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Title
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Permalink
https://escholarship.org/uc/item/5tt8d1rh

Journal
Journal of Clinical Microbiology, 53(8)

ISSN
0095-1137

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Publication Date
2015-08-01

DOI
10.1128/jcm.00581-15

Peer reviewed

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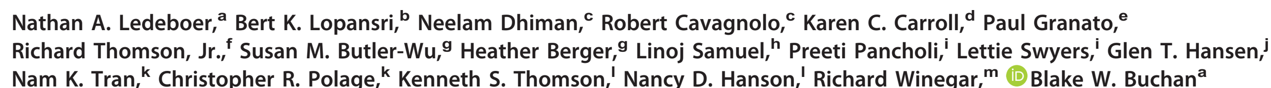

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Bloodstream infection is a serious condition associated with significant morbidity and mortality. The outcome of these infections can be positively affected by the early implementation of effective antibiotic therapy based on the identification of the infecting organism and genetic markers associated with antibiotic resistance. In this study, we evaluated the microarray-based Verigene Gram-negative blood culture (BC-GN) assay in the identification of 8 genus or species targets and 6 genetic resistance determinants in positive blood culture broths. A total of 1,847 blood cultures containing Gram-negative organisms were tested using the BC-GN assay. This comprised 729 prospective fresh, 781 prospective or retrospective frozen, and 337 simulated cultures representing 7 types of aerobic culture media. The results were compared to those with standard bacterial culture and biochemical identification with nucleic acid sequence confirmation of the resistance determinants. Among monomicrobial cultures, the positive percent agreement (PPA) of the BC-GN assay with the reference method was as follows; *Escherichia coli*, 100%; *Klebsiella pneumoniae*, 92.9%; *Klebsiella oxytoca*, 95.5%; *Enterobacter* spp., 99.3%; *Pseudomonas aeruginosa*, 98.9%; *Proteus* spp., 100%; *Acinetobacter* spp., 98.4%; and *Citrobacter* spp., 100%. All organism identification targets demonstrated >99.5% negative percent agreement (NPA) with the reference method. Of note, 25/26 cultures containing *K. pneumoniae* that were reported as not detected by the BC-GN assay were subsequently identified as *Klebsiella variicola*. The PPA for identification of resistance determinants was as follows; bla_{CTX-M}, 98.9%; bla_{KPC}, 100%; bla_{NDM}, 96.2%; bla_{OXA}, 94.3%; bla_{VIM}, 100%; and bla_{IMP}, 100%. All resistance determinant targets demonstrated >99.9% NPA. Among polymicrobial specimens, the BC-GN assay correctly identified at least one organism in 95.4% of the broths and correctly identified all organisms present in 54.5% of the broths. The sample-to-result processing and automated reading of the detection microarray results enables results within 2 h of culture positivity.

Bloodstream infection (BSI) is a serious and life-threatening condition that has been associated with 25% to 80% mortality (1, 2). The outcome of BSI can be dependent on host factors, such as underlying comorbidities, and microbiological factors, including the type of infecting organism and its susceptibility to antibiotics. It is estimated that up to 30% of hospital-acquired BSI are attributable to Gram-negative organisms (3). Infections caused by these bacteria, particularly when acquired in the hospital, have been associated with 15% to 29% increased crude mortality rates compared with those of the case controls (4, 5). This is particularly true for infections with multidrug-resistant organisms, including those harboring extended-spectrum β-lactamases (ESBLs) or carbapenemases, which have been associated with prolonged hospital stay and increased 30-day mortality (6, 7).

Perhaps the most important intervention in the management of BSI is the early implementation of effective antibiotic therapy (8, 9). In one study, failure to administer effective antibiotics within 24 h of sepsis onset resulted in a two-day-longer hospital stay, which was statistically significant, and was associated with a higher mortality rate (10). In a similar study, ineffective antimicrobial therapy was associated with a statistically significant 33% increase in mortality (8). Of note, both studies reported that up to one-third of patients initially received antibiotics that were ineffective against the infecting

