

First Notification of Positive Blood Cultures and the High Accuracy of the Gram Stain Report[∇]

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When blood cultures turn positive, the attending physicians are usually notified immediately about Gram stain findings. However, information on the accuracy of Gram staining is very limited. We examined the accuracy of preliminary blood culture reports provided by a regional laboratory in an observational study including the years 1996, 2000 to 2001, and 2003. We used data from computer files and technicians' laboratory notes. The study was restricted to cultures with one morphological type. Using cultural identification as a reference, we estimated the sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) for the following defined morphological groups: gram-positive cocci in clusters, gram-positive cocci in chains or diplococci, gram-positive rods, gram-negative cocci, gram-negative rods, and yeasts. We further evaluated the Gram stain and wet mount findings for the most frequent bacterial species/groups. We obtained 5,893 positive blood cultures and the following results for the defined groups: sensitivity, range of 91.3 to 99.7%; specificity, 98.9 to 100%; PPV, 94.6 to 100%; and NPV, 99.0 to 100%. The sensitivity for the most frequent species was in the range 91.3 to 100%, with nonhemolytic streptococci having the lowest value (sensitivity, 91.3%; 95% confidence interval, 86.2 to 94.9%). Wet mount reports were less accurate (sensitivity of 30 to 70% for species with peritrichous motility), and *Enterobacteriaceae* (notably *Salmonella* spp.) accounted for 25% of the reports stating polar motility. In conclusion, we demonstrated a high accuracy of Gram stain reports, whereas wet mount microscopy was generally less accurate.

Bacteremia is a serious condition with an overall in-hospital mortality above 20% (15, 19). Early administration of appropriate empirical antibiotic treatment has repeatedly been associated with improved survival in patients with bacteremia (5, 15, 27), yet up to 40% of all patients with bacteremia receive inadequate antibiotic treatment until the first notification of a positive blood culture (5, 6, 22, 24). Therefore, an important task for the microbiological laboratory is to provide expedient reports on positive blood cultures that may guide antibiotic therapy.

The first notification of a positive blood culture is typically based on the Gram stain result. At this time, 12 to 20% of the patients may not have started antibiotic treatment, and in another 30 to 45% of patients, the Gram stain result is followed by a change in the empirical treatment (2, 7, 19, 22, 24). The Gram stain report has been shown to have a much greater impact on antimicrobial treatment than provision of cultural identification and antimicrobial susceptibility test results (17, 22), and recently Hautala et al. (12) reported that combining Gram stain results with information on whether the infection was hospital or community acquired could further improve the appropriateness of the antibiotic treatment. Besides the direct implications for antibiotic treatment, the Gram stain result may also prompt further diagnostic and therapeutic interventions.

Despite the acknowledged importance of the first notification,

the accuracy of the Gram stain result has only been addressed sporadically, and the studies have mainly focused on distinction of either contaminants from true bacteremia (3, 13) or staphylococci from streptococci (1, 28). Therefore, we conducted this study to evaluate the accuracy of the preliminary blood culture reports based on Gram stain and wet mount microscopy.

MATERIALS AND METHODS

Setting. We conducted this observational study in North Jutland County, Denmark (population of approximately 500,000), using blood culture data from the years 1996, 2000, 2001, and 2003. We restricted the study to blood cultures with one morphological type: for patients with bacteremia, only the first positive blood culture was included. Patients were admitted to one of seven public hospitals, of which one (Aalborg University Hospital) served as both the district and referral hospital. The Department of Clinical Microbiology, Aalborg Hospital, provided bacteriological services, including blood cultures, for the entire county.

Blood cultures. The BacT/Alert blood culture system (bioMérieux, Marcy l'Etoile, France) was used throughout the study period. Blood cultures were obtained due to a physician's suspicion of an infection, and in adult patients three blood culture bottles were routinely inoculated at bedside using one needle. In 1996, a blood culture included two standard aerobic (SA) bottles and one standard anaerobic (SN) bottle; during the other 3 years, one SA bottle was substituted for by an aerobic FAN bottle. The nominal volume of blood per set was 28 to 32 ml for adults. For infants and preschool children, one pediatric aerobic FAN bottle was used.

