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Original Research Article

Detection of Biofilm Formation and Virulence Markers Amongst the Cons Isolates in a Tertiary Care Center in Bihar.

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Abstract

Background: The aim of the study was to speciate Coagulase negative staphylococci [CoNS] from blood samples, ascertain the antibiogram and to determine the expressions of these virulence markers and biofilm production in the CONS isolates.

Materials and Methods: A total of 350 blood samples were collected from clinically suspected cases of BSI [during the fever spikes] for routine blood culture. A panel of standard biochemical tests was used to identify the CoNS upto species level. Phenotypic detection of virulence markers was done by standard test. Biofilm production was screened by tissue culture plate [TCP], Tube method [TM] & Congo red agar [CRA] and brain heart infusion agar [BHIA] with 6% sucrose method. Antibiogram was detected by modified Kirby Bauer method as per CLLSI guidelines.

Results: 32.3% [45/139] isolates produced biofilm by standard tissue culture plate [TCP] assay. 5.7% [8/139] strains by tube method [TM] method followed by 2.1% [3/139] each by and Congo red agar [CRA] method & brain heart infusion agar [BHI] 6% suc method were strong biofilm producers in comparison to TCP method 15.1% [21/139]. The sensitivity & specificity of TA method was [53.3% & 81.9%]. However, sensitivity of CRA & BHIsuc6% method were much lower being, 28.8% & 20.0% whereas the specificity was 93.6% & 89.3%.. Production of DNase, lipase, caesinase and gelatinase was common to all the seven species, DNase being more common in of Staphylococcus epidermidis 42.8% [15/35].

Conclusions: The TCP method was found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation. Biofilm forming capacity and elaboration of various virulence determinants followed by multi-drug resistance by CONS will facilitate

its colonizing ability. Hence importance should be laid for routine identification of CONS and determining its antimicrobial susceptibility pattern.

Key words: Tissue culture plate, Tube adherence, Congo red agar, BHI6% suc

Introduction

Little information is available on the characterization and prevalence of Coagulase-negative staphylococci [CONS] and its associated antimicrobial resistance pattern in this geographical region. In the present scenario, CONS has emerged from a contaminant to opportunistic pathogens, with predilection for colonizing immunosuppressed and long-term hospitalized patients. The ability to form biofilm facilitates this organism to adhere and colonize artificial materials. [1,2]

Blood culture is the gold standard for the diagnosis of neonatal septicaemia. Incidences of increased antimicrobial resistance of CONS in neonates underscore the need to understand the role of CONS as pathogens in blood stream infections. [3] CONS are a heterogeneous group of bacteria, consisting of approximately 40 species, of which, several species have been recognized as potential pathogens to humans. Blood stream infections in neonates with IV catheter and orthopaedic wound infections with implant devices are usually associated with Staphylococcus epidermidis. Staphylococcus lugdunensis is mainly involved in causation of prosthetic valve endocarditis. Staphylococcus haemolyticus is the second most frequently encountered CONS species in the clinical laboratory. Staphylococcus saprophyticus is the second most common cause of urinary tract infections in females of reproductive age group. Many laboratories donot put up a detailed panel of biochemical tests nor use expensive automated systems required for speciation of CONS routinely. This underscores the need to properly identify the species of CONS. Studies on species diversity, antibiotic resistance, and virulence factors of CONS are of great significance because of their emerging role as pathogens in blood stream infections.

Aims and Objective

The aim of the study was to look for the local distribution of CONS isolated from neonatal sepsis along with antibiotic susceptibility pattern and distribution of virulence markers & biofilm formation.

Materials and Methods

This cross-sectional study included all patients admitted with signs of sepsis in Paediatrics Department of Katihar Medical College and Hospital. The study was conducted over a period of two years from May 2019 to April 2021, after obtaining Clearance from Institutional Ethics Committee vide memo no. IEC/IRB No: KMC/IEC/Dept. Res./011/2019-2022 [Microbiology]; dated 20.04.2019.

