ORIGINAL ARTICLE

Speciation and Biofilm Production of Coagulase Negative Staphylococcal Isolates from Clinically Significant Specimens and their Antibiogram

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Abstract:

Background: Coagulase Negative Staphylococci (CONS) are increasingly recognized as significant nosocomial pathogens. Their ability of biofilm formation and multiple drug resistance are causing serious human infections. *Aim and Objectives:* To isolate, identify, speciate clinically significant CONS from various specimens, to study antibiotic resistance pattern and biofilm production. *Material and Methods:* Specimens were collected aseptically, processed and identified upto the species level by a simple scheme of tests urease, novobiocin resistance, mannose and mannitol fermentation, ornithine decarboxylase. Antibiotic sensitivity was done with special reference to methicillin resistance. Biofilm formation was detected by Congo Red Agar (CRA) method and Tube Method (TM). *Results:* Study group-Of 100 isolates majority were pus (40), followed by urine (28), blood (16), CSF (5), body fluids (4) and catheter tips and implants (7). The most common species isolated was *S. epidermidis* (40%) followed by *S. haemolyticus* (26%), *S. saprophyticus* (15%), *S. schleiferi* (13%), *S. simulans* (2%), *S. cohnii* (2%) and *S. warneri* and *S. capitis* each 1%. Resistance to penicillin was 91% followed by ampicillin (79%), cotrimoxazole (67%). Methicillin resistance was 72%. Biofilm producers were 69% by CRA method and 33% by TM with majority species *S. epidermidis* (82.5%-CRA and 55%-TM). Biofilm production was significantly associated with MRCONS (p value 0.0036). Control group-Of 30 isolates were *S. epidermidis* 66.6% followed by *S. haemolyticus* (16.66%). Biofilm producers were 53.33% by CRA method and 26.65% by TM with majority species *S. epidermidis* (65%-CRA and 30%-TM).Methicillin resistance was 26.6%. *Conclusion*: Clinical significance of CONS is increasing day by day, so there is a need for accurate identification to species level and their antibiogram to avoid multidrug resistance. Biofilm producing CONS species pose a risk and CRA method for screening biofilm can be used in all conventional microbiology laboratories.

Keywords: Coagulase Negative Staphylococcus, Speciation, Methicillin resistance, Biofilm, Congo Red.

Introduction:

Coagulase Negative Staphylococci (CONS) are common colonizers of the skin, anterior nares and ear canals of human beings [1].They are opportunistic pathogens that cause infection in debilitated or compromised patients. CONS have emerged as predominant pathogens in hospital acquired infections and vary in pathogenic potential [2].More than 30 species of CONS are recognised but only a few are commonly incriminated in human infections [3]. *S. epidermidis* is most frequently isolated from infections of wound, urogenital tract, respiratory tract, meninges, conjunctiva and skin [4]. *S.saprophyticus* was shown to be an important cause of urinary tract infections in young females [5]. Identification of CONS by reference method Kloos and Schleifer is cumbersome and requires expensive reagents not available in most clinical laboratories [6]. Commercial kit identification

systems and automated systems are available but still out of reach in most laboratories in developing countries. Multiple antibiotic resistance is a common finding among clinical CONS isolates indicating its potential pathogenecity [7] these strains are not only resistant to multiple antibiotics, but also act as reservoirs for drug resistance gene [8]. Methicillin resistance among CONS is particularly important due to cross resistance to virtually all β-lactam agents and other anti-microbial classes [9].

CONS are capable of forming biofilm on polymeric surfaces. Biofilm is a structured community of microorganism encapsulated with a self-developed polymeric matrix and adherent to a living or inert surface. Biofilm consists of multi-layered cell clusters embedded in a matrix of extracellular polysaccharide which facilitates the adherence of these microorganisms to biomedical surfaces and protect them from host immune system and antimicrobial therapy [10].A working knowledge of the biology and antimicrobial susceptibility of CONS may be necessary to distinguish infections from contaminating isolates and to device appropriate therapy [11].

In the present study an attempt was made to isolate and speciate CONS with simple, inexpensive and easy to perform tests selected from the scheme of Kloos and Schleifer [6]. Antibiotic resistance patterns and biofilm production was done for all the isolates.

Material and Methods:

The present study was conducted on 100 clinical isolates and 30 healthy isolates of CONS in the Department of Microbiology, Rangaraya Medical College, Kakinada from April 2013 to July 2014 after obtaining Institutional Ethics Committee clearance.

