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Comparison of Phenotypic with Genotypic Procedures for Confirmation of Coagulase-Negative *Staphylococcus* Catheter-Related Bloodstream Infections

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We sought here to review the present definition of catheter-related bloodstream infections (CR-BSI) due to coagulase-negative staphylococci (CNS) by comparing the routine phenotypic methods with a genotypic procedure that considers different morphotypes. Our phenotypic characterization of CNS isolates included routine identification with biotype and antibiotype. The genotypic diagnosis was based on longer incubation periods with the consideration of all morphotypes and molecular typing by pulsed-field gel electrophoresis techniques. We prospectively selected 61 episodes of suspected CR-BSI by CNS occurring during 1 year, based on the presence of a compatible clinical setting and the isolation of one or more CNS from blood and catheter tip. Of these episodes, 47 (77%) were identified as true episodes of CR-BSI based on the presence of microorganisms of the same genotype in the blood and on the catheter tip. The sensitivity, specificity, positive predictive, negative predictive, accuracy, positive likelihood ratio, and negative likelihood ratio values obtained by different phenotypic microbiological approaches to establish the diagnosis of CR-BSI were as follows: identity at species level (78.7%, 85.7%, 94.9%, 54.5%, 80.3%, 5.51, and 0.25, respectively); identity of species and biotype (59.6%, 92.9%, 96.6%, 40.6%, 67.2%, 8.34, and 0.44, respectively); identity of species and antibiotype (61.7%, 92.9%, 96.7%, 41.9%, 68.8%, 8.64, and 0.41, respectively); and identity of species, biotype, and antibiotype (48.9%, 92.9%, 95.8%, 35.1%, 59%, 6.85, and 0.55, respectively). Our study demonstrates the inaccuracy of the diagnosis of CNS CR-BSI when the current definition based on conventional routine microbiological practice is followed. A new definition of CNS CR-BSI is necessary, at least as an epidemiological and research tool.

Catheter-related bloodstream infections (CR-BSI) are the main cause of documented sepsis (4, 5) in modern hospitals (2, 20, 27) and are mainly produced by coagulase-negative staphylococci (CNS) (6, 26, 32). The confirmed definition of a CR-BSI requires the isolation of one or more identical microorganism/s from blood and the catheter tip (19). However, present definitions are far from clear regarding the demonstration of such identity, particularly in the case of CNS. Most CNS colonies look alike after 24-h incubation, and laboratory routine involves taking a single colony for identification and antimicrobial susceptibility tests from those CNS growing in blood and on catheter tip cultures. The identity of both isolates is usually established on a phenotypic basis when the same genus, species, and antibiotype of the isolates in both samples are present. It is well known, however, that CNS growing on catheter tips may be frequently polyclonal (6, 12), especially when colonies are observed after longer periods of incubation. Moreover, biotype and antibiotype may have a weak correlation with genotype in CNS (22, 30, 34).

We compare here the yields of two procedures for the characterization of CNS CR-BSI: "phenotypic" characterization, carried out in microbiology laboratories on a daily basis, and a procedure by which the different morphotypes are obtained after prolonged incubation and the application of genotyping with molecular techniques.

MATERIALS AND METHODS

Selection of Patients with CR-BSI. During a one-year period, we prospectively selected all consecutive patients fulfilling the following criteria: (i) age of ≥18 years; (ii) clinical signs of nosocomial sepsis (patients who had been in the hospital for more than 48 h when the first episode of fever was recorded and with one or more of the following signs or symptoms: temperature of >38°C, heart rate of >90 beats per min, breathing rate of >20 breaths per min, leukocyte count of >12,000 per mm^3, edema or eythema surrounding the insertion catheter point); (iii) one or more blood cultures with CNS; and (iv) concomitant cultures of a catheter tip suspected as the origin of the infection with CNS isolated in significant counts. All catheters were central venous lines.

Microbiological cultures. Peripheral blood cultures were processed using the automatic system Bactec 9000 (Becton-Dickinson Microbiology Systems). Catheter tips were cultured using the roll-plate method (3, 18) (a significant growth was defined as ≥15 CFU).

