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ORIGINAL RESEARCH

INTEGRATIVE ASSESSMENT OF PHENOTYPIC AND MOLECULAR APPROACHES FOR EVALUATING BIOFILM FORMATION IN CLINICAL ISOLATES OF COAGULASE-NEGATIVE STAPHYLOCOCCI

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ABSTRACT

Several bacterial species once dismissed as harmless commensals are increasingly implicated in human infection. Coagulase-negative staphylococci (CoNS) are a prominent example, causing a broad spectrum of disease in hospitalised patients and, in particular, those in intensive care. Biofilm formation is a key virulence attribute that promotes the survival and dissemination of CoNS. This study undertook an integrated phenotypic and molecular assessment of biofilm formation in clinical CoNS isolates. Clinical CoNS isolates were evaluated for biofilm-forming ability by two approaches: a quantitative phenotypic assay using the microtitre-plate method, and molecular detection of the *ica* genes responsible for biofilm formation by polymerase chain reaction (PCR). Taking the genotypic method as the reference standard, the sensitivity and specificity of the phenotypic method were determined. Most CoNS were recovered from urine and blood cultures. *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus* were the predominant species. Of 522 isolates, 489 (93.7%) carried at least two biofilm-associated genes and were classified as biofilm producers by the genotypic method. Against this reference standard, the microtitre-plate method was 95.7% sensitive and 100% specific for the detection of biofilm formation. Advances in molecular biology have identified specific genes governing virulence traits such as biofilm formation, clarifying the mechanisms of pathogenicity; however, molecular methods remain costly for routine use. These findings indicate that the simple, inexpensive microtitre-plate technique offers good sensitivity and specificity and can be readily adopted in diagnostic laboratories for the detection of biofilm formation in CoNS.

Keywords: Biofilm; Coagulase-negative staphylococci; Molecular techniques; Phenotypic method.

INTRODUCTION

The aetiology of infectious disease is undergoing a shift, with classical 'pervasive' pathogens increasingly accompanied by 'opportunistic' organisms. The latter long regarded as commensals, saprophytes, or microbes without pathogenic significance were historically overlooked when recovered from clinical specimens. An opportunistic pathogen has been defined as a microbe of limited or no pathogenic capacity under normal circumstances that is nonetheless able to cause serious disease when favoured by an underlying illness or its treatment [1].

Coagulase-negative staphylococci (CoNS) are among these opportunists and pose a particular challenge in hospitalised patients, in whom they are an important cause of healthcare-associated infection [2]. CoNS infections occur most often in individuals with impaired immunity, recipients of broad-spectrum antimicrobial therapy, and patients with indwelling medical devices [3]. In such hosts, CoNS may produce systemic disease involving the heart, bones, joints, and central nervous system [4].

CoNS constitute a heterogeneous group of staphylococci distinguished from the recognised pathogen *Staphylococcus aureus* by a negative coagulase test. As a group, they possess comparatively few of the aggressive virulence determinants that characterise *S. aureus* [5].

The ability to produce biofilms on biotic and abiotic surfaces is considered the main virulence factor of CoNS. Biofilm is a firm, highly structured, multicellular microbial community formed on a substratum and embedded in an extracellular polymeric matrix that itself is produced by the biofilm cells [6]. The ability of CoNS to form biofilms endows them with valuable characteristics such as resistance to antimicrobial agents and avoidance of host defenses [7,8].

There are several in-vitro methods which can be used to detect the formation of biofilm such as the tube adherence test, the Congo red agar and the tissue-culture (or microtitre-plate) assay. Polymerase chain reaction (PCR)-based molecular methods to amplify genes that are associated with the production of biofilms are also commonly used [9]. The present study aimed to perform an integrated assessment of phenotypic and molecular methods used to assess the ability of clinical isolates of CoNS to form biofilms.

MATERIALS AND METHODS

Study Setting

The study was carried out at People's College of Medical Sciences and Research Centre, Bhopal in the Department of Microbiology. This protocol was approved by the Institutional Ethics Committee.

A total of 522 clinical CoNS were tested for their biofilm-forming properties. S. epidermidis ATCC 12228 and S. warneri ATCC 10209 were used as negative (non-biofilm-forming) controls, while S. epidermidis ATCC 35983 and S. simulans ATCC 27851 were used as positive (biofilm-forming) controls. Clinically recovered CoNS isolates were identified using the catalase test, the coagulase test and Gram staining.

Identification of CoNS

The genus Staphylococcus was separated from Micrococcus using the separation method described by Baker et al. [10] and the simplified sugar-utilisation scheme of Cunha et al. [11] was used for the differentiation of species of CoNS. Isolates were subcultured onto nutrient agar slants and kept at −70 °C until the biofilm assays were carried out.

