ORIGINAL RESEARCH PAPER

Clinical Microbiology

STUDY OF SLIME PRODUCTION AND ANTIMICROBIAL SUSCEPTIBILITY OF VARIOUS CLINICAL ISOLATES OF COAGULASE –NEGATIVE STAPHYLOCOCCI

KEY WORDS: CoNS-Coagulase- negative Staphylococci,Slime, congo red agar method ,Antibiotic susceptibility.

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ABSTRACT

Background - CoNS are one of those amongst the commonly isolated organisms in the clinical microbiology laboratory and Slime production is considered to be significant virulence factor for CoNS. The importance of slime is further increased by its frequent association with reduced antibiotic susceptibility. **Aims and Objectives-** The present study was undertaken with an aim to identify the species of coagulase negative staphylococci isolated most commonly from clinical specimens, to detect production of slime by them and to find their resistance to antimicrobial agents . **Material and Methods -**The present study was carried out in Department of Microbiology, at our tertiary care hospital during Nov 2012 to Oct 2014. **Inclusion Criteria-**All properly collected, well labeled samples of the indoor patients .All samples were processed,species identification done,slime production were compared by using three methods, & their antibiotic susceptibility were compared. **Result -** Out of the total 234 isolates, CoNS infections were more common in males i.e. 138 (58.97%) cases, and 96 (41.03%) were from females. Slime production in CoNS isolates was assessed by tube method, microtitre plate method and Congo red agar method. Sensitivity and specificity of tube method and congo red agar method as compared to microtitre plate method was assessed. It shows sensitivity of 58.22% and 20.55% in tube method and congo red agar method respectively, and specificity of 95.45% and 96.59% in tube method and congo red agar method respectively as compared to microtitre plate method. Antimicrobial susceptibility pattern of CoNS isolates on Kirby Bauer disk diffusion. Maximum CoNS isolates were resistant to penicillin 229 (97.86%) followed by amoxicillin-clavulanic acid 187 (79.91%), cotrimoxazole 171 (73.07%), ceftazidime164 (70.08%), gentamycin 154 (65.81%), cefoxitin 153 (65.38%), ofloxacin 152 (64.96%), erythromycin 129 (55.13%), rifampacin 82 (35.04%). **Conclusion-**The study documents the importance of CoNS as important Gram-positive pathogen special in hospital settings, resistant to commonly used antibiotics. There is a need to have the knowledge of slime production, monitoring of antibiotic sensitivity pattern and formulation of definite antibiotic policy to improve the empirical approaches to the therapy of serious infections caused by CoNS.

INTRODUCTION

Micro-organisms are still the important factors which are worsening the living conditions of man despite decades of dramatic progress in the prevention and treatment of diseases. To add to the misery of mankind, even the commensals are now being increasingly identified as the cause of diseases in special conditions like decreased host resistance. One such group of commensals is coagulase negative staphylococcus (CoNS).[1]

Staphylococcus is the second most commonly isolated microorganism from the clinical specimens in the microbiology laboratory with the exception of enterobacteriacae. Coagulase positive staphylococcus i.e. Staphylococcus aureus is well equipped with variant of virulence factors and is by far the most important pathogen amongst the staphylococci. Historically, S. aureus has been regarded as opportunistic pathogen whereas CoNS have been generally regarded as non pathogens.[2].

In hospital microbiology laboratory, staphylococcus isolation is often limited to coagulase test for S. aureus while non S. aureus isolates are simply reported as CoNS.[3] CoNS are one of those amongst the commonly isolated organisms in the clinical microbiology laboratory. The ubiquity of these organisms does present problems when one is faced with making a decision as to whether their isolation in clinical laboratory represents true infection or merely contaminant[4].

CoNS as opposed to S. aureus are not equipped with wide spread spectrum of virulence factors.[5] The postulated reasons for their current prevalence, their clinical importance and the resistance pattern include their great number, their selection as a result of widespread usage of broad spectrum antibiotics in the hospital, their ability to adhere to and form biofilms on the surface of devices and their meager nutritional requirement.[1] In CoNS, a loosely bound exopolysaccharide layer (slime) has been found in addition to capsule which has been associated with sepsis, intravenous catheter related bacteraemia and other prosthetic device infections.[6,7] Slime production is considered to be significant virulence factor for CoNS.[8,9] The importance of slime is further increased by its frequent association with reduced antibiotic susceptibility.[10]

The determination of antimicrobial susceptibility of clinical isolates is often crucial for optimal therapy of infected patients. This is particularly important considering the increase of resistance and emergence of multidrug resistant organisms.[11] CoNS strains are commonly multi-resistant to various groups of antibiotics. In reports from different parts of Europe, methicillin resistance in CoNS varies between 70 to 80 % whic Thus it has become important to identify CoNS to species level and to determine their antimicrobial susceptibility for effective therapeutic intervention.h is similar to that of U.S, Canada and Latin America.[12-14]

Regarding to slime production and the consequent biofilms formation, many aspects still need to be explored since divergences exist in the literature. Therefore, further studies about the formation of biofilms by these micro organisms are necessary, as well as the improvement of detection methods and treatments to prevent their production.[15]

AIMS AND OBJECTIVES

The present study was undertaken with an aim to identify the species of coagulase negative staphylococci isolated most commonly from clinical specimens, to detect production of slime by them and to find their resistance to antimicrobial agents .This aim was achieved by the following objectives:

1) Isolation, genus identification and species identification of infection/colonization strains of coagulase negative staphylococci by conventional test methods.
2) Demonstration of slime production by the isolated species of coagulase negative staphylococci and comparison of various methods for the detection of slime

production.

