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Short Communication

Prevalence of methicillin-resistant, coagulase-negative staphylococci in neonatal intensive care units: findings from a tertiary care hospital in India

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This study was undertaken to determine the antimicrobial resistance pattern and species of coagulase-negative staphylococci (CNS) isolated from the blood and skin of neonates with clinical suspicion of late-onset septicaemia (>72 h post-delivery) admitted to neonatal intensive care units, with particular reference to the phenotypic and genotypic expression of methicillin resistance. Blood culture specimens were collected by venipuncture from 660 such neonates in brain heart infusion broth. Skin swabs from axillae were obtained from 60 neonates and inoculated on mannitol salt agar. All CNS thus obtained were further identified and antibiotic sensitivity was performed according to NCCLS recommendations. PCR for the *mecA* gene was carried out on 54 randomly selected isolates. *Staphylococcus haemolyticus* was the commonest species (34 %) followed by *Staphylococcus epidermidis* (24 %) amongst blood isolates. All blood isolates were sensitive to glycopeptides. Resistance to penicillin and methicillin was 94 and 66 %, respectively. Similar biotypes and antimicrobial resistance patterns were observed in skin isolates. All phenotypically methicillin-resistant isolates had the *mecA* gene and two of the phenotypically methicillin-sensitive isolates were also positive for *mecA*. A PCR assay for detection of the *mecA* gene in CNS may be a beneficial adjunct to standard susceptibility testing for timely and reliable detection of methicillin resistance. Given the large number of methicillin-resistant CNS, inclusion of vancomycin in empiric therapy for neonates with late-onset septicaemia may be justified.

Introduction

Advances in neonatal intensive care units (NICUs) have led to improved survival of very low birth weight infants, though late-onset nosocomial neonatal septicaemia (>72 h post-delivery) continues to be an important cause of morbidity and mortality among these infants (Stoll *et al.*, 1996). Although Gram-negative bacilli were the principal concern in previous decades, coagulase-negative staphylococci (CNS) are now the most common organisms associated with late-onset septicaemia, accounting for more than 50 % of cases (Stoll *et al.*, 2002; Nataro *et al.*, 1994; Freeman *et al.*, 1987). These CNS isolates show multiple antibiotic resistance, including resistance to methicillin. Vancomycin is often required for adequate therapy (Stoll *et al.*, 1996; Baumgart *et al.*, 1983). CNS are also among the most common cause of blood-culture contaminants, since they are the most common micro-organisms found colonizing the skin and mucous membranes of neonates (Nataro *et al.*, 1994; Klein, 1990; Freeman *et al.*, 1987; Carr & Kloos, 1977). Skin colonization by CNS can be demonstrated in over 90 % of NICU admissions; these organisms may then gain entry to the blood during an invasive procedure and result in sepsis (Dear, 1999). It has been shown that endemic strains of CNS can be maintained in NICUs for many years, up to a decade (Huebner *et al.*, 1994). It remains difficult to determine which blood isolates of CNS represent true infection and which are contaminants. If CNS are responsible for >50 % of late-onset septicaemia, it would be useful to know the various biotypes prevalent amongst these strains and the level of methicillin resistance, regardless of their status as true bacteraemic agents. This may help in formulating an effective empirical antibiotic policy and controlling vancomycin overuse.

Thus, the present study was undertaken with the primary aim of studying antimicrobial resistance patterns and species of CNS isolated from the blood and skin of neonates with late-onset septicaemia admitted to the NICU with particular reference to phenotypic and genotypic expression of methicillin resistance.

Abbreviations: CNS, coagulase-negative staphylococci; NICU, neonatal intensive care unit.

Methods

This study was carried out in the Department of Microbiology and NICU at Gandhi Memorial and Associated Hospitals of King George's

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Medical University, Lucknow, India, from August 2002 to April 2003. This hospital is a 2000-bed tertiary care teaching hospital. The study included 660 neonates admitted to the NICU with clinical suspicion of late-onset septicaemia, including temperature instability, feeding difficulties, respiratory distress, jaundice and convulsions (Dear, 1999).

After skin preparation with 70 % alcohol and 2 % iodine solution, 1·5 ml blood was collected by venipuncture and inoculated into blood culture bottles containing 10 ml brain heart infusion broth with sodium polyanetholesulphonate as an anticoagulant (Hi Media Laboratories). On delivery to the hospital microbiology laboratory, bottles were processed according to a standard protocol (Collee *et al.*, 1996). Skin swabs from the axillae of 60/660 neonates were also taken and inoculated on to mannitol salt agar. These neonates were divided into three groups: (i) blood-culture positive for CNS; (ii) blood-culture positive for organisms other than CNS; and (iii) blood-culture sterile.