Received 3 March 2015 **Returned for modification** 2 April 2015
Accepted 14 May 2015
Accepted manuscript posted online 20 May 2015
Citation Ledeboer NA, Lopansri BK, Dhiman N, Cavagnolo R, Carroll KC, Granato P, Thomson R, Jr, Butler-Wu SM, Berger H, Samuel L, Pancholi P, Swyers L, Hansen GT, Tran NK, Polage CR, Thomson KS, Hanson ND, Winegar R, Buchan BW. 2015. Identification of Gram-negative bacteria and genetic resistance determinants from positive blood culture broths by use of the Verigene Gram-negative blood culture multiplex microarray-based molecular assay. J Clin Microbiol 53:2460–2472. doi:10.1128/JCM.00581-15.
Editor: P. Bourbeau
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doi:10.1128/JCM.00581-15

organism. These data underscore the importance of early organism identification and, when possible, the detection of genetic markers associated with antibiotic resistance to aid in directing specific therapy for BSI. Further, early narrowing of broad-spectrum antibiotic treatment based on definitive organism identification is a goal of many hospital stewardship programs and may further reduce the incidence of iatrogenic conditions, including *Clostridium difficile*-associated diarrhea and colonization with multidrug-resistant organisms (11–13).

Several approaches to the rapid detection of bacteria and genetic markers of resistance have been applied to positive blood culture broths. These include fluorescence *in situ* hybridization (FISH), matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and nucleic acid amplification or detection assays (14–23). While each approach has specific strengths and weaknesses, all have demonstrated their potential to positively impact key indicators, such as laboratory turnaround time, length of hospital stay, cost of care, and overall mortality associated with BSI. Of the available methods, only three tests are currently FDA cleared and capable of multiplex identification and differentiation of >4 individual targets in positive blood culture broths. These include the FilmArray blood culture identification (BCID) assay (BioFire Diagnostics, Salt Lake City, UT) and the Verigene Gram-positive blood culture (BC-GP) and Gram-negative blood culture (BC-GN) assays (Nanosphere, Northbrook, IL).

The FilmArray BCID is a sample-to-result assay for the analysis of positive blood culture broths. BCID relies on two-stage nested-PCR for identification of 8 Gram-positive, 11 Gram-negative, and 5 yeast targets in addition to the *mecA*, *vanA/B*, and bla_{KPC} resistance determinants on a single panel. Clinical evaluations of BCID have demonstrated an overall sensitivity of >97% for targets present on the panel and a specificity of 97% to 100% for individual targets (22, 23). In contrast, the Verigene BC-GP and BC-GN assays are nonamplified tests that rely on nucleic acid extraction from positive blood cultures, followed by microarray-based detection using capture and detection probes. The BC-GP assay is specific for 12 Gram-positive bacterial identification targets and 3 associated resistance markers (*mecA*, *vanA*, and *vanB*), while the BC-GN assay is specific for 8 Gram-negative bacterial identification targets and 6 key resistance markers (bla_{CTX-M}, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA}). Selection of the BC-GP or BC-GN assay is based on the primary Gram stain result of a positive blood culture broth. Several studies have reported on the performance of the BC-GP assay, which has a sensitivity ranging from 96% to 100% and >98% specificity for the majority of targets (18, 21, 24, 25). The BC-GN assay has also been evaluated; however, studies to date have included <150 blood cultures or have included primarily simulated specimens (20, 26–30).

Here, we evaluate the Verigene BC-GN assay for the identification of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Pseudomonas aeruginosa*, and *Acinetobacter* spp. in positive blood culture broths containing Gram-negative bacilli. We also assess the ability of the BC-GN assay to detect 6 genetic markers associated with resistance to cephalosporins (bla_{CTX-M}) and carbapenems (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA}) in these organisms. This study was conducted at 13 clinical centers within the United States, from which a total of 1,847 blood cultures were evaluated. This includes both prospectively collected cultures and simulated cultures seeded with less common clinical isolates or resistance genes to fully assess the performance of the BC-GN assay.

