

## Coagulase-negative staphylococci: clinical, microbiological and molecular features to predict true bacteraemia

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Coagulase-negative staphylococci (CNS) are frequently isolated from blood cultures, where they may be only a contaminant or the cause of bacteraemia. Determining whether an isolate of CNS represents a true CNS bacteraemia is difficult, and there is no single criterion with sufficient specificity. The aim of this study was to assess those clinical, microbiological, pathogenic and genotypic features that characterize true CNS bacteraemia. Twenty patients having two or more blood cultures positive for CNS and 20 patients with only one positive blood culture were studied. Significant bacteraemia was defined according to clinical and laboratory criteria. Incubation time for blood cultures to become positive, macroscopic appearance of colonies, species determination, biotype, susceptibility to antimicrobials, PFGE pattern and adherence capacity were all studied. Clinical bacteraemia was present in 16/20 patients with two or more positive blood cultures and in 2/20 patients with only one positive blood culture. A significant difference was seen in the median time to positivity between the 18 clinical bacteraemias and 22 contaminations (23.6 versus 29.2 h;  $P = 0.04$ , Wilcoxon). There was also a significant difference between the two groups in the median absorbance of the slime test (1.36 versus 0.58;  $P = 0.005$ ). All significant bacteraemias with two or more positive blood cultures had the same species identified, the same antimicrobial susceptibility pattern and the same PFGE pattern. In two patients with true bacteraemia with only one positive blood culture, the incubation time for the culture to turn positive was <24 h and the slime production absorbance was >2.5. The most useful parameters for the diagnosis of true CNS bacteraemia for patients with two positive blood cultures were incubation time until positive, species identification, antimicrobial susceptibility pattern, slime production and PFGE pattern. For patients with only one blood culture positive for CNS, the useful parameters for prediction of true bacteraemia were incubation time until positive and slime production, both of which are simple, low-cost tests.

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## INTRODUCTION

Coagulase-negative staphylococci (CNS) are a group of micro-organisms that are increasingly implicated as a cause of significant infection (Gemmell, 1986; Weinstein *et al.*, 1997). They are one of the main causal agents of bacteraemia in patients with indwelling medical devices such as central and peripheral venous catheters, valvular prostheses, artificial heart valves, pace-makers and orthopaedic prostheses and other infections involving biofilm formation on implanted biomaterials (Huebner & Goldmann, 1999). The

species most frequently associated with bacteraemia is *Staphylococcus epidermidis*. This may necessitate the removal of these devices, which, in turn, may cause high morbidity and mortality and elevated costs (Bates *et al.*, 1991).

Several indicators have been investigated in order to differentiate true bacteraemia from contamination, including number of positive blood cultures, species of CNS and biotype, quantitative antimicrobial susceptibility testing, similarity in colony morphology and clonality. These variables have the following two problems: none has a high positive predictive value and they are useful only when two or more blood cultures in one series of such cultures are

Abbreviations: CNS, coagulase-negative staphylococci; PFGE, pulsed-field gel electrophoresis.

positive. This is because they are based on a demonstration that the strains isolated are identical and that the probability of contamination is very low.

The occurrence of more than one positive blood culture has been used as a good predictor of true bacteraemia; nevertheless, publications from the last decade indicate that about 34 % of patients with nosocomial bacteraemia had only one positive blood culture (MacGregor & Beaty, 1972). The use of this as the sole diagnostic determinant may lead to underdiagnosis of true bacteraemia (Martin *et al.*, 1989; Peacock *et al.*, 1995; Mirret *et al.*, 1993; Herwaldt *et al.*, 1996). In our experience, up to 13 % of blood cultures positive for CNS were true bacteraemias (García *et al.*, 1999). Isolating the same species of CNS in more than one blood culture also increases the probability of true bacteraemia. However, since *S. epidermidis* is the most frequently isolated species in blood cultures and is the most important representative of the skin flora, this strategy is especially useful when a species other than *S. epidermidis* is isolated and when the microbiology laboratories are able to identify the species. This approach is costlier and requires additional time, unless automated identification systems are available. Similar data were obtained by analysing the CNS biotype. Herwaldt *et al.* (1996) found a strong association between identical biotypes found in the same series of blood cultures and significant CNS bacteraemia, although sensitivity was 85 % and specificity 45 % (Herwaldt *et al.*, 1996). This technique requires sophisticated, commercially available methods for identification.