Study population:

Based on the total number of samples received during the study period, a calculation was done using confidence level of 95% and confidence interval of 4.0. Hence a total of 350 blood samples was collected by simple random sampling method from clinically suspected cases of BSI [during the fever spikes] before empirical antibiotic therapy was started. Inoculation was done into Trypticase soya broth [TSB], allowing a 1:10 dilution. CONS isolates as mixed growth with no clinical correlation, were excluded from the study.

Isolation & identification:

The blood culture bottles containing specimens thus collected were incubated at 350C for 7 days. After 12-18 hours following incubation, the bottles were examined for appearance of turbidity and subculture was done from the bottles on Chocolate agar, Blood agar and MacConkey agar plates. If there was growth, the CONS isolates were identified as per standard protocol based on colony morphology, Gram's staining findings and a negative slide coagulase, followed by Tube coagulase test. The strains were further speciated by using battery of biochemical tests that included Urease production, Acetoin production, Voges Proskauer test, Pyrrolidonyl arylamidase [PYR], Alkaline phosphatase, Polymyxin B susceptibility, Novobiocin sensitivity, Nitrate reduction, Fermentation of maltose, sucrose, mannitol, lactose, mannose, trehalose and xylose. [4]

Tests for detection of virulence markers in CONS:

Various virulence markers of CONS viz: haemolysin, deoxyribonuclease, phenolphthalein phosphatase, gelatin liquefaction, caseinase and lipase was detected using standard protocol. [5,6,7]

Congo red agar method [CRA]

CRA was prepared with Brain heart infusion [BHI] broth, sucrose, agar and Congo Red indicator. CRA plates were inoculated with test organisms and incubated at 37ºC for 24 hours aerobically. The biofilm forming strains produced black colonies with dry crystalline consistency while non-forming strains developed red colonies on CRA after 24 hrs of incubation. [8]

Tube adherence method

Trypticase Soya Broth with 1% glucose [TSBGlu] was inoculated with a loopful of bacterial suspension and incubated for 24 h at 370C. The tubes were gradually decanted and were washed with phosphate buffer solution pH 7.3. After drying, the tubes were stained with 0.1% crystal violet. The presence of a layer of stained material adhered to the inner wall of the tubes was considered as positive for biofilm formation. [8]

Detection of biofilm formation

Trypticase soya broth with 5% glucose & BHI broth with 6% sucrose were inoculated with the test strains and incubated overnight at 370C. Culture was diluted 1:20 in the same media. 200 µl of this suspension was used to inoculate sterile 96 well polystyrene microtiter plates followed by static incubation for 24 h at 370C. Later, wells were washed with PBS, dried and stained with 1% crystal violet for 15 min. Absorbance was determined at 490 nm after adding 200 µL of 95% ethanol into each well. The isolates were classified into three categories based on optical density [OD] as non-adherent [OD equal to or lower than 0.111]; weakly adherent [OD higher than 0.111 or equal to or lower than 0.222] and strongly adherent [OD higher than 0.222] as per protocol. [8]

Antimicrobial Susceptibility Testing:

Antibiotic sensitivity testing was done by modified Kirby-Bauer's disc diffusion test on Mueller-Hinton agar [MHA; HiMedia, Mumbai, India] as per the Clinical and Laboratory Standards Institute. For detection of MRSA strains, MHA supplemented with 1 µg oxacillin and 30 µg cefoxitin discs and 4% NaCl was used. [9] For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. The zone of inhibition was determined after 24 h of incubation at 350C. Zone size was interpreted according to CLSI, treating zone size ≥ 13 mm as Sensitive [S]; 11-12 mm as Intermediate [I], and ≤ 10 mm as Resistant [R] for oxacillin and ≥ 18 mm as Sensitive [S]; 11-17 mm as Intermediate [I], and ≤ 14mm as Resistant [R] for cefoxitin.

Statistical analysis:

For sensitivity and specificity, biofilm formation by Tube method [TM], CRA method [CRAM], Brain heart infusion agar

with 6% sucrose [BHIAS] were calculated using Microtiter Plate Assay [MPA] as a gold standard. Variables measured were the number of true positives [TP], number of true negatives [TN], number of false positives [FP], and number of false negatives [FN]. Sensitivity was calculated as TP/[TP+FN], specificity was calculated as TN/[TN+FP], the PPV was calculated as TP/[TP+FP] and NPV was calculated as TN/[TN+FN].