MATERIALS AND METHODS

Collection of blood cultures. Blood cultures were prospectively collected at 12 of the 13 clinical centers throughout the United States. Aerobic cultures using Bactec Plus Aerobic/F (852 [46.1%]) and Bactec Standard/10 Aerobic/F (292 [15.8%]) (Becton Dickinson, Franklin Lakes, NJ), BacT/Alert FA FAN (357 [19.3%]), BacT/Alert SA (20 [1.1%]) and BacT/Alert SN (17 [0.9%]) (bioMérieux, Marcy l'Etoile, France), and the VersaTREK Redox1 40 ml (122 [6.6%]) and VersaTREK Redox1 80 ml (187 [10.1%]) (Thermo Fisher Scientific, Waltham, MA) broth culture bottles met the inclusion criteria and were included in the study. To qualify for study enrollment, prospectively collected cultures had to (i) be removed from the blood culture cabinet within 8 h of signaling positive, (ii) contain Gram-negative bacilli upon Gram stain, and (iii) be tested using the BC-GN assay within 12 h of initial positive detection by the automated blood culture instruments. Only one specimen per patient per septic episode was enrolled to reduce redundancy in testing. Upon enrollment, two aliquots (2 ml each) of the blood culture broth were made in sterile plastic tubes. One tube was used for BC-GN testing (see below), and the other was immediately frozen at or below −70°C for use in repeat testing or discrepant analysis, as needed. The positive blood culture broth was also used to inoculate a Trypticase soy agar plate with 5% sheep blood (BAP) and a MacConkey agar plate (MAC). The BAP and MAC plates were incubated at 35°C in an atmosphere of 5% CO_2 for up to 48 h. The resulting bacterial growth was used to make two heavy suspensions in Trypticase soy broth with 20% glycerol (glycerol stocks). If multiple colony morphologies were observed, the isolated colonies were streaked for purity prior to making the glycerol stocks. All glycerol stocks were stored at or below −70°C until they were shipped to a central reference laboratory for identification (see below). Retrospectively collected and simulated specimens were frozen within 8 h of signaling positive. Frozen specimens were allowed to thaw completely at room temperature and were vortexed and run within 2 h of thaw. All specimens (blood cultures) were deidentified prior to enrollment and tested in accordance with site-specific institutional review board (IRB)-approved protocols.

Design of Verigene BC-GN assay capture probes. For each of the nucleic acid sequences detected by the BC-GN assay, at least two sets of oligonucleotides are required: (i) capture oligonucleotides (captures) and (ii) mediator oligonucleotides (mediators). Captures are printed onto the glass slide (microarray) and are designed to specifically bind to one region of the target sequence. Mediators bind specifically to a second region of the same sequence and are conjugated to nanoparticles utilized for target detection. The accession numbers of the reference sequences for each identification or resistance marker target were downloaded from the Lahey Clinic (http://www.lahey.org/Studies/other.asp) (Table 1). Full alignments (not the consensus sequence) were analyzed to generate oligonucleotides from conserved regions. For the same start and end positions on the alignments, all possible variant oligonucleotides were generated. Several sets of oligonucleotides for each resistance target were tested empirically, and the best (thermodynamic stability, assay compatibility, and target specificity) was chosen to be used in the BC-GN test. For diverse markers, such as bla_{CTX-M} and bla_{IMP}, multiple capture and mediator oligonucleotides were used to ensure inclusivity.

Verigene BC-GN testing. Specimens were tested on the Verigene system using the BC-GN assay within 12 h of culture positivity by the automated blood culture monitoring system utilized by the study site. A BC-GN test cartridge, utility tray, and extraction tray were loaded into the Verigene Processor *SP*. A 700-μl portion of the positive blood culture was transferred to the specimen well located in the BC-GN assay extraction tray, and the Verigene Processor *SP* was started. Following automated nucleic acid extraction and hybridization to the glass array, the Verigene Processor *SP* was opened, and the array was transferred to the Verigene Reader for analysis and automated reporting of qualitative results. The

TABLE 1 Identification of Gram-negative organisms in monomicrobial cultures using BC-GN assay