Biofilm Formation

The ability to form biofilm was evaluated by two complementary approaches, namely a quantitative phenotypic assessment carried out in a microtitre-plate biofilm assay [8] and molecular detection of ica genes involved in the formation of the biofilm by PCR.

Microtitre-plate Method (Phenotypic)

Flat-bottomed 96-well plates were used. Each well received 0.2 mL of a bacterial suspension containing 10^5 CFU/mL in trypticase soy broth (TSB). After aerobic incubation at 37 °C for 24 h, the well contents were aspirated and the plates washed twice with phosphate-buffered saline (PBS, pH 7.2). Adherent biofilm was stained with 0.1% crystal violet for 2 min, and the optical density (OD) of each well was read at 492 nm using a microplate reader (BioTek ELx808). Sterile TSB served as the negative control. To limit error, each assay was performed at least in duplicate and the OD values were averaged. Biofilm production was graded against the OD of the negative control (cut-off OD, ODc) using the classification of Stepanović et al. [12]: no biofilm, OD ≤ ODc; weak, ODc < OD ≤ 2×ODc; moderate, 2×ODc < OD ≤ 4×ODc; and strong, OD > 4×ODc.

Detection of ica Genes by PCR (Molecular)

The biofilm-associated *ica* genes were detected by the method of Arciola et al. [13], comprising nucleic-acid extraction, PCR amplification, and visualisation of the amplification products by agarose-gel electrophoresis.

Statistical Analysis

The phenotypic and genotypic methods were compared by calculating sensitivity and specificity, with the genotypic method taken as the reference standard. Isolates carrying at least two of the *ica* genes (*icaA*, *icaB*, or *icaC*) were

considered biofilm-positive by the genotypic method. Sensitivity was defined as the proportion of genotype-positive isolates that were also positive by the phenotypic method, and specificity as the proportion of genotype-negative isolates that tested negative phenotypically. The association between specimen type and CoNS isolation was examined using the Z-test for proportions, with $P < 0.05$ considered statistically significant.

RESULTS

The specimen-wise distribution of CoNS is shown in Table 1. Most isolates were obtained from urine and blood cultures, which together accounted for 70% (365/522) of the total. Isolation was significantly higher from urine than from CSF, pus, pleural fluid, vaginal swabs, and miscellaneous specimens (Z-test, $P < 0.00001$), whereas the difference between urine and blood cultures was not significant (Z-test, $P > 0.05$).

Table 1. Specimen-wise distribution of coagulase-negative staphylococci (n = 522).

Clinical specimen	No. of isolates (%)
Urine	197 (37.7)
Blood	168 (32.2)
Pus	57 (10.9)
Vaginal swabs	12 (2.3)
Pleural fluid	19 (3.6)
CSF	24 (4.6)
Miscellaneous samples	45 (8.6)
Total	522 (100)

Nine CoNS species were identified across the clinical specimens (Figure 1). *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus* were the most common, accounting for 219 (42.0%), 151 (28.9%) and 73 (14.0%) of isolates, respectively.

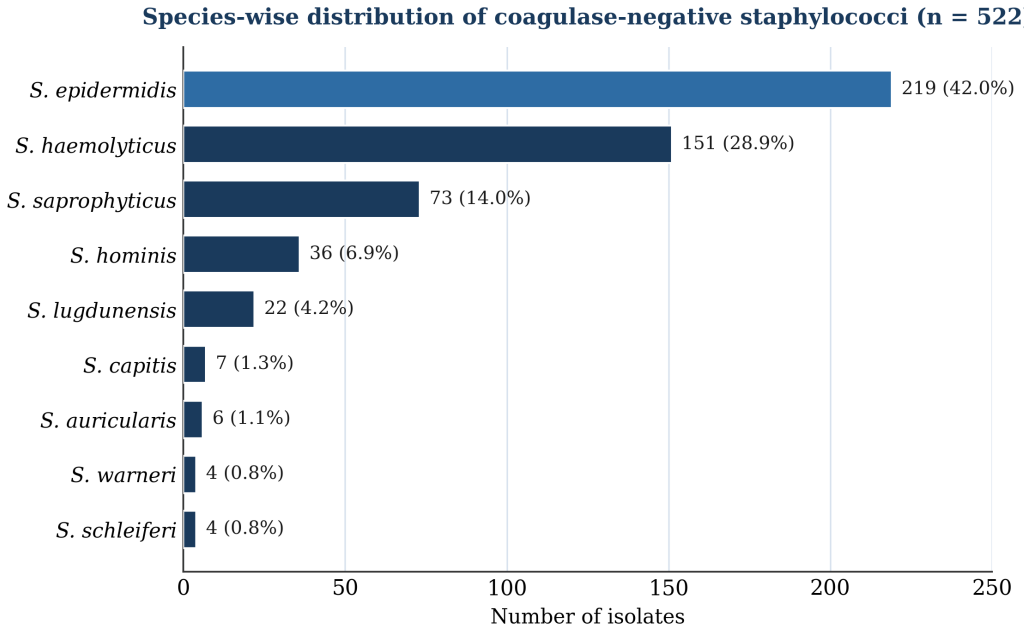

Figure 1. *Species-wise distribution of coagulase-negative staphylococci isolated from clinical specimens (n = 522).*