Results

This cross-sectional study was undertaken in a tertiary centre of northern Bihar, India. [39.7%; 139/350] CONS were isolated from blood samples submitted to the laboratory for processing. Species identification was done using a battery of biochemical tests as mentioned in **Table 1**. Staphylococcus epidermidis [38.8%; 54/139] was isolated as the common species followed by Staphylococcus haemolyticus [15.8%; 22/139], Staphylococcus saprophyticus [13.6%; 19/139], Staphylococcus lugdunensis [10.7%; 15/139], Staphylococcus warneri [9.3%; 13/139], Staphylococcus hominis [7.9%; 11/139] and Staphylococcus capitis [3.5%; 5/139].

In the standard TCP assay, only 32.3% [45/139] isolates displayed biofilm positive phenotype. However, the rate of biofilm formation after addition of glucose in TSBglu-5%, increased to 48.9% [68/139] & 57.5% [80/139] after 24 & 48 hours of incubation, respectively. Further enhancement of biofim formation using sucrose in BHIsuc-6% medium was noted viz: 53.2% [74/139] & 60.4% [84/139] after 24 & 48 hours, respectively **[Table 2]**.

Using standard TCP method 15.1% [21/139] were found to be strong biofilm producer, 17.2% [24/139] were moderate biofilm producers and 67.6% [94/139] were non biofilm producers. On the other hand, a slightly lower number of strains were detected by TA method, that included 5.7% [8/139] strong, 11.5% [16/139] moderate, and 12.2% [17/139] weak biofilm producers. This was followed by CRA & BHI6% suc method that could detect only 2.1% [3/139] each as strong biofilm producers. The number of strains producing weak biofilm was 7.1% [10/139] by BHI6%suc method and 4.3% [6/139] by CRA method **[Table2]**. Overall, amongst all the methods used to detect biofim, TA method, more or less, was found close to TCP method for moderate biofilm production. By CRA method, different biofilm test interpretations were made. It was seen that of all strains that were positive by TCM, did not display black to brown colonies indicative of biofilm producers.

Of the 139 strains analysed for biofilm formation data was obtained using TCM as a standard method, to evaluate TA method followed by CRA and BHIsuc 6% methods respectively. The sensitivity & specificity of TA method was 53.3% & 81.9% respectively. The PPV was 58.5% & and NPV was 78.5% respectively. However, sensitivity of CRA & BHIsuc 6% method were much lower being, 28.8% & 20.0%, whereas the specificity was 93.6% & 89.3%. 53.3% [24/45] strains were found to correlate with TCP method for biofilm formation by TA method. This was followed by CRA method where 28.8% [13/45] strains producing biofilm correlated with TCP method. A total of 33 strains [that included 17 strains of TA method, 10 strains of BHIsuc 6% method & 6 strains of CRA method] were false positive. 36 strains by BHIsuc 6% method, 32 strains by CRA method and 21 strains by TA method were false negative **[Table 3]**.

The commonest species showing haemolysis to sheep R.B.C & human R.B.C. was Staphylococcus epidermidis being 34.6% [27/78] & 79.1% [19/24]. Production of DNase, lipase, caseinase and gelatinase was common to all the seven species, being more common in Staphylococcus epidermidis 42.8%;

[15/35], 30.4% [7/23], 40.7% [11/27] and 38.4% [15/39] respectively **[Table 4]**.

In the hemagglutination tests, 50.0% [9/18] followed by 42.8% [15/35] strains of Staphylococcus epidermidis agglutinated human "O" group and "B" group RBCs. None of the CONS strains agglutinated human "A" group RBCs **[Table 4]**.

A total of 41.0% [57/139] isolates were found to be Methicillin resistant CONS [MRCONS] as compared to 58.9% [82/139] Methicillin sensitive CONS [MSCONS]. Of the MRCONS strains, resistance was more common to ampicillin [100%] followed by amoxy-clavulanic acid [73.6%] and ciprofloxacin & vancomycin [66.6% each] respectively. Likewise, the MSCONS showed maximum resistance to ampicillin [62.1%; 51/82] and ciprofloxacin [59.7%; 49/82]. Least resistance was shown to linezolid [3.6%; 3/82]; **[Table 5]**.