Target	Specimen type	No. with result[a]:				Total no.	% (95% CI) for[b]:	
		TP	FP	TN	FN		PPA	NPA
E. coli[c]	Fresh	322[d]	4	377	0	703	100.0 (98–100)	99.0 (97–100)
	Frozen	304	2	456	0	762	100.0 (98–100)	99.6 (98–100)
	Simulated	49	0	285	0	334	100.0 (91–100)	100.0 (98–100)
	Total	675	6	1,118	0	1,799	100.0 (99–100)	99.5 (99–100)
K. pneumoniae	Fresh	103	0	588	12	703	89.6 (82–94)	100.0 (99–100)
	Frozen	103	1	645	13	762	88.8 (81–94)	99.8 (99–100)
	Simulated	133	0	200	1	334	99.3 (95–100)	100.0 (98–100)
	Total	339	1	1,433	26	1,799	92.9 (90–95)	99.9 (99–100)
K oxytoca	Fresh	21	6	675	1	703	95.5 (75–100)	99.1 (98–100)
	Frozen	38	1	722	1	762	97.4 (85–100)	99.9 (99–100)
	Simulated	4	0	329	1	334	80.0 (30–99)	100.0 (99–100)
	Total	63	7	1,726	3	1,799	95.5 (86–99)	99.6 (99–100)
Enterobacter spp.	Fresh	49	0	654	0	703	100.0 (91–100)	100.0 (99–100)
	Frozen	61	6	694	1	762	98.4 (90–100)	99.1 (98–100)
	Simulated	34	0	300	0	334	100.0 (87–100)	100.0 (98–100)
	Total	144	6	1,918	1	1,799	99.3 (96–100)	99.7 (99–100)
P. aeruginosa	Fresh	84	0	619	0	703	100.0 (95–100)	100.0 (99–100)
	Frozen	51	1	708	2	762	96.2 (86–99)	99.9 (99–100)
	Simulated	41	0	293	0	334	100.0 (89–100)	100.0 (98–100)
	Total	176	1	1,620	2	1,799	98.9 (96–100)	99.9 (99–100)
Proteus spp.	Fresh	25	0	678	0	703	100.0 (83–100)	100.0 (99–100)
	Frozen	47	2	713	0	762	100.0 (91–100)	99.7 (99–100)
	Simulated	3	0	331	0	334	100.0 (31–100)	100.0 (97–100)
	Total	75	2	1,722	0	1,799	100.0 (94–100)	99.9 (99–100)
Acinetobacter spp.	Fresh	13	0	690	0	703	100.0 (72–100)	100.0 (99–100)
	Frozen	19	2	740	1	762	95.0 (73–100)	99.7 (99–100)
	Simulated	28	0	306	0	334	100.0 (85–100)	100.0 (98–100)
	Total	60	2	1,736	1	1,799	98.4 (90–100)	99.9 (99–100)
Citrobacter spp.	Fresh	8	1	694	0	703	100.0 (60–100)	99.9 (99–100)
	Frozen	18	0	744	0	762	100.0 (78–100)	100.0 (99–100)
	Simulated	32	0	312	0	344	100.0 (87–100)	100.0 (98–100)
	Total	58	1	1,750	0	1,799	100.0 (92–100)	99.9 (99–100)

[a] TP, true positive, FP, false positive, TN, true negative, FN, false negative.
[b] PPA, positive percent agreement; NPA, negative percent agreement; 95% CI, 95% confidence interval.
[c] Includes *Shigella* spp., which cannot be differentiated by the BC-GN assay.
[d] One of 322 was identified as *Shigella* sp. by culture.

BC-GN assay contains an internal control for nucleic acid extraction and array hybridization. If a specimen test (i) yielded a result of (i) no call or (ii) preanalysis error, or (iii) no residual DNA was present after testing, the specimen was retested a single time within 24 h of the initial blood culture positivity. If a specimen could not be retested within 24 h, the specimen aliquot was frozen until the repeat test could be performed. At the conclusion of each BC-GN test, the residual extracted nucleic acid was removed from the extraction tray to a microcentrifuge tube and stored frozen at or below −70°C for molecular characterization of the resistance determinants (see below). Removal of the residual nucleic acid was necessary only for this study protocol and is not performed by the end user in routine use.