Inclusion criteria:

Clinically significant CONS isolates.

Exclusive criteria:

Sputum, stool, throat and vaginal swabs were excluded.

For study group a total of 100 clinically significant CONS were isolated from aseptically collected different clinical samples. For control group informed consent was obtained and samples were collected using sterile cotton wool swab moistened with normal saline from anterior nares, ear, skin and inoculated into nutrient broth. All specimens were inoculated onto nutrient agar, blood agar and nutrient broth. The isolates were considered clinically significant when isolated in pure culture from infected sites. The isolates collected were initially identified by colony morphology, Gram staining, catalase, slide and tube coagulase test (Fig. 1, 2) and anaerobic acid formation from mannitol (Fig.5) [2, 12]. Speciation of CONS was done by urease test, mannose fermentation test, Novobiocin sensitivity test, ornithine decarboxylase test [3] (Fig. 3, 4, 6, 7). These simple, inexpensive and easy to perform tests were selected from the scheme of Kloos and Schleifer to identify CONS species [3] (Table 1). Biofilm production was performed by culture of the CONS isolates on Congo Red Agar (CRA) plates as proposed by Freeman *et al* which is an alternative method of screening biofilm formation which requires the use of a specially prepared solid medium-Brain Heart Infusion (BHI) broth supplemented with 5% sucrose and Congo red (Fig.8) [13].

A qualitative assessment of biofilm formation was determined as previously described by Christensen *et al* [15]. A positive result was defined as the presence of a layer of stained material adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid air interface was not considered to be positive (Fig. 9). The antimicrobial susceptibility profiles of all isolates were done by Kirby Bauer Disc Diffusion Method (KBDDM) according to CLSI guidelines (Fig. 10).