Positive bottles were unloaded at 8:00 a.m., 11:00 a.m., 14:00 p.m., and 8:30 p.m. and immediately examined by a technician. Technicians with less than 2 years of experience were supervised by more experienced colleagues. The compound microscopes were equipped with $\times 100$ achromatic oil objectives also suited for phase-contrast microscopy, and Koehler illumination was checked daily. Wet mount preparations were immediately examined by phase-contrast microscopy, and smears for Gram staining were fixed by flame fixation and stained using acetone for decolorization and safranin as counterstain. The motility (wet mount), Gram stain reaction, morphology, and bacterial arrangement

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TABLE 1. Distribution by calendar year of the 5,893 positive blood cultures with one morphological type on Gram stain grouped according to Gram stain characteristics, arrangement, and morphology

Yr	No. (%) ^a :						Total
	Cocci			Rods		Yeasts	
	Gram positive, clusters	Gram positive, chains/diplococci	Gram negative	Gram positive	Gram negative		
1996	428 (35.0)	241 (17.5)	8 (0.7)	104 (8.5)	467 (38.2)	3 (0.3)	1,224 (100)
2000	595 (37.5)	198 (12.5)	8 (0.5)	246 (15.5)	511 (32.2)	28 (1.8)	1,586 (100)
2001	603 (38.6)	210 (13.4)	11 (0.7)	156 (10.0)	563 (36.0)	20 (1.3)	1,563 (100)
2003	481 (31.6)	211 (13.9)	11 (0.7)	114 (7.5)	662 (43.6)	41 (2.7)	1,520 (100)
Total	2,107 (35.8)	833 (14.1)	38 (0.6)	620 (10.5)	2,203 (37.4)	92 (1.6)	5,893 (100)

^a For patients with bacteremia, only the first positive blood culture was included.

were recorded on a laboratory note. The positive blood cultures were subcultured onto plate media selected in accordance with the Gram stain result, and isolates were routinely identified by a combination of conventional and commercial methods (18).

The laboratory's proficiency was assured by participation in the UK External Quality Assessment Scheme as well as national quality control programs. All isolates of streptococci, pneumococci, meningococci, and yeasts were referred to Statens Serum Institut (Copenhagen, Denmark) as part of a national surveillance program. All microbiological information was recorded in a laboratory information system (ADBakt, Autonik, Ramstra, Sködinge, Sweden).

Data on positive blood cultures. We defined bacteremia as bacterial or fungal growth in blood culture, where a combined clinical and microbiological assessment effectively ruled out contamination (30). Coagulase-negative staphylococci, *Corynebacterium* spp., *Bacillus* spp., and *Propionibacterium acnes* were regarded as contaminants unless they were isolated from two or more separate blood cultures or special risk factors were known to be present. All episodes of bacteremia (and fungemia) in the county since 1981 have been registered in the North Jutland County Bacteremia Registry (23, 24), which we used to identify the first positive blood culture for all episodes of bacteremia occurring during the 4 years studied.

Information on contaminated blood cultures was retrieved from the laboratory information system, and for all cultures, we abstracted information on Gram stain result, bacterial motility, and species diagnosis from the technician's notes; these data were tabulated independently of the main investigator. We defined six main groups according to Gram stain characteristics and morphology, namely: gram-positive cocci in clusters, gram-positive cocci in chains or diplococci, gram-positive rods, gram-negative cocci, gram-negative rods, and yeasts. Bacteria were classified by motility as peritrichous, polar, or nonmotile.

Data analysis. We evaluated the accuracy of Gram staining and wet mount microscopy using the results obtained by cultural identification as a reference standard. For each of the six defined groups, we estimated the performance characteristic of Gram staining (sensitivity, specificity, and positive and negative predictive values [PPV and NPV, respectively]) (9). Using gram-negative rods as an example, sensitivity refers to the proportion of gram-negative rods identified by culture that

were determined as such by Gram stain. The specificity describes the ability of the initial Gram stain to rule out a certain combination of Gram staining and morphology. For gram-negative rods, the specificity refers to the proportion of blood cultures with isolates other than gram-negative rods that were classified accordingly in the initial Gram stain examination (i.e., the numerator was the number of blood cultures not identified as being gram-negative rods in the initial Gram staining and the denominator was the number of all blood cultures that were not classified as gram-negative rods by cultural identification). The PPV is the probability that gram-negative rods seen on Gram stain were identified as such by culture. The NPV is the probability that a morphotype different from that of gram-negative rods is not identified as gram-negative rods by culture.

