *et al.,* 2010; Naseer, and Sundsfjord, 2011; Wachino, and Arakawa, 2012).

Thus the extensive utilization of antibiotics has been accountable for the development of numerous problems including the emergence of multidrug resistant bacteria, increased number of hospital-acquired and community-acquired infections and increased health care costs (Akelere, *et al.,* 2013). A penicillin MIC of ≤0.12 mg/L is formally in the sensitive range, but the CLSI recommend that supplementary testing should be carried out for the detection of penicillinase or lactamase (Wikler, 2006). Furthermore, in the increasing importance of CoNS, *S. haemolyticus* has the maximum level of antimicrobial resistance among all the CoNS (Fredheim, *et al.,* 2009). This limits the therapeutic options available to treat the *S. haemolyticus* infections (Akelere, *et al.,* 2013), and forcing the scientists to look for the most successful ways to fight *S. hemolyticus* infections.

Indeed, *S. haemolyticus* was the first gram-positive pathogen to attaingly copeptide resistance prior to the other staphylococcal and enterococci species, and has been recommended to be single among CoNS in being predisposed to develop glycopeptide resistance which in this species may be multifactorial (Vignaroli, *et al.,* 2006). Therefore the present study was aiming at, antibiotic drug use, monitoring and evaluation of resistance (ADMER) in Pakistan. This study was conceived to find out the prevalence of the Multidrug resistant (MDR) forms of *S. hemolyticus* among the unassuming entire population, strengthen microbiology and surveillance of antibiotic resistance, ultimately to improve awareness of antimicrobial use in Pakistan.

Materials and methods

Sampling site and collection

A total of 280 clinical samples of urine, pus, blood, high vaginal swab (HVS), stool, ear, throat, cerebrospinal fluid and ascitic fluid and pleural fluid from different patients of age and gender (male and female) were collected at different health care units and clinical laboratories of Khairpur and Sukkur

cities for the isolation and antimicrobial sensitivity profiling of CoNS isolates.

Sample from each patient was collected in 150 ml sterile containers and transported to the Postgraduate Research Laboratory (PGRL), Department of Microbiology, Shah Abdul Latif University, Khairpur, within two hours of collection under ice cold conditions.

Isolation and characterization of bacterial isolates

The streaking plate technique was used in replicates for the isolation of staphylococci onto the surface of nutrient agar plates. Subsequently all the inoculated plates were subjected to incubation at 37°C for 18-24 hours and all the bacterial isolates obtained were stained using Gram's staining procedure as described by Cheesborough (2006) for the preliminary identification of the bacterial isolates. Furthermore, the coagulase test was performed and screening for the multidrug-resistant coagulase negative staphylococci (CoNS) was carried out. All the CoNS isolates were preliminarily identified based on cultural characteristics, microscopic characteristics, sugar fermentation and biochemical characteristics. All the growth media and biochemical test reagents were purchased from Oxoid (Oxoid, UK) and Sigma-Aldrich (Sigma-Aldrich, USA).

Molecular characterization by 16S rRNA gene sequencing

The bacterial isolate selected on the basis of its highly multi-drug resistant profile was subjected to molecular identification through commercial sequencing of the target gene, i.e.16S rRNA, from Macrogen Inc., Seoul, Korea. For this, a pair of universal primers viz. 27F and 1492R having nucleotide sequences 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-TACGGYTACCTTGTTACGACTT-3', respectively, were used for the amplification of target gene followed by purification of the amplified genetic sequences, while another pair of universal primers viz. 518F and 800R, i.e.5'-CCAGCAGCCGCGGTAATACG-3' and 5'-TACCAGGGTATCTAATCC-3', respectively, was used for sequencing of the amplified gene using ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystem, USA).

Finally, an automated ABI PRISM 3730 × l DNA Sequencer (Applied Biosystems, USA) was used to complete the electrophoresis of sequencing reaction.

Phylogenetic correlation analysis

After receiving the partial nucleotide sequence of 16S rRNA gene of the selected bacterium from Macrogen Inc., it was further compared with database of nucleotide sequences of other taxa using Basic Local Alignment Search Tool (BLAST) program availableon the National Center for Biotechnology Information (NCBI) website. The sequence was analyzed in order to confer the percentage sequence similarities. The Maximum Likelihood method was opted to infer the evolutionary history according to Tamura-Nei model (Tamura K, and Nei M, 1993). The phylogenetic tree was re-constructed after performing the evolutionary analyses using MEGA software (Version 6.0) according to Tamura (Tamura, *et al.,* 2013).