By the microtitre-plate (phenotypic) method, 468 of 522 isolates (89.7%) formed biofilms. Among these, strong biofilm formation was observed in 134 isolates (28.6%), moderate in 179 (38.2%), and weak in 155 (33.1%). The species- and grade-wise distribution is presented in Table 2. Strong biofilm formation predominated in *S. epidermidis*, whereas a moderate grade was the most frequent pattern among *S. saprophyticus*; *S. capitis*, *S. auricularis* and *S. warneri* produced only weak biofilms.

Table 2. Species and grade wise distribution of biofilm-forming coagulase-negative staphylococci.

CoNS isolate (N)	Weak n (%)	Moderate n (%)	Strong n (%)	Biofilm formers
S. epidermidis (219)	34 (15.8)	86 (40.0)	95 (44.2)	215
S. haemolyticus (151)	78 (52.3)	41 (27.5)	30 (20.1)	149
S. saprophyticus (73)	23 (36.5)	32 (50.8)	8 (12.7)	63
S. hominis (36)	10 (45.5)	11 (50.0)	1 (4.5)	22
S. lugdunensis (22)	6 (42.9)	8 (57.1)	–	14
S. capitis (7)	2 (100)	–	–	2
S. auricularis (6)	1 (100)	–	–	1
S. warneri (4)	1 (100)	–	–	1
S. schleiferi (4)	–	1 (100)	–	1
Total (522)	155 (33.1)	179 (38.2)	134 (28.6)	468

N denotes the total number of isolates of each species; grade-wise percentages are calculated relative to the number of biofilm-forming isolates of that species (final column). – indicates no isolates in that grade.

By the genotypic method, 489 isolates (93.7%) carried at least two biofilm-associated genes and were classified as biofilm producers. Taking the genotypic method as the reference standard, the microtitre-plate method was 95.7% sensitive and 100% specific for the detection of biofilm formation in CoNS.

DISCUSSION

Coagulase-negative staphylococci were historically regarded as harmless inhabitants of the skin and mucous membranes but are now recognised as important agents of infection, particularly opportunistic and healthcare-associated disease. The present study found CoNS to predominate in urinary-tract and bloodstream infections. Consistent with earlier reports, CoNS are a notable cause of urinary-tract infection, especially in young women, elderly men, catheterised or hospitalised patients, and those with a history of urinary-tract surgery [14,15]. They are also among the common causes of bloodstream infection and infective endocarditis, although the frequency of infective endocarditis attributable to different CoNS species in patients with bloodstream infection has received limited attention [16].

S. epidermidis, *S. haemolyticus* and *S. saprophyticus* were the species most frequently isolated, representing 219 (42.0%), 151 (28.9%) and 73 (14.0%) of CoNS, respectively. In most infections, the relative frequency of CoNS species mirrors their density within the cutaneous flora; accordingly, *S. epidermidis* the most abundant CoNS of human skin is also the species most often isolated clinically. Over recent decades, *S. epidermidis* and other CoNS have emerged as major causes of nosocomial infection, particularly among immunocompromised hosts such as premature neonates and patients with leukaemia or other malignancies. The predominance of *S. epidermidis* among CoNS has been documented previously [15].

Among the virulence determinants that distinguish pathogenic from non-pathogenic CoNS, biofilm formation is the most important. By forming multilayered cellular aggregates encased in a polysaccharide slime, CoNS persist on foreign bodies and exhibit reduced antimicrobial susceptibility; this capacity favours adhesion to and colonisation of implanted materials and is considered the principal virulence factor of the group [17].

In this study, 468 of 522 isolates (89.7%) formed biofilms by the phenotypic method, with almost all species demonstrating some biofilm-forming ability and strong biofilm formation predominating in *S. epidermidis*. The PCR-based molecular method detected biofilm-associated genes in 489 isolates (93.7%). Relative to the genotypic reference standard, the microtitre-plate method was 95.7% sensitive and 100% specific, indicating close agreement between the two approaches.

CONCLUSION

Coagulase-negative staphylococci particularly *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus* are emerging opportunistic pathogens in hospitalised patients with comorbidities and in those undergoing aggressive therapeutic or surgical intervention. Virulence traits such as biofilm formation increase the likelihood of disseminated infection. Although genotypic methods are regarded as the reference standard, they are costly and labour-intensive. The simple and inexpensive microtitre-plate technique demonstrated good sensitivity and specificity in this study and can serve as a practical tool for the routine assessment of biofilm formation in CoNS.

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