Different procedures. Our laboratory methods were divided into a phenotypic procedure and a genotypic procedure, which were performed by different and blinded investigators, as described below.

(i) Phenotypic procedure. Blood agar plates with blood and catheter isolates were incubated for 24 to 48 h at 37°C in a 5% CO_2 atmosphere. Representative colonies from blood and catheter isolates were selected for final identification and antimicrobial susceptibility tests.

Phenotypic identification was performed by evaluating colony characteristics. Identification and susceptibility tests of strains was carried out using catalase and coagulase tests and commercially available panels (Pos Combo Panel Type 2S; Dade Behring/MicroScan, Sacramento, CA). Antibiotyping patterns were interpreted according to Clinical and Laboratory Standards Institute criteria (9)

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Differences in the antibiotic profiles (differences in two dilutions of MIC or differences in one dilution of MIC that implicated changes of interpretation (sensitive, intermediate, or resistant) in oxacillin, cotrimoxazole, ciprofloxacin, vancomycin, or teicoplanin) for isolates of the same patient were confirmed by the disk diffusion technique (9)

(ii) Genotypic procedure. As previously established, plates from the cases selected were referred to a second blinded investigator. Plates were always observed after ≥72 h of incubation. Plates were observed under an ocular lens (×10) to select different colony morphotypes. Colonies representing all the different morphotypes were isolated for genotyping.

Molecular characterization of the different morphotypes isolated in blood and catheter tip samples was performed by pulsed-field gel electrophoresis (PFGE) (16, 21, 28). Single colonies isolated from cultured plates were inoculated into Luria broth and incubated at 37°C with shaking. When the culture reached an optical density of 0.4 to 0.6 (absorbance at 600 nm) (12), incubation was stopped in a ice-water bath. Then, 1 ml of culture was centrifuged, and the pellet was resuspended in "clean-cut" agarose (contour-clamped homogeneous electric field [CHEF] genomic DNA plug kit; Bio-Rad, Richmond, CA) to constitute agarose plugs. Each plug was lysed with 375 μl of lysozyme (3.75 mg/ml), 100 μl of lysostaphine (300 μl/ml), and 525 μl of lysis buffer (50× Tris-EDTA, 1 M NaCl, 0.2% sodium deoxycholate, 0.5% Sarkosyl, 10% Brij 58, and water) for 5 h at 37°C. Next, after removing the lysis buffer, 1 ml of proteinase K buffer was added (100 μl of proteinase K [1 mg/ml] and 900 μl of buffer [100 mM EDTA, 0.2% sodium deoxycholate, 1% Sarkosyl]) before incubation at 50°C overnight. Four 1-h washes at room temperature with Tris-EDTA buffer 1× were performed, and phenylmethane sulfonyl fluoride was added to the second wash. Plugs were digested with 30 units of SmaI in 300 μl of final volume, with incubation for 18 h at 25°C. Restricted DNA fragments were separated by PFGE in a CHEF electrophoresis system (Bio-Rad) using 1% agarose gels and 0.5× Tris-borate-EDTA buffer. Gels were run for 24 h at 195 V (6 V/cm) and 14°C, with pulses ranging from 5 to 15 s. The typing profiles obtained were compared visually on the same gel and processed by using the software package bioNumerics 3.0 (AppliedMaths, Belgium) to design the similarity dendrogram using the band-base Dice correlation coefficient, a tolerance (proportion of common bands or different bands between isolates) of 1 to 1.2%, and an optimation (i.e., the distance of migration between bands) of 10%. Strains with differences in more than one macrorestriction fragment were assigned to different clones (21, 28). Using these conditions, all of the isolates clustered by the program shared identical PFGE types. Therefore, different isolates were defined as a clone when they shared identical PFGE types (21, 28).

Definitions. (i) Definition of CR-BSI according to genotypic criteria. We required the presence of one or more microorganisms of the same clone in blood and in the catheter tip. We considered as the same clone isolates with the same PFGE type (considering the coefficient and values of tolerance and optimation described above).