To quantify the maximum impact of a potential selection bias caused by missing data, we repeated the analyses assuming that all missing data had been incorrect. We further evaluated the Gram stain and wet mount results for predominant pathogens at the species level. Bacterial motility was assessed for the most frequent motile gram-negative species.

Estimates are presented with 95% confidence intervals (CI). Statistical analyses were performed using Stata Statistical Software v.9.0 (Stata Corp., College Station, TX).

RESULTS

Among 6,461 positive blood cultures obtained during the study period, 438 (7%) were polymicrobial with more than one morphological type and 130 (2%) records lacked information on either bacterial morphology or Gram stain reaction. Thus, our study sample included 5,893 blood cultures, of which 1,985 (34%) were contaminants. The distribution of recovered isolates grouped according to Gram stain characteristics is shown in Table 1. Sensitivity, specificity, PPV, and NPV of the Gram stain are given in Table 2. These estimates remained stable

TABLE 2. Performance characteristics of the Gram stain with culture-based identification as reference

Pathogen ^a	No. of correct Gram stain evaluations/total	% Sensitivity (95% CI)	% Specificity (95% CI)	% PPV (95% CI)	% NPV (95% CI)
Cocci					
Gram-positive, clusters	2,101/2,129	99.7 (99.4–99.9)	99.3 (98.9–99.5)	98.7 (98.1–99.2)	99.8 (99.7–99.9)
Gram-positive, chains/diplococci	707/818	96.8 (95.4–97.8)	99.8 (99.6–99.9)	98.7 (97.6–99.3)	99.5 (99.3–99.7)
Gram negative	35/37	92.1 (78.6–98.3)	100 (99.9–100)	94.6 (81.8–99.3)	100 (99.9–100)
Rods					
Gram positive	566/584	91.3 (88.8–93.4)	99.7 (99.5–99.8)	96.9 (95.2–98.2)	99.0 (98.7–99.2)
Gram negative	2,175/2,217	98.7 (98.2–99.2)	98.9 (98.5–99.2)	98.1 (97.5–98.6)	99.2 (98.9–99.5)
Yeasts					
	90/92	97.8 (92.4–99.7)	100 (99.9–100)	100 (96.0–100)	100 (99.9–100)

^a Pathogens are grouped according to their Gram stain characteristics, morphology, and arrangement.

TABLE 3. Evaluation of Gram stain results for predominant bacterial pathogens or groups

Pathogen	No. of correct Gram stain evaluations/total	% Sensitivity (95% CI)
<i>S. aureus</i>	592/592	100 (99.4–100)
Streptococci		
Hemolytic	151/152	99.3 (96.4–100)
Nonhemolytic	167/183	91.3 (86.2–94.9)
Enterococci	103/106	97.2 (96.7–99.4)
Pneumococci	386/392	98.5 (96.7–99.4)
Meningococci	33/34	97.1 (84.7–99.9)
Enterobacteria	1841/1859	99.0 (98.5–99.4)
<i>E. coli</i>	1275/1282	99.5 (98.9–99.8)
<i>Citrobacter</i> spp.	23/23	100 (85.2–100)
<i>Enterobacter</i> spp.	110/111	99.1 (95.1–100)
<i>Klebsiella</i> spp.	296/305	97.1 (94.5–98.6)
<i>Morganella morganii</i>	19/20	95.0 (75.1–99.9)
<i>Proteus</i> spp.	72/72	100 (95.0–100)
<i>Serratia marcescens</i>	24/24	100 (85.8–100)
<i>Salmonella</i> serovar	42/42	100 (91.6–100)
Other enterobacteria ^a	7/7	100 (59–100)
<i>Pseudomonas aeruginosa</i>	117/118	99.2 (95.4–100)

^a Includes two *Hafnia alvei* isolates, one *Pantoea agglomerans* isolate, two *Yersinia enterocolitica* isolates, and one unidentified enterobacterium.

across the 4 years studied (data not shown). Assuming that all of the 130 excluded records were false negative and distributed as in Table 1, this led to the following sensitivities: gram-positive cocci in clusters, 95.5% (95% CI, 96.8 to 98.2%); gram-positive cocci in chains/diplococci, 94.8% (95% CI, 93.1 to 96.2%); gram-negative cocci, 89.7% (95% CI, 75.8 to 97.1%); gram-positive rods, 89.3% (95% CI, 86.6 to 91.6%); gram-negative rods, 96.6% (95% CI, 95.7 to 97.3%); and yeasts, 95.7% (95% CI, 89.5 to 98.8%).