Table 1: Biochemical tests for identification of CoNS isolates and their interpretation

CoNS isolates		Staphylococcus epidermidis	Staphylococcus saprophyticus	Staphylococcus hemolyticus	Staphylococcus lugdunensis	Staphylococcus warneri	Staphylococcus hominis	Staphylococcus capitis
Biochemical tests		Percentage of strains						
Acid Production from	Trehalose	0	100	100		100	0	0
	Mannitol	0	0	0	0	0	0	100
	Mannose	90	0	0	0	0	0	90
	Xylose	0	0	0	0	0	0	0
	Maltose	100	100	100	100	100	100	0
	Sucrose	100	100	90	90	90	90	90
	Lactose	100	100	90	90	90	90	90
Urease production		100	100	0	0	90	90	0
Acetoin production		100	90	95	0	95	0	0
Novobiocin		S	R	S	S	S	S	S
Polymyxin B		R	S	S	S	S		S
Slide coagulase		0	0	0	0	0	0	0
Tube coagulase		0	0	0	0	0	0	0
Alkaline phosphatase		95	0	0	0	0	0	0
Pyrrolidonyl arylamydase		0	0	95	0	0	0	0
Ornithine decarboxylase		0	0	0	0	0	0	0

Table 2: Screening of CONS for biofilm formation by TCP method in different media and at 24 and 48 hours of incubation

Biofilm formation [OD]570nm	No. of isolates					
	TSB				BHI suc	
	Incubation period [hour]					
	24	48	24	48	24	48
High [> 0.240 ± 0.022]	21	24	29	34	31	37
Moderate [0.120-0.240 ± 0.020]	24	26	39	46	43	47
Non [< 0.12 0 ± 0.012]	94	89	71	59	65	55

Biofilm production	Distribution of Detection of Biofilm Production by the Three Methods Detection method							
	TCP [TSB at 24 hrs]		TM		CRA		BHI agar with 6% sucrose	
	n	[%]	n	[%]	n	[%]	n	[%]
High	21	15.1	8	5.7	3	2.1	3	2.1
Moderate	24	17.2	16	11.5	10	7.1	6	4.3
Weak	0	0	17	12.2	6	4.3	10	7.1
Non	94	67.6	98	70.5	120	86.3	120	86.3

Table 3: Statistical evaluation of different methods for detection of biofilm formation in CONS using the tissue culture plate method as the standard method

Biofilm detection methods		Interpretation	Tissue culture plate method	
			Positive	Negative
Congo red agar method		Positive	[TP] 13	[FP] 6
		Negative	[FN] 32	[TN] 88
Tube adherence method		Positive	[TP] 24	[FP] 17
		Negative	[FN] 21	[TN] 77
BHI 6% sucrose		Positive	[TP] 9	[FP] 10
		Negative	[FN] 36	[TN] 84
Screening method	Test characteristics			
	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Congo red agar method	28.8%	93.6%	68.4%	73.3%
Tube adherence method	53.3%	81.9%	58.5%	78.5%
BHI with 6% sucrose	20.0%	89.3%	47.3%	70.0%

Table 4: Detection of various virulence markers among CONS

Species	**Virulence factors among CONS**							**Hemagglutination**		
	Hemolysis on Sheep blood agar	**Hemolysis on human blood agar**	**Phosphatase enzyme**	**DNase test**	**Lipase**	**Serine caesinase**	**Gelatinase**	**Human group O**	**Human group B**	**Human group A**
Staphylococcus epidermidis	27[34.6%]	19[79.1%]	19[42.2%]	15[42.8%]	7[30.4%]	11[40.7%]	15[38.4%]	9[50.0%]	15[42.8%]	0
Staphylococcus haemolyticus	17[21.7%]	3[12.5%]	11[24.4%]	7[20.0%]	5[21.7%]	7[25.9%]	11[28.2%]	5[27.7%]	9[25.7%]	0
Staphylococcus saphrophyticus,	9[11.5%]	2[8.3%]	7[15.5%]	6[17.1%]	5[21.7%]	3[11.1%]	3[7.6%]	2[11.1%]	4[11.4%]	0
Staphylococcus lugdunensis	7[8.9%]	0	0	2[5.7%]	1[4.3%]	2[7.4%]	4[10.2%]	2[11.1%]	4[11.4%]	0
Staphylococcus hominis	5[6.4%]	0	0	2[5.7%]	1[4.3%]	2[7.4%]	4[10.2%]	0	3[8.5%]	0
Staphylococcus capitis	0	0	0	1[2.8%]	1[4.3%]	1[3.7%]	1[2.5%]	0	0	0
Staphylococcus warneri	6[7.6%]	0	8[17.7%	2[5.7%]	3[13.0%]	1[3.7%]	1[2.5%]	0	0	0
TOTAL	78	24	45	35	23	27	39	18	35	