Sloos *et al.* (2000) compared colony morphology and quantitative antimicrobial susceptibility testing with pulsed-field gel electrophoresis (PFGE) to assess clonality in two positive blood cultures. Quantitative antimicrobial susceptibility testing, using a mathematical formula to obtain a similarity coefficient based on inhibition zone diameters, showed a good correlation with PFGE (Sloos *et al.*, 2000). For demonstrating clonality of two CNS isolated from blood cultures at the same febrile episode, PFGE is considered the reference method; however, it is laborious, time-consuming (results take up to 4 days) and expensive.

All of the methods described above may be clinically useful when the patient has at least two positive blood cultures. However, there are a significant number of patients with true clinical bacteraemia who have only one positive blood culture. In these cases, incubation time until cultures become positive and the presence of bacterial virulence factors such as production of capsular polysaccharide (also known as slime) might be useful for the diagnosis of true bacteraemia.

Polysaccharide production by CNS is related to their ability to adhere to biomaterials and is one of their main virulence factors (Ishak *et al.*, 1985; O'Gara & Humphreys, 2001). Microbiological methods that allow assessment of slime production have been described. For example, Christensen's method or the Congo red test were used in the 1980s to correlate microbiological findings with true CNS infections (Christensen *et al.*, 1982; Davenport *et al.*, 1986; Kotilainen 1990; Hussain *et al.*, 1992; Baldassarri *et al.*, 1993; Ammen-

doia *et al.*, 1999). Christensen's method is based on the ability of CNS to adhere to plastic material and the quantification of this adherence (Christensen *et al.*, 1982). The original test was poor, because it relied on a subjective interpretation of the results. This has since been replaced by a modification that uses spectrophotometric readings (Baldassarri *et al.*, 1993). The Congo red test is based on the ability of this dye to stain polysaccharides black, so, if a strain is able to synthesize capsular polysaccharide and Congo red is incorporated in the culture medium, the colony will be black (Freeman *et al.*, 1989). Although these publications showed that most pathogenic strains of CNS produced slime, more recent data show that the specificity of this test is inadequate (Herwaldt *et al.*, 1996). Genetic control of polysaccharide synthesis is mediated by *icaA* and *icaD*, which encode an *N*-acetylglucosaminyl transferase enzyme that catalyses the synthesis of the capsular polysaccharide ( $\beta$ -1,6-glucosamine glycan) from *N*-acetylglucosamine (Arciola *et al.*, 2001). This provides new insights into CNS pathogenicity, since a strain that carries the genes that encode capsular polysaccharide will potentially be pathogenic.

The object of this study was to examine the clinical, microbiological, molecular typing and virulence characteristics of CNS isolated from blood cultures so as to be able to differentiate true bacteraemia from contamination in patients with one or two positive blood cultures.

## METHODS

**Patients.** From September 2000 to June 2001, 20 patients who were hospitalized in the Hospital Clínico de la Universidad Católica were studied. They had two or more blood cultures positive for CNS in one series of blood cultures and were selected randomly for study. An independent investigator visited the patient or reviewed the clinical charts to determine whether or not the case was a true CNS bacteraemia.

**Definition of true bacteraemia.** The criteria of Bates (Bates *et al.*, 1990, 1997) and Herwaldt (Herwaldt *et al.*, 1996) were used: patients with a suggestive clinical sepsis, a temperature above 38 °C, chills, white blood cell count greater than 12 000 mm<sup>-3</sup> with a left shift, initiation of specific therapy (vancomycin), all other causes of bacteraemia having been ruled out and/or having had a central venous catheter or medical device removed.

**Blood cultures.** Blood cultures were taken according to routine procedures of the Health Network of Universidad Católica. Each blood culture was obtained from a different venipuncture site, after careful cleansing and disinfection of the skin. Ten millilitres of blood was drawn per bottle. The specimen was inoculated into BacT/Alert bottles (bioMérieux), which were then incubated for 7 days in a BacT/Alert automated blood culture system. The incubation time (in hours) before the culture became positive was recorded. Each positive bottle was subcultured onto blood, chocolate and MacConkey agar plates and incubated at 37 °C for 24–48 h.

**Bacterial identification, antimicrobial susceptibility and biotype.** Every suspicious colony was Gram stained and tested for coagulase. If the coagulase test was negative, a MicroScan Gram-positive panel was inoculated (Dade). This panel allowed identification and biotyping of the species. Susceptibility to 20 antimicrobials was tested by broth

micro-dilution. Panels were read in an AutoScan instrument (Dade) after 24 h.