Antibiotic sensitivity profiling

Antimicrobial sensitivity profiling (ASP) was carried out using disk diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines (Dowzicky and Park, 2008; NCCLS, 2012). For this, Kirby-Bauer's disk-diffusion assay was performed by using Muller-Hinton agar against selected antibiotics namely: Amoxicillin clavunic acid (AMC), Sparfloxacin (SPX), Gentamycin (GM), Fosfomycin (FOS), Moxifloxacin (MXF), Fusidic acid (FD), Enoxacin (EN), Azomax (AZM), Piperacillin-Tazobactam (TZP) and Sulbactam (SCP). The results were interpreted according to the CLSI (Wikler MA, 2006 and NCCLS, 2012).

Results

The 280 clinical samples (162 from male and 118 from female) were randomly collected at different health care facilities of Khairpur and Sukkur cities of Pakistan. The uniqueness of present study was the very high samples number in order to accomplish the promising results. The maximum number of samples were collected from the pus followed by the urine whereas the minimum number of samples were

collected from the ascetic fluid and ear swab as shown in table 1.

Table 1. List of collected samples and gender-wise percent distribution of the samples.

S. No.	Sample site	Number of samples (% Distribution)		
		Male	Female	Total
1	Pus	75(46.3)	61(51.7)	136(48.6)
2	Blood	11(6.8)	3(2.5)	14(5)
3	Stool	3(1.9)	3(2.5)	6(2.1)
4	Pleural fluid	13(8)	5(4.2)	18(6.4)
5	ear	2(1.2)	1(0.8)	3(1.1)
6	Throat	7(4.3)	1(0.8)	8(2.9)
7	CSF*	4(2.5)	0(0)	4(1.4)
8	Ascitic	2(1.2)	1(0.8)	3(1.1)
9	HVS**	0(0)	10(8.5)	10(3.6)
10	Urine	45(27.8)	33(28)	78(27.9)
Total		162(100)	118(100)	280(100)

NOTE: **CSF- cerebrospinal fluid; *HVS-high vaginal swab.

The forty four isolates of staphylococci (16%) were isolated, separated and purified by repetitive streak plate method on the surface of nutrient agar. The frequency distribution of staphylococci in different clinical samples revealed maximum prevalence in throat (80%) followed by the ear swab (75%) > HVS (20%) > pus (19.1%)> stool (14.3%) > urine (7.5%) (fig. 1).

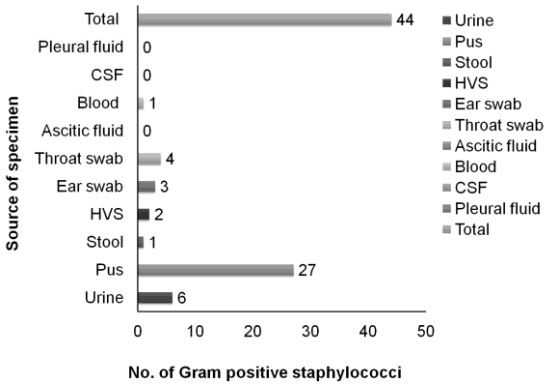

Fig. 1. Frequency distribution of Gram positive staphylococci in different samples of clinical origin.

The gender-wise prevalence of staphylococci indicated that in case of throat, urine and pus samples, the prevalence was significantly higher in male patients (i.e. 100%, 75% and 59%, respectively), while the HVS, stool and ear swab (100%, 100%, and 66.7%) samples showed higher staphylococcal infections in female patients. On the Contrary, the cerebral spinal fluid (CSF), ascitic fluid and in blood samples did not show any prevalence of staphylococci.

The throat sample of male patients displayed 100% prevalence of staphylococci, while prevalence in female was observed as zero. Moreover, the overall prevalence of staphylococci was higher in samples of female patient than the male patients (fig. 2).

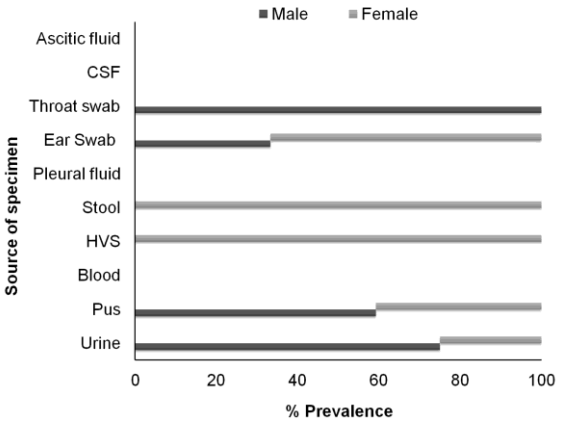

Fig. 2. Frequency distribution of staphylococci based on source of specimen and gender in different clinical samples.