(ii) Definition of CR-BSI according to phenotypic criteria. A case of CR-BSI by the standard method was defined according to different categories of analysis, as follows. For the species level, identity was considered at the species level when isolates of the same CNS species were obtained in one or more blood cultures and from the catheter tip. For the biotype level, CR-BSI was considered when isolation of the same CNS species and same biotype were isolated from one or more blood cultures and from the catheter tip. For the sake of biotype identity, we allowed up to one variation in the fermentation of two sugars. For the antibiotype level, we considered CR-BSI as fulfilling these criteria when the microorganisms isolated from blood and catheter tip were from the same species and had the same antimicrobial susceptibility pattern. We accepted as identical isolates those with a maximal variation of two dilutions in MICs to macrolides, lincosamines, chloramphenicol, tetracycline, and gentamicin. For the identity of species, biotype, and antibiotype, CR-BSI was confirmed when the isolates from blood and catheter tip fulfilled the three previous criteria.

Statistical analysis. Qualitative variables are described as the percentage of frequencies and are presented with their frequency distribution. Quantitative variables are summarized as the mean and standard deviation (SD). In all cases, the distribution of the variable was checked against the theoretical models. The validity indexes of the tests for the different levels of phenotypic procedures were calculated against genotypic results considered as our reference standard and included the sensitivity, specificity, positive predictive value, negative predictive value, accuracy, and positive and negative likelihood ratios (LR+ and LR−).

The association between the sensitivity and specificity of the phenotypic tests and the genotypic test was assessed by using the χ^2 test or the Fisher exact test as appropriate. Odds ratios (OR) and their confidence intervals were calculated at 95% (95% CI) according to Cornfield's method. In all circumstances the null hypothesis was rejected with an alpha error of <0.05. The calculations were performed using SPSS for Windows (version 12.0) and Epidat (version 3.1).

TABLE 1. Comparison of phenotypic and genotypic procedures

Method[a]	No. (%) of samples[b]				Accuracy (%)
	TP	TN	FP	FN	
Genotypic	47 (77)	14 (22.9)	0 (0)	0 (0)	
Phenotypic					
SP	37 (60.6)	12 (19.6)	2 (3.2)	10 (16.3)	80.3
SP+BIO	28 (45.9)	13 (21.3)	1 (1.6)	19 (31.1)	67.2
SP+AB	29 (47.5)	13 (21.3)	1 (1.6)	18 (29.5)	68.8
SP+BIO+AB	23 (37.7)	13 (21.3)	1 (1.6)	24 (39.3)	59

[a] SP, species; BIO, biotype; AB, antibiotype.
[b] TP, true positive; TN, true negative; FP, false positive; FN, false negative.

RESULTS

Overall, 61 episodes of presumptive CR-BSI were selected for our study, according to our preestablished criteria. They had clinical manifestations compatible with CR-BSI and had one or more CNS isolates in both blood and catheter tip cultures. The numbers of positive blood cultures in these episodes were 3 in 37 episodes (61%), 2 in 13 episodes (21%), and 1 in 11 episodes (18%). Most isolates were *Staphylococcus epidermidis* (70%) and the remainder were other CNS, mainly *S. hominis-hominis* and *S. haemolyticus*.

Genotypic method. The study of the 61 episodes of suspected CR-BSI enabled us to analyze 368 morphotypes. Of these, 105 different clones were obtained after molecular analysis. According to our molecular definition of CR-BSI, we had 47 episodes with true CR-BSI (77%) and 14 episodes (23%) in which we could not find a similar clone on the catheter tip and in blood. These were not considered as CR-BSI. Of the 47 episodes of true CR-BSI, 44 (95%) were caused by a single clone, and 3 (5%) were caused by two clones.

Comparison of phenotypic procedures with the genotypic method. The different levels of phenotypic identification of CR-BSI achievable by a phenotypic procedure were compared with the genotypic method (Table 1). The accuracy of different levels of phenotypic microbiological diagnosis with the genotypic method (considered our reference standard) was as follows: species level (80.3%); species and biotype (67.2%); species and antibiotype (68.8%); and species, biotype, and antibiotype (59%). The results of the evaluation of the different phenotypic microbiological techniques with the genotypic procedures are presented in Table 2.