Table 5: Antibiotic sensitivity/resistant pattern of CONS with reference to biofilm formation

Antibiotics	MSCONS, n=82		MRCONS, n=57	
	Sensitive n[%]	Resistant n[%]	Sensitive n[%]	Resistant n[%]
Amoxycillin	31[37.8]	51 [62.1]	0	57 [100]
Amoxy-clavulunic acid	37[45.1]	45[54.8]	15[26.3]	42[73.6]
Ciprofloxacin	33[40.2]	49[59.7]	19[33.3]	38[66.6]
Aztreonam	54[65.8]	28[34.1]	46[80.7]	11[19.2]
Nalidixic acid	49[59.7]	33[40.2]	35[61.4]	22[38.5]
Netilmycin	51[62.1]	31[37.8]	29[50.8]	28[49.1]
Erythromycin	55[67.0]	27[32.9]	27[47.3]	30[52.6]
Amikacin	47[57.3]	35[42.6]	22[38.5]	35[61.4]
Gentamicin	39[47.5]	43[52.4]	21[36.8]	36[63.1]
Clindamycin	48[58.5]	34[41.4]	31[54.3]	26[45.6]
Vancomycin	65[79.2]	17[20.7]	19[33.3]	38[66.6]
Linezolid	79[96.3]	3[3.6]	45[78.9]	12[21.0]

Discussions

Uncertainties regarding the significance of CONS isolated from blood cultures may result in over-diagnosis and overuse of vancomycin, which may contribute to the development of resistance and thereby amplify the likelihood of morbidity and mortality. [10] Species identification of CONS will also help to ascertain the epidemiology of CONS in our region since information related to spread of these organisms among population is very limited.

A total of 39.7% [139/350] CONS were isolated from blood samples in the present study. Other authors reported a lower rate of isolation of CONS species 12.4% [62/500] from different samples like pus [40.3%], followed by urine [29%], blood [24.2%] and least isolated from indwelling devices like central line tip [4.8%] and UVC tip [1.6%]. The species distribution in their study was similar to our test results that included S. epidermidis [20 isolates, 32.3%] S. haemolyticus [15 isolates, 24.2%], S. saprophyticus [9 isolates, 14.5%], S. lugdunensis [7 isolates, 11. 3%], S. hominis [7 isolates, 11.3%], S. schlieferi [2 isolates, 3.2%], S. warneri [1 isolate, 1.6%], and S. warneri [1 isolate, 1.6%]. [8] In contrast to the finding of our study, Jain A et al. isolated S. haemolyticus [58%] as the most common isolate, followed by S. epidermidis [17%].[11]

In the TCP assay with TSB medium, only 32.3% [45/139] tested CONS isolates produced biofilm. Similar observations were noted wherein biofilm producing isolates were much lower [4.6%; 7/152] using this medium. On the other hand, the rate of biofilm formation in TSBglu-5% & TSBsuc-6% increased to 57.5% [80/139] & 60.4% [84/139] respectively. Likewise, other findings showed biofilm formation increased to 80 [52.6%] and 82 [53.9%] after incubation for 18 hour and 24 hours respectively, following supplementation of TSB with glucose. Prolonged incubation followed by sugar supplementation improved the rate of biofilm formation. [8]