ASP of CoNS was determined by Kirby- Bauer disk diffusion method. It was observed that all the CoNS isolates were resistant to at least more than five antibiotics tested. The results of preliminary identification based on morphology, microscopy, biochemical tests and sugar fermentation profile displayed that out of nine (09) CoNS isolates, three (03) isolates were *S. haemolyticus*, two (02) isolates each of *S. epidermidis* and *S. hominis,* and one (01) isolate each of *S. caprae* and *S. saprophyticus* (Table 2).

Among the isolates, *S. haemolyticus* NS2 (CoNS5 isolate) isolated from pus displayed complete resistance against all the antibiotics tested (Table 3), thus it was subjected to molecular characterization using 16S rRNA sequence homology. The phylogenetic correlation studies revealed that the amplified 16S rRNA gene sequence of CoNS5 isolate shared 98% similarity with *Staphylococcus haemolyticus* ATCC 29970 strain (GenBank accession no. D83367) available in NCBI GenBank database (fig. 3).

Table 2. Morphological and biochemical characteristics of the CoNS bacteria isolated in this study.

Bacterial isolate	ID test	CoNS1	CoNS2	CoNS3	CoNS4	CoNS5	CoNS6	CoNS7	CoNS8	CoNS9
Morphology & Biochemical Characteristics	CS	+	+	-	-	-	-	-	-	-
	SS	-	-	-	-	-	-	-	-	-
	M	-	-	-	-	-	-	-	-	-
	CAT	+	+	+	+	+	+	+	+	+
	CoAG	-	-	-	-	-	-	-	-	-
	Ox	-	-	+	+	+	+	+	+	+
	I	-	-	-	-	-	-	-	-	-
	MR	-	-	-	-	-	-	-	-	-
	VP	+	+	-	-	-	-	-	-	-
	CIT	-	-	-	-	-	-	-	-	-
	NR	+	+	+	+	+	+	+	+	+
	UT	+	+	+	-	-	-	+	+	-
Sugar fermentation	Glu	+	+	+	+	+	+	+	+	+
	Lac	+	+	-	+	+	+	-	-	+
	Mal	+	+	+	+	+	+	+	-	+
	Man	-	-	-	-	+	-	-	-	-
	Suc	+	+	+	+	+	-	+	-	+
Tentative identification		*S. epiderm-idis*	*S. epiderm-idis*	*S. hominis*	*S. haemoly-ticus*	*S. haemolyt-icus*	*S. saprophy-ticus*	*S. hominis*	*S. caprae*	*S. haemolytic us*

CS	Capsule Staining	CAT	Catalase	VP	Vogesproskaur	Lac	Lactose
SS	Spore staining	CoAG	Coagulase	CIT	Citrate	Mal	Maltose
M	Motality	Ox	Oxidase	NR	Nitrate reduction	Man	Mannitol
I	Indole	UT	Urease test	Suc	Sucrose		
MR	Methyl red	Glu	Glucose				

Table 3. The antimicrobial sensitivity profile of the coagulase negative staphylococci (CoNS).

Specimen	Isolate	Antibiotics and their sensitivity										% Sensitivity* *N* (%)
		AMC	SPX	CN	FOS	MXF	FD	EN	AZM	TZP	SCP	
Urine	CoNS1	S	R	R	S	R	R	R	R	S	R	3 (30%)
Urine	CoNS2	R	S	R	R	R	R	R	R	S	R	2 (20%)
Pus	CoNS3	R	S	R	R	S	R	R	R	S	R	3 (30%)
Pus	CoNS4	R	R	R	R	R	R	R	R	S	R	1 (10%)
Pus	CoNS5	R	R	R	R	R	R	R	R	R	R	0 (0%)
Pus	CoNS6	R	S	R	R	S	R	R	R	S	S	4 (40%)
Pus	CoNS7	R	R	R	R	R	R	R	S	R	R	1 (10%)
Ear swab	CoNS8	S	R	R	R	S	R	R	S	R	R	3 (30%)
Throat swab	CoNS9	R	S	R	R	R	R	R	R	S	R	2 (20%)
% Resistance** *n* (%)		7(77.8%)	5(55.6%)	9(100%)	8(88.9%)	6(66.7%)	9(100%)	9(100%)	7(77.8%)	3(33%)	8(88.9%)	

*(aE/aT*100 = No. of effective antibitoitcs/total no. of antibiotics tested X 100, where aT=10); *N* = number of effective antibiotics