Fig. 1: Slide Coagulase Test

Fig. 2: Tube Coagulase Test

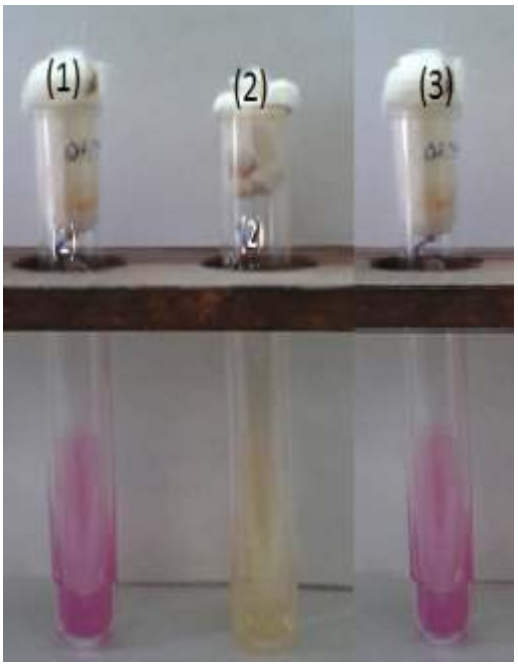


Fig. 3: Urease Test
(1) Test Positive
(2) Negative Control
(3) Positive Control

Fig. 4: Ornithine Decarboxylase Test
(1) Positive
(2) Negative

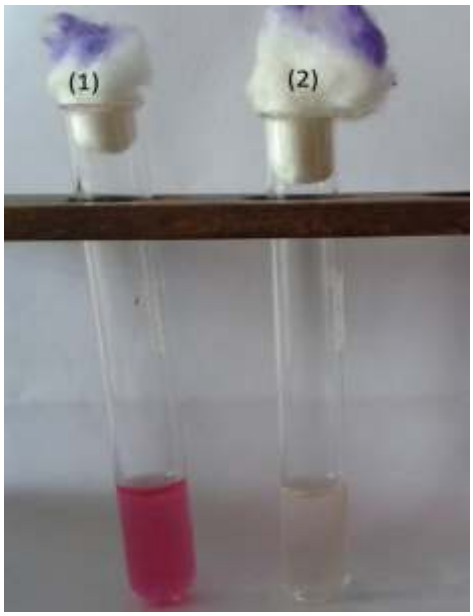


Fig. 5: Mannitol Test
(1)Positive
(2)Negative

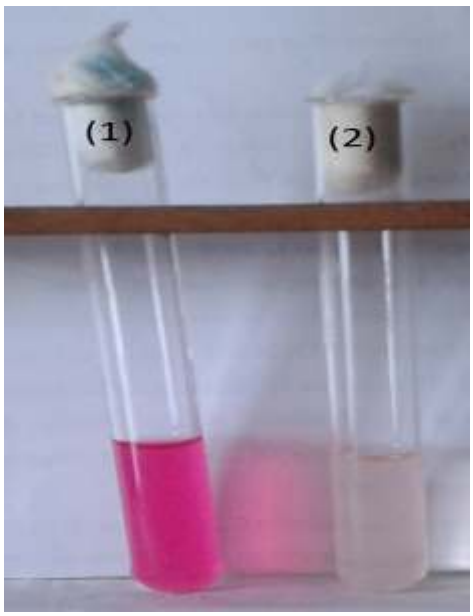
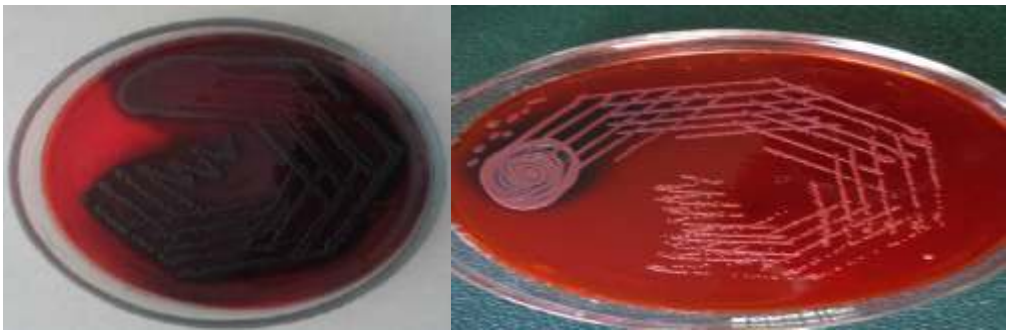


Fig. 6: Mannose Fermentation
(1) Positive
(2) Negative

Fig. 7: Novobiocin Sensitivity Test



Positive **Negative**

Fig. 8: Biofilm Production (Congo Red Agar)

Fig. 9: Biofilm Production (Tube method)

Fig. 10: Antibiotic Susceptibility Test

Results:

Among 100 isolates of CONS, 40 were isolated from pus, 28 from urine, 16 Blood, 05 CSF, 04 Body fluids and07 Catheter Tips and implants (Table 1). Majority of isolates were from males 63 (63%) and remaining were from females 37 (37%). Most common age group in both males and females was 15-45 years. Simple scheme for the Identification of CONS (Table-2) showed *S. epidermidis* as the most common isolate (40%),followed by *S. haemolyticus* 26 (26%), *S. saprophyticus* 15 (15%), *S. schleiferi* 13 (13%), *S. simulans* and *S. cohnii* each 02 (2%) and *S. warneri* and *S. capitis* each 01 (1%). Maximum isolates of *S. epidermidis* were from pus 30 (75%), *S. saprophyticus* from urine 12 (80%), *S. haemolyticus* from blood 08 (53.85%) (Table 2). Majority showed resistance to penicillin 91 (91%) followed by ampicillin 79 (79%), cefoxitin 72(72%), cotrimoxazole 67(67%). Methicillin resistant CONS were 72 (72%) (Table 3). Of 100 isolates 69 (69%) were Biofilm producers by CRA method and 33% by TM with majority species *S. epidermidis* (82.5%-CRA and 55%-TM) (Table 4). Biofilm production was associated with MRCONS (p – Value 0.0036) (Table 5). In control group, of 30 isolates most common CONS species isolated was *S. epidermidis* 20(66.6%) followed by *S. haemolyticus* 05(16.66%) (Table 6). Majority showed resistance to Penicillin19 (63.3%) followed by cotrimoxazole 13(43.3%), Ampicillin 12(40%) and Erythromycin 10(33.3%). Methicillin resistance CONS were 08 (26.6%) (Table 3). Biofilm producers were 16 (53.33%) and 26.65% by TM with majority species *S. epidermidis (*65%-CRA and 30%-TM) (Table 4).