Since the specificities of the different tests were not significantly different ($P > 0.05$), we calculated a comparison of the different sensitivities. The phenotypic qualification of a CNS-CR-BSI based on identity at the species level of blood and catheter tip isolates was more sensitive than the combination of species and biotype identity (OR = 2.51; 95% CI = 1.02 to 6.15; $P = 0.035$); more sensitive than the combination of similar species and antibiotype identity (OR = 2.29; 95% CI = 0.93 to 5.64; $P = 0.056$); and more sensitive than the combination of identity in the species, biotype, and antibiotype (OR = 3.86; 95% CI = 1.58 to 9.41; $P = 0.002$).

According to the results of the likelihood ratios of the phe-

TABLE 2. Evaluation of the different categories of analysis of phenotypic procedures compared to the genotypic procedure[a]

Procedure[b]	Se (%)	Sp (%)	PPV (%)	NPV (%)	Likelihood ratio (95% CI)	
					+	−
Genotypic	100	100	100	100		
Phenotypic						
SP	78.7	85.7	94.9	54.5	5.51 (1.51–20.05)	0.25 (0.14–0.44)
SP+BIO	59.6	92.9	96.6	40.6	8.34 (1.24–55.95)	0.44 (0.30–0.62)
SP+AB	61.7	92.9	96.7	41.9	8.64 (1.29–57.88)	0.41 (0.28–0.60)
SP+BIO+AB	48.9	92.9	95.8	35.1	6.85 (1.01–46.32)	0.55 (0.41–0.70)

[a] Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.
[b] SP, species; BIO, biotype; AB, antibiotype.

notypic tests, the best equilibrium between positive and negative ratios is achieved by the characterization at species level in the phenotypic microbiological procedures (LR+ = 5.51; 95% CI = 1.51 to 20.05; and LR− = 0.25; 95% CI = 0.14 to 0.44).

DISCUSSION

Our study demonstrates the inaccuracy of the diagnosis of CNS CR-BSI when the current definition based on conventional phenotypic microbiological practice is followed. A new, genotypic definition is required, al least as a research tool, as CR-BSI are a common and increasing problem in hospitals all over the world (2) and are caused mainly by CNS (7), especially *S. epidermidis*.

The current definition of CR-BSI requires the isolation of the "same organism (species and antibiogram)" from the catheter tip and a peripheral blood sample (8). The first difficulty is found in the correct phenotypic identification of the CNS isolates. Schleifer and Kloos's CNS classification is tedious and cumbersome and has been largely replaced by automatic systems and simple diagrams (1, 10, 14). The correlation between species identification, even including biotypes with typing characterization by genotypic methods (e.g., PFGE), is largely imprecise (15). At the same time, the determination of the sensitivity pattern as a typing method is also inaccurate (13, 17, 22, 23, 31).

Another difficulty in phenotypic microbiological characterization of CNS CR-BSI is the differentiation of morphotypes after incubation periods shorter than 48 h. Different morphotypes are frequently observed after only 72 h of incubation and frequently reflect differences at the genotypic level (11, 12, 17, 23, 25, 29, 33). The possibility of a sampling error in phenotypic diagnosis when a single colony of the culture is being studied may be high (24).

Our genotypic approach is time-consuming and expensive and cannot be recommended to substitute for phenotypic methods. Nevertheless, the genotypic approach highlights the inaccuracy of our day-to-day practice in the characterization of CNS CR-BSI.

Our study suggests that phenotypic methods underestimate the diagnosis of CNS CR-BSI, and rule out, as non catheter related, many BSI that are really due to catheter colonization. This is misleading for physicians, who feel obliged to search for alternative sources of the BSI or interpret the positive blood as simple contaminants.

Our final recommendation would be to maintain the simplest and most sensitive definition of CNS CR-BSI for phenotypic practice, based on the identity at the species level of the CNS obtained in blood and the catheter tip. Nevertheless, a new definition based on genotypic identity of the CNS isolated from blood and catheter tip is necessary, at least for epidemiological and research purposes.

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