3) Demonstration of antimicrobial susceptibility of isolated species of coagulase negative staphylococci to various classes of antimicrobials.

MATERIAL AND METHODS

The present study was carried out in Department of Microbiology, at our tertiary care hospital during Nov 2012 to Oct 2014.

All properly collected, well labeled samples of the indoor patients coming to the department for microbiological investigations were included in our study.

I] Collection, Transportation And Processing Of Specimen In The Laboratory

Clinical specimen i.e. urine, blood, pus, CSF and body fluids received in microbiology laboratory of our tertiary care center were collected aseptically in accordance with the standard recommendations from the patients admitted in various wards of Government Medical College and Hospital.

The labelled specimens were transported immediately, along with the requisition form, to the Microbiology laboratory for processing.

The specimens received in the Microbiology laboratory were processed in accordance with the recommended procedures for the isolation and identification of bacteria.[16] After initial scrutiny and screening process the samples used for final processing were blood (bacterial endocarditis, septicaemia), pus, urine (significant bacteriuria), cerebrospinal fluid, fluids(pleural, ascitic, drain-microscopically significant), catheter tips and aspirates.

II] Isolation, Presumptive Identification And Storage Of Isolates / Strains

1) Isolation

In accordance with the recommended procedures, the received samples were screened by microscopy and specimen showing gram positive cocci in cluster were inoculated on Blood agar and Mac Conkey agar.[17]

2) Presumptive Identification

The growth was identified by colony morphology, gram staining and biochemical reactions:

a) **Blood agar:** Large white / cream / yellow, 1 - 3 mm diameter, smooth, low convex / dome shaped , with entire edges, opaque, showing beta-hemolysis / no hemolysis, butyrous in consistency on blood agar plates were studied by Gram stain.

b) **Mac Conkey agar:** very small colonies, < 0.5 mm in diameter, opaque, lactose fermenting/ non lactose fermenting.

Gram Stain

Gram positive cocci uniform in size, appearing characteristically in groups, but also seen singly and in pairs were further identified by the scheme described for the identification of the gram positive cocci arranged in clusters using tests given below.

3) Storage

All the isolates /strains, presumptively identified were stored at minus 70°C in Tryptic soy broth for future retrieval to perform various tests, slime production testing and antibiotic sensitivity testing. All strains were sub cultured at monthly intervals. Subsequently, all the 234 cultures presumptively identified as coagulase negative staphylococci were retrieved for testing.[16]

III] Identification Scheme Of Coagulase Negative Staphylococci

1) Genus Identification

Staphylococcal colonies were subjected to

a) Catalase Test[7]

This demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. Staphylococcus and Micrococcus are catalase positive.

A part of isolated colony was picked up with a sterile clean glass rod or wooden applicator stick and inserted into a small clean test tube containing 3% hydrogen peroxide solution. Immediate production of effervescence indicated positive catalase test.

Positive control- Staph aureus

Negative control- Streptococci

b) Modified Oxidase Test;[7]

Staphylococcus are modified oxidase negative whereas Micrococcus is modified oxidase positive.

Principle

The cytochrome oxidase system of Micrococcus contains cytochrome C, which yields a colored end product in presence of modified oxidase reagent.

Procedure

Few drops of reagent were added to the filter paper strip. A colony from nutrient agar plate was smeared onto the reagent zone of the filter paper.

Interpretation

Positive organism turned blue - purple within 30 seconds and negative remained colorless.

Positive control- Staphylococci

Negative control- Micrococci

c) Coagulase Test[17]

Staphylococcal coagulase is a protein of unknown chemical composition that has prothrombin-like activity, which can convert fibrinogen into fibrin, forming a visible clot. Both slide and tube coagulase tests were done in every staphylococcal isolate with positive & negative controls.

i) Slide Coagulase Test:[17]

Principle: Slide coagulase determines the bound coagulase (clumping factor). Clumping factor is attached to bacterial cell wall and is not present in culture filtrates. Fibrin strands are formed between the bacterial cells when suspended in plasma (fibrinogen), causing them to clump into visible aggregates. The test is falsely negative in 5-10 % Staphylococcus aureus strains which are otherwise positive by tube coagulase test.

Procedure: Two drops of saline were placed in two separate circles drawn on the slide with a wax pencil. Colony material from the organism to be identified was gently emulsified in saline in each circle. After checking for absence of autoagglutination, a drop of undiluted heparinised plasma was added to the emulsion in one of the circles and mixed well. A drop of saline was added to another circle as a control. The slide was rocked back and forth, and observed for agglutination of the test suspension.