Table 1: Frequency of Clinically Significant CONS in Different Clinical Samples

Species	Pus	Urine	Blood	CSF	Body Fluids	Catheter Tips and Implants
***S. epidermidis* (40)**	30(75)	04(10)	01(2.50)	01 (2.50)	00	04(10)
***S. saprophyticus* (15)**	03(20)	12(80)	00	00	00	00
***S. haemolyticus* (26)**	04(15.38)	10(38.46)	08(30.76)	01(3.85)	02(8.34)	01(7.69)
***S. schleiferi* (13)**	02(15.38)	01(7.69)	07(53.85)	02(15.38)	00	01(7.69)
***S. simulans* (02)**	01(50.0)	01(50.0)	00	00	00	00
***S. cohnii* (02)**	00	00	00	00	02(100.0)	00
***S. warneri* (01)**	00	00	00	00	00	01(100.0)
***S. capitis* (01)**	00	00	00	01(100.0)	00	00
Total	**40**	**28**	**16**	**05**	**04**	**07**

Table 2: Simple Scheme for the Identification of CONS

Species	Clumping factor	Tube Coagulase	Ornithine Decarboxylase	Urease	Mannose	Mannitol	Novobiocin (5 µg)
S. epidermidis	-	-	+	+	+	-	S
S. saprophyticus	-	-	-	+	-	+	R
S. haemolyticus	-	-	-	-	-	V	S
S. schleiferi	-	-	-	-	+	-	S
S. simulans	-	-	-	+	±	V	S
S. cohnii	-	-	-	+	+	+	R
S. warneri	-	-	-	+	-	V	S
S. capitis	-	-	-	-	+	V*	S

Note: V = 50-80 % isolates positive V = 20-50% isolates positive*

Table 3: Resistance Patterns of CONS to Different Antibiotics in Study(S) and Control(C) groups

Species	P		AMP		E		CX		LZ		VA		AK		G		CIP		COT	
	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
***S. epidermidis* (40)**	33	10	29	07	22	06	31	05	01	00	09	00	07	02	06	03	17	03	25	10
***S. saprophyticus* (15)**	11	00	09	00	08	00	09	00	03	00	04	00	00	00	06	00	06	00	10	00
***S. haemolyticus* (26)**	25	03	22	02	21	02	18	01	02	00	05	00	04	00	00	01	07	00	17	02
***S. schleiferi* (13)**	16	04	13	03	07	02	13	02	01	00	01	00	02	00	03	00	05	00	10	01
***S. simulans* (02)**	02	01	02	00	00	00	00	00	00	00	00	00	00	00	00	00	00	00	01	00
***S. cohnii* (02)**	02	00	02	00	01	00	00	00	00	00	00	00	02	00	02	00	01	00	02	00
***S. warneri* (01)**	01	00	01	00	00	00	00	00	00	00	00	00	00	00	00	00	01	00	01	00
***S. capitis* (01)**	01	00	01	00	01	00	01	00	00	00	00	00	00	00	00	00	00	00	01	00
Total S-100, C-30	**91**	**19**	**79**	**12**	**60**	**10**	**72**	**08**	**07**	**00**	**19**	**00**	**15**	**02**	**17**	**04**	**37**	**03**	**67**	**13**

Table 4: Detection of Biofilm in CONS by Two Phenotypic Methods in Study(S) and Control (C) Groups

	CONS Species									
	S. epidermidis		***S. saprophyticus***	***S. haemolyticus***		***S. schleiferi***		***S. simulans***	**Total**	
Biofilm Method	**S**	**C**	**S**	**S**	**C**	**S**	**C**	**C**	**S**	**C**
Congo Red Agar Method										
Positive	33(82.5)	13(65)	12(80)	18(69.2)	02(40)	06(46.1)	01(25)	00	69	16
Negative	07(17.5)	07(35)	03(20)	08(30.7)	03(60)	07(53.8)	03(75)	1(100)	25	14
Tube Method										
Positive	22(55)	06(30)	05(33.3)	04(15.3)	02(40)	02(15.3)	00	00	33	08
Negative	18(45)	14(70)	10(66.6)	22(84.6)	03(60)	11(84.6)	04(100)	01(100)	61	22
Total	40	20	15	26	05	13	04	01	94	30

Table 5: Detection of Biofilm (CRA method) in Methicillin Resistant and Sensitive Isolates of CONS in Cases

Types	MRCONS(72)	MSCONS(28)	Total(100)
Biofilm producers	56 (77.7%)	13(46.42%)	69
Biofilm non-producers	16(22.2%)	15(53.57%)	31

p –0.0036. Biofilm production was associated with MRCONS (chi-square test)