Bacterial isolates obtained were identified by colony morphology, Gram staining and biochemical reactions. All CNS isolates (catalase-positive, tube coagulase-negative, Gram-positive cocci) were further analysed.

Species identification. All CNS isolates were identified to species level based on the scheme of Kloos & Bannerman (1994). The tests were performed according to standard methodology (Kloos & Bannerman, 1999; Koneman *et al.*, 1997).

Antimicrobial susceptibility test. This was performed on Mueller–Hinton agar with the following antibiotic discs (Hi Media Laboratories) using the Kirby Bauer disc diffusion method: penicillin (10 U), cefotaxime (30 μg), vancomycin (30 μg), teicoplanin (30 μg), gentamicin (10 μg), amikacin (30 μg) and ciprofloxacin (5 μg). An oxacillin disc (1 μg) was used to detect methicillin resistance. Sensitivity was read after incubation for 24 h at 35 °C. Isolates were regarded as sensitive or resistant according to NCCLS criteria (NCCLS, 2000).

Detection of the *mecA* gene by PCR. Two to four colonies of overnight bacterial subculture were suspended in 100 μl lysis buffer (10 mM Tris/HCl, pH 8·0, 2 mM EDTA, 0·4 % NaCl, 0·1 % Triton X-100), vortexed for 5 min and boiled for 15 min followed by centrifugation at 3000 r.p.m. for 10 min at room temperature. The supernatant was used for DNA amplification. The primers used were: forward, 5′-CATTTTGAGTTCTGCACTACC-3′, and reverse, 5′-GCAATACA ATCGCACATACATTAATAG-3′ (Bangalore Genie) (Ryffel *et al.*, 1992). The reaction mixture (50 μl total) containing 5 μl extracted DNA, 10 mM Tris/HCl, pH 9·0, 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM each dNTP, 1·0 U *Taq* DNA polymerase and 0·5 μM each primer was used for PCR to amplify the *mecA* gene in an automated thermal cycler (Progene Techne). The amplified product of 967 bp was detected by ethidium bromide staining following 1·5 % agarose gel electrophoresis (Jaffe *et al.*, 2000).

Results and Discussion

A total of 108 CNS strains were recovered from blood specimens of 660 neonates with late-onset septicaemia and CNS were isolated from 59/60 skin swabs.

Of the total 108 blood isolates of CNS, eight were lost during processing. The remaining 100 CNS were identified. *Staphylococcus haemolyticus* was the commonest species (34 %) followed by *Staphylococcus epidermidis* (24 %), *Staphylococcus saprophyticus* (8 %), *Staphylococcus cohnii* (6 %), *Staphylococcus hominis* (4 %), *Staphylococcus warneri* (3 %), *Staphylococcus sciuri* (2 %), *Staphylococcus simulans* (1 %) and *Staphylococcus capitis* (1 %). CNS isolated from skin did not show any significant difference in biotype distribution within the three groups of neonates. Overall, *S. haemolyticus* was the commonest isolate (34/59, 58 %) followed by *S. epidermidis* (10/59, 17 %) and one isolate each (2%) of *S. saprophyticus* and *S. capitis*. Seventeen CNS strains from blood and 13 from skin could not be identified in our study using conventional biochemical tests.

Antimicrobial susceptibility testing revealed that all the blood isolates were sensitive to vancomycin, while 66 % were resistant to methicillin. Similar findings were observed in another study from India on neonatal septicaemia (Kumhar *et al.*, 2002), although in that study, CNS was not a major isolate. Penicillin resistance was frequent (94 %), while amikacin resistance was relatively rare (19 %). Resistance to antibiotics was seen more in the methicillin-resistant isolates compared with those that were methicillin sensitive (Table 1). The resistance pattern of isolates from skin did not show any significant difference when compared with the blood isolates for any of the tested antibiotics (Fig. 1). Detection of the *mecA* gene was carried out in 43 randomly selected blood isolates and 11 skin isolates. The *mecA* gene was present in 37 CNS isolates and its presence correlated with the phenotypic expression of methicillin resistance (Table 2).

The number of multiple-drug-resistant strains, including methicillin-resistant CNS, has increased, and the majority of CNS causing neonatal septicaemia are resistant to the routine antibiotics used to treat newborn infants (Stoll *et al.*, 1996; Baumgart *et al.*, 1983). In the present study, 66 % of isolates from blood were resistant to methicillin using the disc diffusion test. All (100 %) of the methicillin-resistant CNS were penicillin resistant, while only 81·2 % of methicillin-sensitive CNS showed resistance to penicillin (Table 1). A co-existing resistance to a different antibiotic was significantly higher in methicillin-resistant CNS compared with methicillin-sensitive CNS in the present study, with the exception of gentamicin. Similar findings have also been observed by Fluit *et al.* (2000).