Interpretation: Appearance of coarse clumps within 10-15 seconds indicates the positive test. The test was considered as negative if no agglutination was observed after two minutes. The saline control should remain smooth and milky. If the control suspension agglutinates as well, the test was as un-interpretable.

All isolates were further processed by tube coagulase test.

Positive control- Staph aureus

Negative control- Staph epidermidis

ii) Tube Coagulase Test :[17]

Principle: Tube coagulase test determines the free coagulase. Free coagulase is a thrombin - like substance present in culture filtrates. When a suspension of coagulase - producing organism is prepared in plasma in a test tube, a visible clot is formed as the result of coagulase reacting with a serum substance, CRF (coagulase -reacting factor) to form a complex that, in turn, reacts with fibrinogen to produce fibrin clot.

Procedure

Tube coagulase test was performed by any of the following procedures:

a. 1 in 6 dilution of plasma saline was prepared and 1 ml volume of diluted plasma was placed in small tubes. A colony of Staphylococcus under test / loopful of overnight broth was emulsified in a tube of diluted plasma.
b. A small amount of colony growth of the organism was emulsified in a tube containing 0.5 ml of coagulase plasma. Along with the positive and negative control, a tube of unseeded plasma was included to confirm that it did not clot spontaneously. The tubes were incubated at 37^{0} C for up to 4 hours and examined at 1, 2 and 4 hrs for clot formation by tilting the tube through 90^{0}. If negative, tubes were left at room temperature overnight and re-examined.

Interpretation: Any degree of clotting noted was considered as positive tube coagulase test.
Positive control- Staph aureus
Negative control- Staph epidermidis

Detection Of Biofilm (Slime Production)

All the species of CONS were tested for one of the important virulence factor i.e. slime production by three methods viz. microtitre plate method, congo red agar method and tube method.

1) Tube Method (TM) [8]

10mL tryticase soy broth was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with Phosphate-buffered saline (pH 7.3) and dried, dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as absent, weak, moderate or strong

2) Congo- red Agar Method (CRA) [18]

Congo red Agar medium was composed of BHI (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

3) Microtitre Plate Method (TCP) [19]

It is a quantitative assessment of the slime production.

Isolates from fresh agar plates were inoculated in Tripticase soya broth (TSB) with 1% glucose and incubated for 18 hours at 37^{0}C and diluted 1 in100 with fresh medium. Individual wells of sterile 96 well-flat bottom 96 plates were filled with 0.2 ml aliquots of the diluted cultures and plain broth served as control to check sterility and non-specific binding of media.

The microtitre plate was incubated for 24 hours at 37°C. Content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'plank tonic' bacteria. The wells were stained with crystal violet (0.1 % w/v). Excess stain was rinsed off by thorough washing with water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 570 nm. OD readings from sterile medium and dye were averaged and subtracted from all test values. The mean OD value obtained for media control well was deducted from all the test OD values.

Table 1 : Interpretation Of Bio Film Based On OD Value

OD value	Biofilm
<0.120	Non producer
0.120 — 0.240	Moderate producer
>0.240	Strong producer

The comparative statistical analysis for two methods (tube method and congo red agar method) by using 2x2 table was done. Data obtained from microtitre plate method, considered as gold standard for this study was compared with data from tube method and congo red agar methods.

Parameters determined were
Sensitivity = a / (a + c)
Specificity = d / (b + d)
Wherein a, b, c and d refer to number of determinants in which;

True Positives (a) - Biofilm producers by TM and CRA as well as positive by microtitre plate method (gold standard)
False Positives (b) - Biofilm producers by TM and CRA method but negative by microtitre plate method
False Negatives (c) - Non biofilm producers by TM and CRA but biofilm producers by microtitre plate method
True Negatives (d) - Non biofilm producers by all the methods.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was performed as per the CLSI guidelines (2012) by modified Kirby-Bauer method.

1) Medium

Mueller-Hinton agar (Hi-media laboratories Pvt, Ltd. Mumbai) was prepared from dehydrated base as per manufacturer's recommendations.

2) Inoculum

The inoculum was prepared from the primary culture plate by touching with a straight wire the top of the 3-5 colonies of similar appearance of the organism to be tested. This growth was transferred to a tube of sterile saline. The tube was compared with the 0.5 McFarland turbidity standard, and the turbidity of test suspension was adjusted to that of the standard.

3) Procedure

The plates were inoculated by dipping a sterile swab into the inoculum. Excess inoculum was removed by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. The swab was streaked all over the surface of the medium three times, rotating the plate through an angle of 60^{0} after each application. Finally, the swab was passed around the edge of the agar surface.

4) Antibiotic Disks

Commercially available antibiotic disks (Hi-media laboratories Pvt. Ltd. Mumbai) with proper diameter and potency were used. All the strains were tested for their sensitivity to antimicrobial drugs using recommended CLSI guidelines (2012) combined with institutional antibiotic policy and hospital formulary practices for the purpose of reporting to the clinician.