Bacterial reference method testing. Identification of all bacterial isolates was carried out at a central reference laboratory (University of Maryland, Baltimore, MD) using a standardized protocol. Upon receipt of the frozen strains, each isolate was plated onto Trypticase soy agar with 10% sheep blood and incubated at 35°C in an atmosphere of 5% CO_2 for up to 72 h until sufficient bacterial growth was achieved for biochemical testing. Each isolate was initially identified using Gram stain and an oxidase test, followed by Vitek 2 (bioMérieux) and/or Phoenix (BD) automated identification systems to achieve a species-level identification. The final reference identification was based on the results of the Vitek 2 and/or Phoenix systems. The reference laboratory made a secondary set of glycerol stocks from the inoculated BAP during the reference testing process and stored them at or below −70°C for any additional testing or discrepant analysis. Resolution of discrepant results between the BC-GN assay and reference method identification was conducted using bidirectional sequence analysis. Briefly, a PCR using primers external to those used in the BC-GN assay was performed, and the products were run on an agarose gel. Any PCR that yielded an amplification product (band) corresponding to the expected size of the PCR target on the gel were submitted to ACGT, Inc. (Wheeling, IL) for sequencing. Differentiation of *Klebsiella variicola* was based on PCR and sequence analysis of the *yggE* gene.

Construction of simulated specimens. Isolates containing rare targets, such as those with specific resistance gene markers, were obtained as archived glycerol stocks from two clinical laboratories (International Health Management Associates, Schaumburg, IL, or JMI Laboratories, North Liberty, IA) or as lyophilized strains from the American Type Culture Collection (ATCC). All strains were received at Nanosphere and revived onto BAP for the construction of simulated cultures. A suspension of each pure isolate was made in 0.65% saline and was diluted to approximately 10 to 100 CFU/ml. A 0.1-ml aliquot of the bacterial suspension (approximately 1 to 10 CFU) was used to inoculate Bactec Plus/F Aerobic medium bottles along with 8.0 ml of anticoagulated whole blood (Tennessee Blood Services, Memphis, TN) to simulate a clinically relevant specimen. The inoculated broths were incubated in the Bactec system until the bottles reached positivity. Each positive culture was assigned a unique identification number, divided into four 1- to 2-ml aliquots, and frozen within 8 h of signaling positive at −70°C. Specimens were shipped to the study testing sites on dry ice and were stored at −70°C until BC-GN testing was performed.

PCR amplification and bidirectional sequencing confirmation of resistance markers. Residual nucleic acid extracted from the blood culture specimens during BC-GN assay processing steps were removed from the designated well in the extraction tray consumable by manual pipette upon conclusion of the assay and were stored frozen at below −70°C. These extracts were submitted in a blinded fashion to the study internal testing laboratory (Nanosphere) for PCR amplification of 13 resistance marker targets (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}2}$ group, $bla_{CTX\text{-}M\text{-}8}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, $bla_{CTX\text{-}M\text{-}25}$ group, $bla_{OXA\text{-}23}$ group, $bla_{OXA\text{-}40}$ group, $bla_{OXA\text{-}48}$ group, and $bla_{OXA\text{-}58}$ group). Primers used for confirmation of the resistance markers were designed to target genetic sequences disparate from those used for detection of the markers by the BC-GN assay. Following endpoint PCR amplification, each reaction mixture was electrophoresed on an agarose gel. Amplification reactions that resulted in a visible band of expected size and sufficient quantity were sent to a third-party sequencing laboratory (ACGT, Inc., Wheeling, IL) for bidirectional sequencing confirmation of specific resistance determinants. Sequencing templates were prepared by PCR amplification of the original nucleic acid extracts obtained from BC-GN assay sample processing, which had been stored at temperatures below −70°C. Primers used for presequencing target amplification were the same as those used for initial identification of resistance markers at the internal testing laboratory, with the exception of bla_{KPC}, bla_{VIM}, and $bla_{CTX\text{-}M\text{-}9}$. Amplification of these three markers was accomplished using primers with an additional engineered universal M13 sequence, which was also used for subsequent sequencing reactions. The primers for each marker were designed to target conserved regions of the gene based on multiple sequence alignments of all available sequence entries for each target in GenBank at the time of design. The length of the amplicons was optimized to ensure that >200 bases with a Phred quality score of ≥30 would be generated for each sample during sequencing reactions.