**Colony morphology.** Each plate was inspected at 24 and 48 h by two independent investigators. They observed and recorded the colour, size, elevation and nature of the borders of the colonies.

**Molecular typing by PFGE.** Cellular lysis was performed as described by Leonard & Carroll (1997) with a few modifications. A CNS colony cultured on a blood-agar plate for 18–24 h was picked and inoculated into Todd–Hewitt broth and incubated overnight at 37 °C. A 500 µl aliquot from this broth was centrifuged at 4 °C for 3 min at 12 000 r.p.m. and the sediment was resuspended in 1 ml TEN buffer (0.1 M Tris/HCl, pH 7.5, 0.1 M EDTA, 0.15 M NaCl) and centrifuged at 4 °C for 3 min at 12 000 r.p.m. The supernatant was removed and the sediment resuspended in 1 ml TN buffer (10 mM Tris/HCl, pH 8.0, 10 mM NaCl) and centrifuged again. This sediment was then resuspended in 100 µl TN buffer and 10 µl (200 U) achromopeptidase (Wako Bioproducts) plus 100 µl 2 % low-melting-point agarose (Bio-Rad) at 50 °C were added. This mixture was immediately pipetted into a plug mould (Bio-Rad) and allowed to solidify at 4 °C for 30 min. The plugs were removed from the wells and incubated at 50 °C in 300 µl TN buffer for 30 min. They were then washed three times with 2 ml TE buffer (10 mM Tris/HCl, pH 7.6, 1 mM EDTA) for 30 min and incubated at room temperature for 3 h with 30 U *Sma*I (Gibco BRL) and 1× restriction buffer for digestion. The samples were run on an agarose gel (1 % PFGE grade, Bio-Rad) in 0.5× TBE (0.045 M Tris/borate, 0.001 M EDTA). Electrophoresis was performed with CHEF-DR-III equipment (Bio-Rad). The program used was an initial 5 s pulse and a final 35 s pulse for 21 h at 6 V cm<sup>-1</sup>. *Saccharomyces cerevisiae* DNA (Bio-Rad) was used as the molecular mass standard. Gels were stained in 0.5 µg ethidium bromide ml<sup>-1</sup> and visualized on a UV transilluminator.

### Slime production

**Slime test.** Briefly, a bacterial suspension was prepared from a blood-agar plate culture in trypticase soy broth at an opacity of 0.5 MacFarland standard and cultured overnight at 37 °C. The next day, 100 µl of the overnight culture was added to 200 µl tryptose broth and placed in a microtitre tray well, where it was mixed and incubated overnight at 37 °C. The following day, the wells were carefully emptied and washed three times with PBS. The plate was allowed to dry at 60 °C for 1 h and then stained with Hucker's crystal violet (2 g crystal violet, 20 ml 95 % alcohol, 0.8 g ammonium oxalate and 80 ml distilled water). The excess stain was washed off with distilled water, excess water was removed and the plates were read with an ELISA reader at 570 nm (Baldassarri *et al.*, 1993).

**Congo red test.** The medium was prepared with 37 g brain heart infusion broth, 50 g sucrose, 10 g agar and 0.8 g Congo red l<sup>-1</sup>. Congo red stain was prepared as a concentrated aqueous solution, autoclaved at 121 °C for 15 min and then added to the other components of the culture medium when it had cooled to 55 °C. Plates were inoculated with one or more colonies of the original isolate and incubated at 37 °C for 24 h. A result was considered positive when black colonies grew on the surface. Strains that did not produce slime developed red colonies (Freeman *et al.*, 1989).

**PCR.** Bacterial DNA was obtained directly from a colony on blood agar and was added to a reaction mixture (final volume 50 µl) containing 1× buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl), 3 mM MgCl<sub>2</sub>, 200 µM of each deoxynucleotide, 2.5 U *Taq* DNA polymerase (Gibco BRL) and 0.4 µM of each primer [for *icaA*, *icaA1* (5'-TCTCTTGCAGGAGCAATCAA) and *icaA2* (5'-TCAGGCACTAACATCCAGCA); for *icaD*, *icaD1* (5'-ATGGTCAAGCCCAGACAGAG) and *icaD2* (5'-CGTGTTTTCAACATTTAATGCAA); Freeman *et al.*, 1989]. The first pair of primers amplifies a 188 bp region and the second pair a 198 bp region. The

reaction underwent 40 amplification cycles (95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s). *S. epidermidis* ATCC 12228 was used as a negative control. The amplified products were electrophoresed on a 1.5 % agarose gel, the gel was stained with 0.5 µg ethidium bromide ml<sup>-1</sup> and visualized on a UV transilluminator and product sizes estimated by comparison with a 50 bp DNA ladder (Gibco BRL).