- Penicillin 10 IU
- Amoxicillin-clavulanic acid 30 µg
- Cefoxitin 30 µg
- Ceftazidime 30 µg
- Erythromycin 15 µg

- Vancomycin 30 µg
- Linizolid 30 µg
- Pristinomycin 15 µg
- Ofloxacin 5 µg
- Gentamycin 10 µg
- Cotrimoxazole 25 µg
- Nitrofurantoin 300 µg

5) Application Of Antibiotic Disks On The Inoculated Plate

The above mentioned antibiotic disks were placed on the agar surface of inoculated plates. A maximum of six disks were placed on 9 cm plate. Each disk was gently pressed down to ensure even contact with the medium which were then incubated at 37°C for 18-24 hours aerobically and observed for zone of inhibition.

6) Reading And Interpretation

Diameter of the circular zone of inhibition including the antibiotic disk was measured. Interpretation as sensitive, intermediate or resistant was done with reference to CLSI guidelines (2012).

RESULTS

The study was conducted during the period from November 2012 to October 2014. Isolates of Coagulase Negative Staphylococci from the various clinical specimens received in the diagnostic bacteriology laboratory from inpatient and outpatient departments were included in this study.

We processed a total of 12358 clinical samples in our laboratory and were able to isolate 234 Coagulase Negative Staphylococcial strains from these specimens

Table no. 2 shows that out of the total 234 isolates, CoNS infections were more common in males i.e. 138 (58.97%) cases, and 96 (41.03%) were from females.

Table No.2 SexWise Distribution Of CoNS Isolates

Sex	Number(234)	Percentage
Male	138	58.97%
Female	96	41.03%

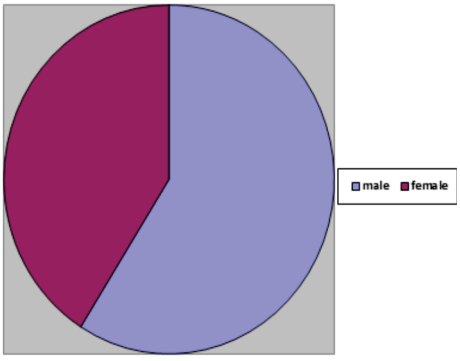
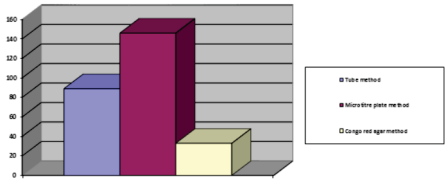


Fig 1 Showing SexWise Distribution Of CoNS Isolates

Slime production in CoNS isolates was assessed by tube method, microtitre plate method and Congo red agar method. Table no. 3 shows detection of slime production in CoNS isolates by different methods. Maximum slime production was shown by microtitre plate method 146 (62.39%) isolates, followed by tube method 89 (38.03%) isolates and 33 (14.1%) by congo red agar method.

Table No. 3 Slime Production In CoNS By Different Method

Method	Number of isolates	Percentage
Tube method	89	38.03%
Microtitre plate method	146	62.39%
Congo red agar method	33	14.10%



Graph 1 Showing Slime Production In CoNS By Different Method

Sensitivity and specificity of tube method and congo red agar method as compared to microtitre plate method was assessed and is shown in Table no 4.

It shows sensitivity of 58.22% and 20.55% in tube method and congo red agar method respectively, and specificity of 95.45% and 96.59% in tube method and congo red agar method respectively as compared to microtitre plate method

Table No. 4 Sensitivity And Specificity Of Different Methods As Compared To Microtitre Plate Method

Method	Sensitivity	Specificity
Tube method	58.22%	95.45%
Congo red agar method	20.55%	96.59%

Table No. 5 Slime Production In Different Species Of CoNS By Microtitre Plate Method

Species	Total number of isolates	Number of slime producers	Percentage
S. epidermidis	109	81	74.31%
S. saprophyticus	26	20	76.92%
S. haemolyticus	61	31	50.82%
S. lugdunensis	20	8	40.00%
S. hominis	6	2	33.33%
S. capitis	8	3	37.50%
S. warneri	0	-	-
S. simulans	1	0	0.0%
S. xylosus	3	1	33.33%
TOTAL	234	146	62.39%

Table no. 5 shows maximum slime production in S. saprophyticus isolates (76.92%), and S. epidermidis isolates (74.31%) followed by S. haemolyticus isolates (50.82%), S. lugdunensis isolates (40.0%). Single isolates of S. simulans which was isolated did not show slime production.

Table no. 6 shows the antimicrobial susceptibility pattern of CoNS isolates on Kirby Bauer disk diffusion. Maximum CoNS isolates were resistant to penicillin 229 (97.86%) followed by amoxicillin-clavulanic acid 187 (79.91%), cotrimoxazole 171 (73.07%), ceftazidime 164 (70.08%), gentamycin 1 5 4 (65.81%), cefoxitin 153 (65.38%), ofloxacin 152 (64.96%), erythromycin 129 (55.13%), rifampacin 82 (35.04%). Nitrofurantoin was tested on only urine isolates of CoNS and it showed resistance in 13 (38.23%) of 34 isolates. Least resistance was noted to pristinomycin 20 (8.55%), vancomycin 1 (0.43%). Linezolid showed 100% susceptibility by Kirby Bauer disk diffusion.