Although others have reported *S. epidermidis* to be the most common species of CNS isolated from the blood of neonates with suspected late-onset septicaemia (Martin *et al.*, 1989; Freeman *et al.*, 1987), we found that *S. haemolyticus* was the commonest, followed by *S. epidermidis*, in accordance with the study done by Mehta *et al.* (1991). This was also true for the skin CNS isolates, and the antimicrobial resistance pattern of CNS isolated from the two sources was similar. The rate of colonization on skin of neonates by CNS was very high (59/60, 98 %), and high resistance to methicillin (42/59, 71·2 %; Fig. 1) points to their probable nosocomial origin. Molecular methods are required to show clonality amongst strains recovered from skin and blood, which we were not able to do in the present study.

The correlation between the presence of the *mecA* gene and phenotypic resistance to methicillin in CNS remains less well defined than in *Staphylococcus aureus*. A small number of *mecA*-negative CNS that are phenotypically methicillin resistant have been reported by Suzuki *et al.* (1992). In the present study, there was 100 % correlation between the

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Table 1. Susceptibility pattern in methicillin-resistant and methicillin-sensitive CNS from blood ($n = 100$)

Numbers in parentheses represent percentages.

Antibiotic	Methicillin-resistant CNS ($n = 68$)*			Methicillin-sensitive CNS ($n = 32$)		
	Sensitive	Resistant	Intermediate	Sensitive	Resistant	Intermediate
Penicillin†	0	68 (100)	0	6 (18·8)	26 (81·2)	0
Cefotaxime†	6 (8·8)	58 (85·3)	4 (5·9)	19 (59·4)	8 (25)	5 (15·6)
Vancomycin	68 (100)	0	0	32 (100)	0	0
Teicoplanin	68 (100)	0	0	32 (100)	0	0
Amikacin†	43 (63·2)	17 (25)	8 (11·8)	27 (84·4)	2 (6·2)	3 (9·4)
Gentamicin	23 (33·8)	37 (54·4)	8 (11·8)	16 (50)	15 (46·9)	1 (3·1)
Ciprofloxacin†	20 (29·4)	42 (61·8)	6 (8·8)	22 (68·8)	10 (31·3)	0

*Two methicillin-intermediate strains are included.

†$P < 0·05$.

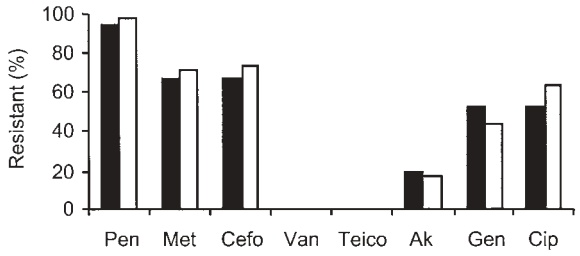

Fig. 1. Antimicrobial resistance patterns of CNS isolated from blood (filled bars) and skin (open bars). Pen, Penicillin; Met, methicillin; Cefo, cefotaxime; Van, vancomycin; Teico, teicoplanin; Ak, amikacin; Gen, gentamicin; Cip, ciprofloxacin.

Table 2. Comparison of phenotypic expression of methicillin resistance and *mecA* gene positivity ($n = 54$)

Presence of *mecA* gene	Methicillin phenotype		
	Resistant	Sensitive	Intermediate
Negative ($n = 17$)	0	16	1*
Positive ($n = 37$)	31	2*	4

*CNS isolates with discrepant results.

presence of the *mecA* gene and expression of methicillin resistance. There were no *mecA*-negative methicillin-resistant isolates, although we found a single strain of *mecA*-negative, moderately methicillin-resistant CNS. Another non-penicillin-binding protein-dependent mechanism such as hyperproduction of β-lactamase, or the presence of other low-affinity penicillin-binding proteins (Geha *et al.*, 1994), could be responsible for this phenomenon. There were two *mecA*-positive methicillin-sensitive isolates, suggesting that the *mecA* gene is not consistently expressed. Similar observations have been reported by Tan (2002). There are studies that support the possibility that certain auxiliary genes such as *femA*, *mecR* and the gene encoding the β-lactamase plasmid control *mecA* expression (Geha *et al.*, 1994).

PCR assays for detection of the *mecA* gene in CNS may be a beneficial adjunct to standard susceptibility testing and allow the identification of intrinsic resistance in a timely and reliable manner. Given the high proportion of CNS resistance to methicillin, vancomycin should be included in empirical therapy for neonates with late-onset septicaemia.

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