In our study, Tube method detected biofilm formation in 29.4% [41/139] isolates followed by CRA method in 13.6% [19/139] and BHI6% suc in 13.6% [19/139], which is similar to study done by Soumya et al, being 50.0% & 31.5% by TM and CRA method. and TCP method in 32.3% [45/139] isolates. Similar findings were reported by Ruchi et al, where biofilm formation was detected in 56 [40.88%] isolates by CRA Method followed by 52 [37.96%] in TM and 37 [27%] in TCP method. [12]

TCP method was the most specific test for detection of biofilm formation in the

present study. The test is easy to perform and assess biofilm forming capacity of an isolate both qualitatively and quantitatively. Subjective error is overcome in this method because the reading of biofilm formation is done using an ELISA reader. The sensitivity by TM was 53.3%, being more than that detected by CRA & BHI suc 6%, yet TM cannot be recommended for general biofilm screening test because of the differences associated with observing results by naked eye visualization.

Using TCP method as the standard method to evaluate other biofilm formation tests, it was seen that the sensitivity of TA method was 53.3%, which is higher than CRA method, being 28.8%. On the other hand, specificity of CRA and BHIsuc 6% method was 93.6% & 89.3% in contrast to that of TA method [81.9%]. However, in other studies, the sensitivity [82.35%] and accuracy [86.5%] of CRA were higher than Tube adherence method [sensitivity = 76.47, accuracy = 82.69%], while no difference was observed in the specificity of these two methods. The PPV of CRA & TAM was 68.4% and 58.5% respectively. [13] Hence these tests cannot be recommended for clinical decision making.

Various virulence factors elaborated by CONS were evaluated. Staphylococcus epidermidis was the predominant strain showing haemolysis to sheep RBC and human RBC, being, 34.6% & 79.1% respectively. Similar results have been reported by other authors. However, authors reported no significant correlation between level of resistance of MRSA isolates and their respective protease, lipase or haemolysin production [P > 0.01]. [14]

Lambe et al reported that most Staphylococcus epidermidis, Staphylococcus warneri, and Staphylococcus hominis strains included in their study produced lipase and DNAse. [14] On the contrary, our results showed Staphylococcus epidermidis, Staphylococcus haemolyticus and Staphylococcus saphrophyticus were the common species producing DNase and lipase. Other authors reported DNase activity to be more pronounced in Staphylococcus *chromogenes* as compared to other species of CONS. However, production of DNase though not a potent indicator of pathogenicity of CONS, yet has been reported in our study isolates. Gelatinase enzyme has been reported in 38.4% of Staphylococcus epidermidis and 28.2% of Staphylococcus haemolyticus in our study. In other studies, a higher percentage of Staphylococcus epidermidis 67.2% strains produced gelatinase. The number of strains of Staphylococococcus epidermidis [40.0% vs 38.4%] followed by Staphylococcus saphrophyticus [11.1% vs 7.1%] & Staphylococcus capitis [3.5% vs 2.7%] producing caseinase was more as compared to gelatinase activity. Dissimilar findings was seen in another study, where 43.3% of Staphylococcus epidermidis strains produced gelatinase and only 3.3% displayed caseinolytic activity.[14]

In our study of the MRCONS strains, resistance was more common to ampicillin [100%], followed by amoxy-clavulanic acid [73.6%] and ciprofloxacin & vancomycin [66.6% each]. Likewise, the MSCONS showed maximum resistance to ampicillin [62.1%; 51/82] and ciprofloxacin [59.7%; 49/82]. Least resistance was shown to linezolid [3.6%; 3/82]. In other studies, greater resistance was observed to all antimicrobial drugs in the biofilm producing isolates, being 85.46% for ampicillin and 66.8% for cefoxitin. Vancomycin and Linezolid resistance was observed in 5.81% and 4.07% of the biofilm producing Staphylococcal isolates, respectively. [15]

Conclusions

this study shows that 39.7% [139/350] strains of CONS were isolated from neonatal sepsis, hence the need for speciation and routine antimicrobial susceptibility testing. This practice will reduce irrational use of antibiotics and the inadvertent emergence of resistant strains.

The best approach to identify the species, characterize the resistant traits, determine the virulence factors is by molecular methods. However, in setup with inadequate molecular resources, performance of key biochemical tests and detection of biofilm formation by TCP method should be recommended.

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