**Statistical analysis.** A comparison of quantitative variables (time until positive and slime production by the modified Christensen method) was carried out using a non-parametric Wilcoxon test.

## RESULTS

Of the 20 patients who had two or more positive blood cultures, 16 (80 %) were classified as having true bacteraemias according to the definition and four (20 %) were considered to have contaminated blood cultures. In the 20 patients with only one positive blood culture, two had true bacteraemias (10 %) and 18 were contaminants (90 %). Using this classification, the patients were divided into those with true bacteraemia (18/40) and patients with contaminated blood cultures (22/40).

The utility of microbiological variables to demonstrate relatedness in CNS strains from patients with two or more positive blood cultures is shown in Table 1. Colony morphology was not a good indicator of true bacteraemia. Species identification and PFGE molecular typing were more specific and sensitive measures. All patients with true bacteraemia had indistinguishable strains. In two of the four episodes of contamination, there were different strains. The biotype had good specificity (only one episode of contamination out of the four had similar biotypes) and sensitivity was 81 %. For sensitivity and specificity, the best results were obtained with antimicrobial susceptibility analysis, where sensitivity was 100 % (16/16) and specificity 75 % (three of four contaminants were different). It is important to highlight that one of the patients considered to have a contaminant had the same species (*S. epidermidis*), the same biotype, the same susceptibility pattern and the same PFGE result for the two strains isolated from the two blood cultures.

The incubation time until positive and the slime production test using Christensen's modified method were applied to all strains, regardless of whether they were from patients with one or two or more positive blood cultures. This was done because it was necessary to find a method that allowed differentiation between contaminant and bacteraemia at the time a blood culture became positive, since several hours sometimes elapse before the second blood culture becomes positive. These results are shown in Table 2, where there is a statistically significant difference in incubation time until positive ( $P = 0.02$ ). For slime production, the median absorbance of the bacteraemia group was significantly higher than that of the group without bacteraemia (0.88 vs 0.29;  $P = 0.0008$ ); nonetheless, there was some overlap in the absorbance values of the slime test. A cut-off at an absorbance of 0.7 allowed a better separation between bacteraemia and contaminants. Similarly, a cut-off of 27 h was established for incubation time until positive to allow better discrimination

**Table 1.** Patients with two positive blood cultures: agreement of CNS microbiological variables for true bacteraemia and contamination (phenotype and genotype) in both blood cultures in one series of blood cultures

Microbiological variable	True bacteraemia ( <i>n</i> = 16)		Contamination ( <i>n</i> = 4)	
	<i>n</i>	%	<i>n</i>	%
Indistinguishable colony morphology	16/16	100	4/4	100
Same species	16/16	100	2/4	50
Same biotype	13/16	81	1/4	25
Same antimicrobial susceptibility test	16/16	100	1/4	25
Indistinguishable PFGE	16/16	100	2/4	50

**Table 2.** Time to positive culture and slime production in CNS obtained from patients with one or more positive blood cultures

Parameter	True bacteraemia	Contamination	<i>P</i> value*
Strains studied ( <i>n</i> )	35	27	
Median time until positive (h)	19.4	22.7	0.02
Median slime production ( <i>A</i> <sub>570</sub> )	0.88	0.29	0.0008

\*Non-parametric Wilcoxon test.

between bacteraemia and contaminants. Using these cut-offs, sensitivity and specificity of incubation time were respectively 85 and 35 % and, for slime production, 57 and 74 % (Table 3). The other methods to assess the adherence ability of CNS, such as Congo red and the presence of genes encoding capsular polysaccharide, gave variable sensitivity and specificity: 46 and 85 % for Congo red and 63 and 74 % for *icaA* and *icaD* PCR (Table 3).

Since no single method was suitable, sensitivity and specificity were calculated for all methods and for pairs of methods. Table 4 shows that, if the four methods were positive for a strain, the specificity was very good (92 %) but the sensitivity

was only 38 %. The best positive and negative predictive values were obtained with a combination of all results or with the time to positive <27 h plus a positive slime test or a positive Congo red test. All three of these combinations gave positive predictive values of better than 80 % and negative predictive values of 53–56 % (Table 5).