**(iR/iT*100 = No. of 'R' isolates/Total no. of isolates X 100, where iT=9); *n* = number of resistant isolates

R	Resistant	AMC	Aoxicillin-Calvulanic acid	EN	Enoxacin
S	Sensitive	SPX	Sparfloxacin	AZM	Aztreonam
iR	No. of Resistant isolates	CN	Gentamycin	TZP	Tazobactam-Piperacillin
iT	Total isolates tested	FOS	Fosfomycin	SCP	Sulbactam
aE	No. of effective antibiotics	MXF	Moxifloxacin		

aT Total antibiotics tested FD Fusidic acid

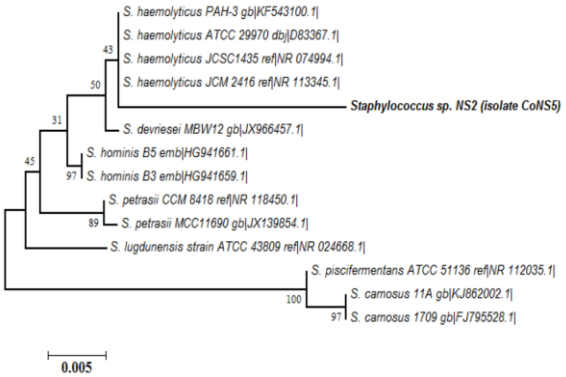

Fig. 3. Molecular phylogeny of coagulase-negative staphylococci isolate CoNS5 (i.e. *Staphylococcus sp.* NS2) with other closely related taxa.

Thepenicillin zone edge test was performed for detection of penicillinase or lactamase production. The results of present study displayed significant lactamase production by *S. haemolyticus* resulting in complete absence of inhibitory zone with penicillin fig. 4).

Various strains were resistant to one or more of the following antibiotics: penicillin, cephalosporins, macrolides, quinolones, tetracyclines, aminoglycosides, glycopeptides and fosfomycin.

Discussion

In order to fight bacterial infections successfully, the rapid recognition of proper treatment modalities are critical. The determination of antibiotic susceptibility and resistance are keys to this process (Khan, *et al.,* 2014). Thus in present study 280 clinical samples were successfully collected in order to investigate the prevalence and antimicrobial resistance profile of *S. haemolyticus.* Han and his fellows (Han, *et al.,* 2013) isolated a total of 32.7% staphylococci isolates from 55 animal samples and all were identified as CoNS. Conversely, in present study, out of forty four (44) isolates of Gram positive staphylococci, only 9 (20%) were found to be CoNS. The results of antibiotic sensitivity profiling of CoNS isolates revealed complete resistance of the isolates against gentamycin, enoxacin and fusidic acid, while the isolates displayed varying resistance rate against rest of the antibiotics tested.

The resistance rates, in the order of higher to lower, were found to be: CN=EN=FD>FOS=SCP (88.9%) > AMC=AZM (77.8%) > MXF (66.7%) > SPX (55.6%) > TZP (33%), according to CLSI guidelines (NCCLS, 2012).

Notably, the disagreement among susceptibility forecast and antibiotic efficiency can occur. Conventional ASP using disk-diffusion and MIC determination techniques may rarely fail to take resistance into account or misreport antibiotic susceptibility and special tests may be essential to perceive resistance mechanisms (Thomson, 2010; Roth, *et al.,* 2012; Thomson, 2013; Khan, *et al.,* 2014). Another example is that the CLSI counsel performing tests to discover β- lactamase production on staphylococci for which penicillin zone diameters are ≥ 29 mm or MIC ≤ 0.12 µg/ml, before reporting isolates as susceptible (NCCLS, 2012), which suggests that additionally taking β-lactamase production into consideration may be important. It is noteworthy to mention here that the *S. haemolyticus* CoNS5 isolate also showed resistance to the choice treatment Vancomycin (fig. 6). Our studies are congruent with previous studies where *S. haemolyticus* was reported to have highest level of antibiotic resistance among the CoNS (Feßler, *et al.,* 2014), where they also found multidrug resistance in *S. haemolyticus* isolates. Various strains were resistant to one or more of the following antibiotics: penicillin, cephalosporins, macrolides, quinolones, tetracyclines, aminoglycosides, glycopeptides andfosfomycin. Cavanagh and his fellows (Cavanagh, *et al.,* 2014) demonstrated that *S. haemolyticus* was the second most frequently isolated CoNS species from human blood cultures, after *S. epidermidis*. Besides, *S. haemolyticus* is often resistant to commonly used antimicrobial agents and is ranked as the most antibiotic-resistant CoNS species (Feßler, *et al.,* 2014 and Cavanagh, *et al.,* 2014).

Conclusions

Our study is the largest epidemiological study carried out in this region of Pakistan. The resistance profile of the CoNS isolates will be important for future

surveillance studies to determine the evolution of resistance levels and mechanisms at national, regional and international level.

The multi-drug resistance profiles obtained for the isolate of coagulase-negative *S. haemolyticus* isolated from diverse centers of Pakistan from both genders of diverse clinical origin of infections indicated that there is a need to constantly monitor the resistance in such strains. At present, we need the new or novel antimicrobial agents in order to cope with the alarming situations.

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