Since the intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher than normal dosage of a drug can be used. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins. For this reason all intermediate resistant were labeled as resistant.[20]

Table No. 6 Antibiotic Resistance Pattern Of CoNS Isolates

Antibiotic	Resistance	Sensitive

Penicillin	229 (97.86%)	5 (2.14%)
Amoxicillin-clavulanic acid	187 (79.91%)	47 (20.09%)
Cefoxitin	153 (65.38%)	81 (34.62%)
Ceftazidime	164 (70.08%)	70 (29.92%)
Erythromycin	129 (55.13%)	105 (44.87%)
Vancomycin	1 (0.43%)	233 (99.57%)
Linizolid	0 (0.00%)	234 (100%)
Pristinomycin	20 (8.55%)	214 (91.45%)
Ofloxacin	152 (64.96%)	82 (35.04%)
Gentamycin	154 (65.81%)	80 (34.19%)
Cotrimoxazole	171 (73.07%)	63 (26.92%)
Rifampacin	82 (35.04%)	152 (64.96%)
Nitrofurantoin *	13 (38.23%)	21 (61.76%)

(*Nitrofurantoin was tested in only urinary isolates of CoNS.)

Table no.7 shows most of CoNS species show 100% resistance to penicillin except single isolate of S. simulans, 2 (25%) of S. capitis and 2 (1.84%) of S. epidermidis were sensitive to penicillin. Maximum resistance was seen in S. haemolyticus to most of antibiotics except linezolid.

Table No. 7 Sensitivity Pattern Of Different Species Of CoNS

Spe.	P*	AC*	CN*	CFZ*	E*	V*	LZ*	PRT*	OF*	G*	CO*	R*	NF*
S.epi	1.84%	22.02%	32.11%	28.44%	45.87%	100%	100%	89.91%	35.78%	33.03%	26.61%	73.40%	50.00%
S.sap	0.00%	34.62%	42.31%	34.62%	36.07%	100%	100%	96.15%	46.15%	57.69%	30.77%	61.54%	68.18%
S.hae	0.00%	8.20%	26.23%	21.31%	36.07%	98.36%	100%	91.80%	16.40%	21.31%	14.76%	47.54%	0.00%
S.lud	0.00%	20.00%	40.00%	45.00%	50.00%	100%	100%	100%	60.00%	55.00%	40.00%	65.00%	75.00%
S.hom	0.00%	16.67%	66.67%	50.00%	33.33%	100%	100%	100%	66.67%	0.00%	33.33%	83.33%	100%
S.cap	25.00%	25.00%	50.00%	50.00%	37.50%	100%	100%	75%	50.00%	25.00%	37.50%	37.50%	-
S.war	-	-	-	-	-	-	-	-	-	-	-	-	-
S.sim	100%	100%	100%	100%	100%	100%	100%	100%	0.00%	100%	0.00%	100%	-
S.xyl	0.00%	33.33%	66.67%	66.67%	33.33%	100%	100%	67.67%	33.33%	67.67%	0.00%	100%	-

(* Please refer appendix for abbreviations of antimicrobials)

DISCUSSION

Coagulase negative staphylococci (CoNS) were generally regarded to be the contaminants, having little clinical significance in the past.[17] Formerly regarded as harmless inhabitants of the skin and mucous linings, CoNS are now recognized as a major cause of nosocomial infections in critically ill patients especially in intensive care units, which leads to morbidity and even mortality.[21]

In our study a total number of 234 clinically significant CoNS strains (1.89%) were isolated from 12,358 processed clinical specimens. Among the 234 CoNS isolates, maximum strains, 112 (47.86%) were isolated from the Blood.

Slime Production In CoNS

Previous reports have suggested that slime has a role in the pathogenesis of CoNS infections. Both slime production and the species of organism appeared to be important factors in the determination of pathogenicity.[6]

Different methods are currently employed to detect slime production, including visual techniques like scanning electron, transmission electron and epifluorescence contrast microscopy, and non-visual methods such as the measurement of impedance and bioluminescence.[22] Qualitative analyses like tube method by Christensen et al[23] and the congored agar method by Freeman et al[18] and quantitative assays, such as microtitre plate method by Christensen et al have also been described. In addition, molecular methods (PCR) are used to provide direct evidence of the genetic basis of slime production.[22]

The most commonly used qualitative method for the analysis of biofilm production is the tube method described by Christensen et al[23]. In a study comparing the reliability of three biofilm detection methods in staphylococcus, a microtitre plate method, a tube method and congo red agar method, Knobloch et al[24] reported good correlation between the tube method and microtitre plate method for strongly biofilm-producing strains, whereas weak producers were not safely discriminated from non-producing strains. Congo red agar showed a low correlation with the tube method and microtitre plate method. Similar results have been reported by Mathur et al[25] who also evaluated these three different methods. The microtitre plate method was highly satisfactory in terms of biofilm-positive phenotype detection. The authors concluded that the microtitre plate method was the most sensitive and accurate method showing good reproducibility for the detection of biofilm formation in staphylococcus.