## DISCUSSION

This study confirms that there is no single test with a high enough accuracy to diagnose a true CNS bacteraemia. The problem of blood culture contamination is relevant and,

**Table 3.** Tests that can be used for CNS isolated from patients with one or more positive blood cultures

Test	With bacteraemia	Without bacteraemia	Sensitivity (%)	Specificity (%)
<27 h incubation until positive*	29/34	16/26	85	35
Positive slime test†	20/35	7/27	57	74
Positive Congo red test	16/35	4/27	46	85
Positive PCR for <i>icaA</i> and <i>icaD</i>	26/35	10/27	63	74

\*Threshold fixed at 27 h incubation.

†Threshold fixed at an *A*<sub>570</sub> of 0.70.

**Table 4.** Combination of two or more methods to differentiate true bacteraemia from contamination in patients with one or more blood cultures positive for CNS

Time(+), blood cultures positive before 27 h incubation; slime(+), CNS strains with  $A_{570} \geq 0.70$ ; CR(+), positive Congo red test; *ica*(+), PCR-positive for amplification of *icaA* and *icaD*. All(+) indicates all methods positive.

Methods	With bacteraemia	Without bacteraemia	Sensitivity (%)	Specificity (%)
All(+)	13/34	2/26	38	92
Time(+) + slime(+)	17/34	4/26	50	85
Time(+) + CR(+)	14/34	3/26	41	88
Time(+) + <i>ica</i> (+)	22/34	7/26	65	73

**Table 5.** Positive and negative predictive values for individual and combined methods

See Table 4 for definitions of positivity criteria. NPV, Negative predictive value; PPV, positive predictive value.

Method(s)	PPV (%)	NPV (%)
Time(+)	65	64
Slime(+)	72	58
CR(+)	80	55
<i>ica</i> (+)	72	64
All(+)	87	53
Time(+) + slime(+)	81	56
Time(+) + CR(+)	82	53
Time(+) + <i>ica</i> (+)	76	61

while the cost of making this differentiation is high, not being able to accomplish this, particularly when there is only one positive blood culture, may lead to even higher costs in terms of morbidity and mortality.

For diagnosis of true bacteraemia due to CNS in patients having two positive blood cultures, it is necessary to identify the species and to evaluate critically the antimicrobial susceptibility testing results. Even though PFGE is expensive and time-consuming, it is undoubtedly the most effective way to discriminate strains of CNS isolated from blood cultures (Kim *et al.*, 2000; Sloos *et al.*, 2000). Up to 20 % of patients with two blood cultures positive for CNS were due to contamination; therefore, the number of positive blood cultures is insufficient as a sole parameter to be considered when predicting CNS bacteraemia (Mirret *et al.*, 1993; Khatib *et al.*, 1995; García *et al.*, 1999). The other parameters used to test for clonality, such as colony morphology and biotype, were not useful for discriminating between bacteraemia and contamination.

It is even more difficult to predict bacteraemia in patients having only one positive blood culture, but the use of simple

methods such as incubation time until positive, slime production by the modified Christensen method and the Congo red test showed adequate positive predictive values (all above 80 %) and they may therefore be useful in clinical decision making. Although the association of time until positive with the Congo red test was slightly less sensitive than the association with slime production, since the Congo red test is less laborious, quicker and requires less equipment than for detecting slime production, it would be very useful in clinical microbiology laboratories.

Some questions remain unanswered about the pathogenicity of CNS. It is well known that the main virulence characteristic of this group of organisms relates to their ability to adhere (Ishak *et al.*, 1985; O'Gara & Humphreys, 2001). This in turn depends on the presence of genes that encode capsular polysaccharide. Nevertheless, strains were isolated that encoded adherence genes, as demonstrated by a positive Congo red test and slime test, but were classified as contaminants. It is possible that the criteria used to define true bacteraemia were not sufficiently specific or sensitive or that there are other virulence factors that play an important role in CNS pathogenicity. The study that described the presence of the *icaA* and *icaD* genes and their role in pathogenicity used strains obtained from the skin flora of healthy persons as a control group (Arciola *et al.*, 2001), and whether these differ from blood culture strains is not known. It will be necessary to continue looking for better predictors of true CNS bacteraemia and to search for other bacterial virulence factors that interact with the host in producing disease.

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