The comparatively low sensitivity for gram-positive rods (91.3%; 95% CI, 88.8 to 93.4%) was mainly caused by *Bacillus* spp. and *Clostridium* spp. Nearly half of the *Bacillus* isolates (45.3%) were recorded as gram-negative rods corresponding to a sensitivity of 48.4% (95% CI, 35.8 to 61.3%). Likewise, 7

of 35 *Clostridium* spp. were reported as gram-negative rods ($n = 6$) or as a mixture of gram-negative and gram-positive rods ($n = 1$). The sensitivity of the preliminary diagnosis for *Clostridium* spp. was 80% (95% CI, 63.1 to 91.6%). Leaving out *Bacillus* spp. and *Clostridium* spp. from the analysis, the remaining gram-positive rods had a sensitivity of 97.3% (95% CI, 95.5 to 98.5%), specificity of 99.7% (95% CI, 99.5 to 99.8%), PPV of 96.6% (95% CI, 94.6 to 98.0%), and NPV of 99.7 (95% CI, 99.6 to 99.9%).

Table 3 shows sensitivity at the species level for the predominant bacterial pathogens. The sensitivity was close to 100% for all listed pathogens, with nonhemolytic streptococci being the only distinctive exception. Sixteen Gram-stained smears with nonhemolytic streptococci were misread and initially reported as gram-negative rods ($n = 2$), gram-positive cocci in clusters ($n = 9$), or gram-positive rods ($n = 5$). Furthermore, all *Acinetobacter* spp. included in the study were reported as gram-negative rods on Gram stain, corresponding to a sensitivity of 100% (95% CI, 85.8 to 100.0%).

Overall, the sensitivity of the wet mount report varied from 30% to 70% for bacterial species with peritrichous motility (Table 4). A total of 100 bacteria were recorded as displaying a polar pattern of motility; one-quarter of these were enterobacteria, of which a *Salmonella* serovar accounted for the major part.

DISCUSSION

In this study of more than 5,800 positive blood cultures, we found that the Gram stain reports were highly accurate and remained so over the years studied. The performance characteristics for the main morphological groups were close to 100% and only slightly lower for gram-positive rods, in accordance with the propensity of both *Bacillus* spp. and *Clostridium* spp. to appear gram negative (4).

The use of wet mounts in association with Gram staining for positive blood cultures to determine the morphology of organisms, gross structure, and motility has a long tradition in Danish clinical microbiology. We cannot determine to what extent the information gained from the wet mounts (beside the information on motility) may have contributed to the technicians' accurate assessment of the Gram stain. It is our impression

TABLE 4. Motility patterns of predominant motile gram-negative bacteria assessed by wet mount microscopy

Species (n)	No. (%) of isolates with motility type:			
	Nonmotile	Peritrichous	Polar	Not stated
Enterobacteria				
<i>E. coli</i> (1,263)	651 (50.8)	602 (47.0)	6 (0.5)	23 (1.7)
<i>Citrobacter</i> spp. (22)	11 (47.8)	8 (39.1)	0	3 (13.1)
<i>Enterobacter</i> spp. (111)	25 (22.5)	69 (62.2)	3 (2.7)	14 (12.6)
<i>Morganella morganii</i> (20)	3 (15.0)	14 (70.0)	0	3 (15.0)
<i>Proteus</i> spp. (72)	19 (26.4)	37 (51.4)	4 (5.6)	12 (16.6)
<i>Serratia marcescens</i> (24)	10 (41.7)	10 (41.7)	0	4 (16.6)
<i>Salmonella</i> serovar (42)	5 (11.9)	23 (54.8)	11 (26.2)	3 (7.3)
Other enterobacteria (7) ^a	4 (57.1)	2 (28.6)	0	1 (14.3)
<i>Pseudomonas aeruginosa</i> (118)	36 (30.5)	10 (8.5)	62 (52.5)	10 (8.6)

^a Includes two *Hafnia alvei* isolates, one *Pantoea agglomerans* isolate, two *Yersinia enterocolitica* isolates, and two unidentified enterobacteria.

that the use of wet mounts aids in the interpretation of Gram stains (mostly with respect to the arrangement of gram-positive cocci and weakly stained gram-negative organisms, which may appear more distinct in wet mounts). In this study, the information on bacterial motility gained from the wet mounts was less accurate and in some instances misleading. Polar motility of gram-negative rods is given particular attention because it may indicate *Pseudomonas aeruginosa* and other aerobic bacteria, which require extended antibiotic coverage. However, considering the variation in motility displayed by *P. aeruginosa*, we find that the absence of polar motility in wet mount microscopy should not be used to rule out *P. aeruginosa*.