Although many studies have found the tube method test for slime production to be simple and reliable in classifying CoNS as slime positive or slime-negative, the test is limited in application due to its subjective and qualitative nature.[26]

Christensen et al[23] noted variability between observers in interpreting tests of observers in interpreting weakly positive strains of CONS. Congo red agar method has its own limitations and many authors have not recommended it as a satisfactory Method.[24,25] In the present study, determination of slime production was done by all three methods.

Table No. 8 : Comparison Of Microtitre Plate, Tube And Congo Red Agar Method For Slime Production

Method	Alcaraz et al 2003	Mathur et al 2006	Arslan et al 2007	Present study 2014
Microtitre plate	57.6%	53.8%	-	62.39%
Tube method	55.6%	41.4%	22.4%	38.4%
Congo red agar	-	5.17%	18.9%	5.17%

Out of 234 Clinically significant CoNS, slime production was seen in 62.39% isolates by microtitre plate method, 38.03% by tube method and 14.1% by congo red agar method. Mathur et al[25] found 53.8% clinically significant CONS to be slime producers by microtitre plate, 41.4% by tube method and 5.17% by congo red agar method. ArsIan and Ozkardes[27] reported 22.4% slime production by tube method and by 18.9% congo red agar method. Alcaraz et al[130] found, 57.6% slime producers by microtitre plate and 55.6% by tube method. Our finding correlate well with Mathur et al[25].

In the present study, it was observed that, of the 89 strains which were slime positive by tube method, except 4 all strain were positive by microtitre plate method. 33 strains were slime positive by congo red agar method of which 30 were positive and 3 strains were negative by microtitre plate method.

Considering the microtitre plate method as gold standard, sensitivity and specificity of tube method and congo red agar method was calculated. Mathur et al[25] calculated sensitivity and specificity by the same parameters. Thus sensitivity and specificity of tube Method was 58.22% and 95.45% respectively and that of congo red agar method was 20.55% and 96.59% respectively. Mathur et al[25] showed 73.6% sensitivity and 92.6% specificity of tube method and 6.8% sensitivity and 90.2% specificity of congo red agar method.

Our study observed high specificity and low sensitivity of both the tube and congo red agar methods as found by Mathur et al[25]. It is thus concluded that even though microtitre plate method is laborious, it is the method which should be followed for testing slime production. Tube and congo red agar method, though easy to perform were found to be less sensitive and many strains might be reported as falsely negative.

Present study found variable amount of slime production in various species. Slime production was seen maximally in S. saprophyticus (76.92%), epidermidis (74.31%) followed by S. haemolyticus (50.82%) and S. lugdunensis (40.00%), S.capitis (37.5%), S. hominis (33.33%) and S. xylosus (33.33%) by microtitre plate method. Alcaraz et al[28] found slime production maximally in S. saprophyticus (83.3%), S. epidermidis (57.7%), S. hominis (42.9%) and S. haemolyticus (37.5%). A single isolate of S.wameri, S. lugdunensis and S. capitis were all slime Producers. Arslan and Ozkardes[27] found slime production maximally in S. saprophyticus (66.7%), S. epidermidis (58.8%) and S. hominis (50%). A single isolate of S. warneri, S. haemolyticus and S. lugdunensis were all positive for slime production.

Antimicrobial Susceptibility Testing

1) Pattern Of Antimicrobial Sensitivity In Clinically Significant CoNS

Widely used antibiotics including Penicillin, particularly semi synthetic penicillins, cephalosporins, macrolids, tetracyclines and aminoglycosides have proven to be ineffective in inhibiting several prevalent species of CoNS, thus augmenting the need for new and effective antimicrobials.[29,30,31]

In the present study, the maximum number of the clinically significant CoNS were sensitive to linezolid (100%), followed by vancomycin (99.57%), pristinomycin (91.45%) and rifampacin (64.96%). Urinary antibiotic nitofurantoin showed 61.76% sensitivity. Maximum resistance of the clinially significant CoNS was seen to penicillin (97.86%), followed by amoxicillin-clavulanic acid (79.91%), cotrimoxazole (73.07%), ceftazidime (70.08%), gentamicin (65.81%), cefoxitin (65.38%) and ofloxacin (64.96%).

Mohan et al[32] showed maximum sensitivity of CoNS isolates to vancomycin (100%), methicillin (71.7%), amikacin (71.3%) and nitofurantoin (66.6%) and maximum resistance to penicillin (90,6%), norfloxacin (77.7%), gentamicin (66,2%), ciprofloxacin (66.2%), erythromycin (64.6%) and chloramphenicol (54.6%). Similar to our study, Singhal et al[33] reported maximum sensitivity for linezolid (100%), vancomycin (100%), teicoplanin (100%). He reported maximum resistance to cotrimoxazoie (74.7%) followed by penicillin (72.3%), methicillin (62.7%), ciprofloxacin (62.7%) and erythromycin (60.2%). Goyal et al[34] showed maximum sensitivity to vancomycin (100%) followed by gentamicin (80%), erythromycin (77%), methicillln (75%) and ciprofloxacin (71%) and maximum resistance to ampicillin (89%) and cefotaxime (59%).