Table 6: Frequency of CONS in Healthy Individuals

Species	Nares	Ear	Skin
***S. epidermidis* (20)**	07	07	06
***S. saprophyticus*(00)**	00	00	00
***S. haemolyticus*(05)**	00	02	03
***S. schleiferi*(04)**	02	01	01
***S. simulans*(01)**	01	00	00
***S. cohnii*(00)**	00	00	00
***S. warneri*(00)**	00	00	00
***S. capitis*(00)**	00	00	00
Total (30)	10	10	10

Discussion:

Infections with CONS have been reported with increasing frequency [9], they must now be individually evaluated as potentially true pathogens and identified to the species level by simple, reliable and preferably inexpensive methods [14]. Many of the CONS species are commonly resistant to antibiotics that are being indicated for Staphylococcal infections. Biofilm may function as a penetration barrier to antibiotics and hence the high level of resistance [13].

In our study majority, i.e., 40(40%) CONS isolates have been from pus, followed by urine 28 (28%), blood 16 (16%), catheter tips and implants 7(7%), CSF 5(5%) and body fluids 4 (4%) correlates to earlier studies by Singh *et al* [15] and Asangi *et al* [12]. *S. epidermidis* has been the most common isolate in 40(40%) and correlates to earlier studies by Singh *et al* (41%) [15] and Shubhra *et al* (40%) [16]. The next most common species in our study has been 26% of *S. haemolyticus* nearly correlates to Usha *et al* (17.64%) [17].

Majority of *S. epidermidis* isolates are from pus (75%) followed by urine (10%), catheter tips and implants (10%) and blood and CSF (2.5%) each which is similar to other studies of Cunha *et al* [18], Sheikh and Mehdinejad *et al* [19].

Most of *S. saprophyticus* isolates have been from urine (80%) followed by pus (20%), similar to other studies by Singh *et al* [15] and Shubha *et al*

[20] and majority of *S. haemolyticus* isolates have been from urine (38.46%) followed by blood (30.76%) which is similar to the study by Asangi *et al* (31.57%) in the urine and (15.78%) in the blood [12].

In our study biofilm production by CRA method is seen in 69 (69%) specimens, which is nearly correlating with the study by Cunha *et al* 73 (73%) [18] and majority of biofilm producers are the species *S. epidermidis* 82.5 (82.5%) which correlates with the study by Shubha *et al* [20], followed by *S. saprophyticus* 80 (80%) and *S. haemolyticus* 69.2 (69.2%). Biofilm production by TM has been 33 (33%) is correlating with the study of Sharvari *et al* 32 (32%) [21].

Antibiotic susceptibility testing has shown variability and multidrug resistance with maximum resistance to penicillin 91 (91%) isolates followed by ampicillin 79 (79%), cefoxitin 72 (72%), cotrimoxazole 67 (67%), erythromycin 60 (60%), ciprofloxacin 37 (37%). Singh *et al*, Sharma *et al*, Bouchami *et al*, Asangi *et al*, Shubha *et al* and Usha *et al* have shown maximum resistance to penicillin and ampicillin which correlates with our study [12,15,17,20, 21,22].

Among 100 isolates 72 (72%) have shown methicillin resistance which nearly correlates with Asangi *et al* (67.7%) of which 56 (77.7%) MRCONS have been biofilm producers with a p-value of 0.00369. It is statistically significant, so biofilm production can be considered as associated with MRCONS. Control group of 30 CONS isolates from nares, ear, and skin from healthy individuals in our study have shown the most common species of *S. epidermidis* 35% which correlates with the study by Shubha *et al* [20]. Antibiotic susceptibility testing has shown multidrug resistance with control group also and methicillin resistant CONS have been 8 (26.6%). Biofilm producers have been 16 (53.33%) by CRA method.

Conclusion:

The clinical significance of CONS is increasing day by day and there is a need for accurate identification to species level by simple, inexpensive methodology and their antibiotic sensitivity to avoid decreased susceptibility to glycopeptides. Pathogenecity of CONS strains may be decided by screening for biofilm production. CRA method is simple and is very easy to perform and interpret, can be used as screening test for biofilm detection in all conventional microbiology laboratories. Biofilm producing CONS species from healthy controls determine the risk of acquiring more infections in hospitals and pose a major concern. Multidrug resistant nature of MRCONS necessitates every effort to be made to eradicate infections by them through a strict hospital policy.

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