The strengths of our study are its large size, coverage of the service for an entire county, and collection of data on blood cultures, bacteremia, and microbiological findings independently of the study, making investigators' bias unlikely. Thus, the study in itself did not influence the diagnostic process, and bias due to differential diagnostic effort was prevented. By excluding repetitious positive blood cultures in patients with bacteremia and Gram stain reports with more than one morphological type, our study focused on those Gram stain reports most likely to influence the clinical decision making. We included all contaminated blood cultures since this is basically a post hoc classification based on multiple criteria including the diagnosis obtained by culture and a clinical assessment (30). Still, other factors could affect the validity of our findings. First, when evaluating the culture-based identification, the technicians were not blinded to the results of Gram stain and wet mount microscopy. This may have led us to overestimate the performance characteristics of Gram stain and wet mount microscopy (16). Second, 2% of the blood culture records were excluded because we lacked information on either the Gram stain result or morphology. This may have been due to problems with the interpretation of the smears and could also cause an overestimation of the accuracy of the Gram stain results. However, even if all of the excluded blood cultures were classified incorrectly by Gram stain (worst case scenario), the sensitivity would still be around or above 90%. Third, because of the retrospective nature of the study, there was no way to systematically determine whether the few observed discrepancies between Gram stain and culture-based identification were mainly due to interpretative or technical errors. Gram stains from the FAN medium may be more difficult to interpret because of the presence of charcoal particles (29), but the accuracy was not negatively affected by the introduction of the FAN medium in 1999. Decolorizing is the most critical part of the Gram staining procedure, and we believe that the use of 100% acetone instead of a 50:50 mixture of acetone and 95% ethyl alcohol, as recommended in the *Manual of Clinical Microbiology* (18), may explain part of the observed decolorization of, especially, *Bacillus* spp. and *Clostridium* spp.

The performance of the Gram stain is dependent on the interpreter, and even though this study was conducted in a routine setting and reflects everyday practice, an important premise is that most technicians undertaking the direct microscopy are highly skilled. Our results may therefore not apply to other settings.

Reports on the accuracy of the Gram stain for blood cultures are very sparse. A study by Cunney et al. (7) reported a discrepancy between Gram stain results and cultural identifica-

tion in 7 of 132 isolates (5%). These results corroborate our results as we observed nonconcordance between the initial Gram stain and the subsequent culture in 119 of the 5,893 blood cultures (2%). This proportion was somewhat lower (57 of 8,253 positive blood cultures) in the study by Rand and Tillan (21), but their study focused only on those errors that had the greatest potential for patient harm. Our findings also agree with the limited data available on the accuracy of differentiating staphylococci and streptococci morphologically on the Gram-stained smear (1, 28). However, only the study by Cunney et al. (7) specified whether contaminants were included in the evaluation. *Acinetobacter* spp. have been reported to stain gram positive despite proper Gram stain technique (11), and in the study by Rand and Tillan (21), an *Acinetobacter* sp. was isolated in 5 of 13 cultures where the Gram stain initially was read as gram-positive cocci or rods. Our data set included 24 *Acinetobacter* spp. which were all reported as gram-negative rods.

Several studies have demonstrated that reporting of blood culture results considerably increases the proportion of bacteremic patients who receive appropriate antibiotic treatment (5, 15, 27). Bouza et al. (5) found that the odds of death increased 1.2-fold for each day until definitive identification was available (odds ratio = 1.2; 95% CI, 1.05 to 1.4%). This and the improvement in antibiotic treatment on the basis of microbiological data underlie the potential benefit of applying rapid microbiological detection and testing methods as previously shown (8, 26). A range of other promising direct tests for rapid identification (including direct inoculation in automated systems, hybridization, and PCR) has been described in recent years (10, 14, 20, 25). Still, our study emphasized that Gram staining performed and interpreted by experienced technicians is inexpensive, fast, and highly accurate.

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