2) Antimicrobial Sensitivity Of Different Species Of Clinically Significant CONS

Resistance to antibacterial agents has been increased among many species of CoNS[27]. Exact identification of CoNS to species level is important since most resistant strains belong to species S. haemolyticus.[35] Present study showed S. simulans to be the most sensitive species having sensitivity to most of the drugs. In our study, maximum resistance was seen to S. haemolyticus, followed by S. epidermidis for most of the antibiotics which is highly comparable to Singhal et al and Arslan and Ozkardes[27].

In present study, maximal sensitivity to penicillin and cefoxitin was shown by S. simulans and minimal sensitivity was shown by S. haemolyticus and S. epidermidis. Price and Flournoy[36] found S. warneri to be the most sensitive species to penicillin and cefoxitin and S. haemolyticus and S. epidermidis to be the minimally susceptible species. Gill et al[37] reported the same finding except that S. saprophyticus was reported to be the minimally susceptible species to penicillin. Del' Alamo et al[38] showed maximum methicillin resistance in S. haemolyticus (95.8%) follow by S. epidermidis (80.8%). Singhal et al[33] showed maximum Methicillin resistance by S. haemolyticus (72.7%) followed by S. epidermidis (71.4%), they also found S. haemolyticus had highest resistance to various antimicrobials namely ciprofloxacin (72.7%), erythromycin (90.9%) and rifampicin (63.6%).

Arciola et al[31] reported resistance levels in 15 species of 193 isolates of CONS infecting orthopedic implants. In the 5 most prevalent species — S. hominis, S. haemolyticus, S. capitis, S. warneri and S. cohnii — resistance to penicillin was similar (51%-66%). Most methicillin resistance was seen in S. haemolyticus whereas no methicillin resistance was present in S. warneri. Multiple antibiotic resistance genes were most likely tobe found in S. haemolyticus, averaging 2.8 for that species.

CONCLUSIONS

From our study it can be concluded that

1. Slime production is very important virulence factor, as it's presence showed reduced susceptibility to various group of Antimicrobials..
2. CoNS showed resistance to most of the antimicrobials in varying proportion except pristinomycin, vancomycin and linezolid.
3. Rifampicin, ceftazidime, and erythromycin are good options as anti CoNS agents (if found susceptible in laboratory testing) reserving vancomycin for life-threatening infections.

The study documents the importance of CoNS as important Gram-positive pathogen special in hospital settings, resistant to commonly used antibiotics. There is a need to have the knowledge of slime production, monitoring of antibiotic sensitivity pattern and formulation of definite antibiotic policy to improve the empirical approaches to the therapy of serious infections caused by CoNS.

REFERENCES

1. Pfaller MA, Herwaldt L. Laboratory, clinical & epidemiological aspects of CONS. Clin Microbiol Rev 1988; 1:281-99.
2. Kloos WE, Schleifer KH. Simplified scheme for routine identification of human staphylococcal species. J Clin Microbiol 1975; 1:82-88.
3. leven M, Verhoeven J, Pattyn SR, Goossens H. Rapid and economical method for species Identification of clinically significant coagulase negative staphylococci. J Clin Microbiology 1995; 33: 1060-3.
4. Jefferson SJ, Parisi JT. Bacteriophage typing of CoNS. J Cin Microbiol 1979; 10: 396-7.
5. Longauerova A. Coaguiase negative staphylococci and their participation in pathogenesis of human infections. Bratisl Lek Listy 2006; 107: 448-52.
6. Ishak MA, Groschel DHM, Mandell GL, Wenzel RP. Association of slime with pathogenicity of CoNS causing nosocomial septicemia. J Clin Microbiol 1985; 22: 1025-9.
7. Sheretz RJ, Falk RJ, Huffman KA, Thomann CA, Mattern WD. Infections associated with subclavian UIdall catheters. Arch Intern Med 1983; 143: 42-56.
8. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-Producing strains of Staphylococcus epidermidis to smooth surfaces. Infect Immun 1982; 37: 318-26.
9. Davenport DS, Massanari RM, Pfaller MA, Bale MJ, Streed SA, Heirholzer W J. Usefulness of a test for slime production as a marker for clinically significant infections with coagulase-negative staphylococcus. J Infect Dis 1986; 153: 332-9.
10. Kloos WE, Bannerman TL. Update on clinical significance of coagulase-negative staphylococci. Clin Microbial Reviews 1994; 7: 117-140.
11. Fluit AC, Jones ME, Schmitz FJ, Acar j, Gupta R, Verkoef J. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from SENTRY. Antimicrobial surveillance programme, 1997 and 1998. Clin Infect Dis. 2000; 30: 454-60.
12. Hanberger H, Diekema D, Fluit A, Jones R, Struelens M, Spenser F, Wolff M. Surveillance of antibiotic resistance in European ICUs. Hosp Infect 2001; 48: 161-76.
13. Vincent JL. Microbial resistance; lessons from EPIC study. Intensive Care Med 2000; 26: 3-8.
14. Diekema DJ, Pfaller MA, Schmitz FJ. Survey of infections due to staphylococcus species; frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin

America, Europe, and the Western Pacific region for the SENTRY antimicrobial surveillance pro, 1997-1999. Clin Infect Dis 2001; 32: 114-32.
15. Oliveira A, Cunha MLR. Bacterial biofilms emphasis on coagulase negative staphylococci. J Venom Anim Toxins Incl Trop Dis 2008; 14: 572-96.
16. Collee J C. Culture of Bacteria, Mackie & McCartney, Practical Medical Microbiology, Elsevier publication 14th Edi Chap 6; 121-128.
17. Winn WC, Allen SD, Janda WM, Koneman EW, Procop GW, Schreckenberger PC, Woods GL. Gram positive cocci: Staphylococci and related gram Positive Cocci. In: Koneman's colour atlas and textbook of diagnostic microbiology. 6th ed. Lippincott Williams and Wilkins; 2006. p 624-73.
18. Freeman DJ, Falkiner FR, Keane CT New method for detecting slime production by coagulase negative staphylococci. J Clin pathal. 1989 Aug;42(8):872-4.
19. Christensen GD Simpson WA, Younger JA, Baddour LM, Barrett FF, Melton DM. Adherence of coagulase negative staphylococci to plastic tissue cultures: a quantitative model for adherence of staphylococci to medical devices. J Clin microbiol1985; 22: 996. 1006.
20. Vas SI .Infection 1 of continuous ambulatory peritoneal dialysis catheters. Infect Dis Clin North Am 1989; 3: 301-28.
21. Huang SY, Tang RB, Chen SJ, Chung RL. Coagulase negative staphylocoaccal bactaeremia in critically III children: risk factors and antimicrobial susceptibility. J Microbiol Immunol Infect 2003; 36: 51-5.
22. Oliveira A, Cunha MLR. Bacterial biofilms with emphasis on coagulase negative staphylococci. J Venom Anim Toxins ind Trop Dis 2008; 14: 572-96.
23. Christensen GD, Baddour LM, Simpson WA. Phenotyr: Staphylococcus epidermidis slime production in vitro arc Infect Immune 1987; 55: 2870-7.
24. Knoblotch JK, Horsekotte MA, Rodhe H, Mack D. Evaluation of different detection methods of biofilm formation in Staphylococcus aureus. Med Microbiol Immunol 2002; 191: 101-6.
25. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol 2006; 24: 25-9.
26. Platter M, Davenport D, Bale M, Barrett M, Koontz F, Massanari RM. Development of the Quantitative Micro-Test for Slime Production by coagulase-negative staphylococci. Eur J Clin Microbiol Infect Dis 1988; 7: 30-3.
27. Arslan S, Ozkardes F. slime production and antibiotic susceptibility in Staphylococci isolated from clinical samples. Mem Inst Oswaldo Cruz,Rio de Janerio 2007; 102: 29-33
28. Alcaraz LE ,Satoress SE, Lucero RM, Puig de Centorbi ON. Species identification ,slime production and oxacillin susceptibility In coagulase negative staphylococci isolated from nosocornial specimens, Braz J Microbiol 2003; 34: 45-51.
29 Cerca N, Martins S, Cerca F. Comparative assessment of antibiotic susceptibility of coagulase--negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. J Antirricrob Chernother 2005; 56: 331-6.
30. Gaudioso de Allori MC, Jure MA, Romero C. Antimicrobial resistance and production of biofilms in clinical isolates of coagulase negative staphylococcus strains. Biol Pharm Bull 2006; 29: 1592-6.
31. Arciola CR, Campocci D. Prevalence and antibiotic resistance of 15 minor staphylococcal species colonizing orthopedic implants. Int J Artif Organs 2006; 29: 395-401.
32. Mohan U, Jindal N and Aggarwal P, Species distribution and antibiotic sensitivity pattern of coagulase negative Staphylococci isolates from various clinical specimens. Indian Journal of Med Microbiol 2002; vol 20, no 1: 45-46.
33. Singhal R, Dhawal S, Mohanty S, Sood S, Das B, Kapil A. Species distribution and antimicrobial susceptibility of CoNS in tertiary care hospital. Indian J Med Res 2006; 123: 569-70.
34. Goyal R, Singh NP, Kumar A, Kaur I, Singh M, Sunita N, Mathur M. Simple and economical method for speciation and resitotyping of clinically significant Coagulase Negative Staphylococci. Indian J Med Microbiol 2006; 24: 201-4.
35. Goldstein FW, Coutrot A, Sieffer A, Acar JF. Percentages and distributions of teicoplanin and vancomycin resistant strains among coagulase negative staphylocooci. Antimicro Agents Chemother 1990; 34: 899-900.
36. Price SB, Flournoy DJ. Comparison of antimicrobial susceptibility patterns among coagulase negative staphylococci. Antimicrob Chemother 1982; 21: 436- 40.
37. Gill VJ, Selepak ST, Williams EC. Species identification Inc antimicrobial susceptibility of coagulase negative staphylococci isolated from clinical specimens. J Clin Microbiol 1983: 18: 1314-9.
38. Del' Alamo L, Cereda RF, Tosin 1, Miranda EA,Sader HS. Antimicrobial susceptibility of coagulase—negative staphylococci and characterization of isolates with reduced susceptibility to of isolates with glycopeptides. Diagn Microbioi Infect Dis 1999: 34: 185-91.