All sequencing reactions were performed in a Bio-Rad Tetrad PCR system thermal cycler, ABI GeneAmp PCR system 9700 thermal cycler, or Bio-Rad C1000 PCR thermal system (Bio-Rad Laboratories, Inc., Hercules, CA). Each sequencing plate contained four negative-control reaction mixtures using the universal primer M13F(-21) and nuclease-free water substituting for DNA template, as well as four positive-control reaction mixtures using pGEM 3Zf(+) plasmid as the control template and M13F(-21) as a primer. The sequencing reaction products were purified with the CleanSEQ purification reagent (ACGT, Wheeling, IL) and were analyzed by the ABI 3730 XL genetic analyzer (Life Technologies, Carlsbad, CA). Each PCR product was sequenced twice from each direction to achieve a total of 4-fold coverage. For a valid sequencing result, specimens were required to have ≥200 consecutive bases sequenced with 2-fold coverage in double strands (4-fold coverage overall) with a Phred quality score of ≥30. After Phred analysis, the sequence data were assembled by the Gene Codes Sequencher software to generate a single contig for each PCR product. Only bases with a Phred quality score of ≥30 were used in the assembly.

Valid sequences were used to establish the identity of specific resistance markers in comparison to known reference sequences of each target downloaded from GenBank based on curations by the Lahey Clinic (http://www.lahey.org/Studies/other.asp). The identity of the sequence was assigned only if the sample sequence met the following criteria: (i) it contained ≥200 consecutive high-quality bases (Phred score of 30 or greater), and (ii) it shared ≥95% sequence similarity to the reference strain across ≥95% of the high-quality sequenced region. All analyzed specimens outperformed the minimum requirements and yielded between 99.8% to 100% identity to the reference strains while containing ≥300 consecutive bases (i.e., Phred 30).

Statistical analysis. Positive percent agreement (PPA), negative percent agreement (NPA), and 95% confidence intervals were calculated using standard formulas and the Newcombe method. Statistical significance was calculated using Fisher's exact test and is presented as a probability value (*P* value). Calculations were completed using the clinical calculator 1 application available at http://vassarstats.net/clin1.html.

RESULTS

Study enrollment and prevalence of markers in prospectively collected blood culture broths. A total of 1,871 blood culture broths collected in 7 types of aerobic culture medium (see Materials and Methods) met the study inclusion criteria and were evaluated using the BC-GN assay. Twenty-four (1.3%) broth cultures were removed from analysis because of failure to generate a valid BC-GN result (e.g., internal control failure, high background signal, etc.). The remaining 1,847 specimens comprised 729 (39.5%) fresh prospectively collected blood culture broths tested at the time of collection, 781 (42.3%) frozen specimens collected both prospectively and retrospectively, which were frozen and tested at a later date, and 337 (18.2%) simulated specimens that were prepared using characterized clinical isolates. The study cohort included 48 (2.6%) cultures found to be polymicrobial by reference culture, including 26/729 (3.6%) prospectively collected fresh cultures.

Among 703 monomicrobial fresh prospectively collected broth cultures, 645 (91.7%) contained an organism included on the BC-GN panel. The most common target by reference culture was *E. coli* (321 [45.7%]), followed by *K. pneumoniae* (115 [16.4%]), *P. aeruginosa* (84 [11.9%]), *Enterobacter* spp. (49 [7.0%]), *Proteus* spp. (25 [3.6%]), *K. oxytoca* (22 [3.1%]), *Acinetobacter* spp. (13 [1.8%]), and *Citrobacter* spp. (8 [1.1%]). The most common organisms not present on the BC-GN panel included *Serratia* spp. (19 [2.7%]), non-*aeruginosa Pseudomonas* (10 [1.4%]), and *Stenotrophomonas maltophilia* (6 [0.9%]).

$bla_{CTX\text{-}M}$ was the most common genetic resistance determinant detected in the fresh prospectively collected cultures. Overall, $bla_{CTX\text{-}M}$ was identified in 46/703 (6.5%) fresh broths, including 39/321 (12.1%) broths containing *E. coli*. The remaining $bla_{CTX\text{-}M}$-positive broths contained *K. pneumoniae* (4/103 [3.9%]), *K. oxytoca* (1/21 [4.8%]), *Proteus* sp. (1/25 [4.0%]), and *Enterobacter* sp. (1/49 [2.0%]). Of the 46 $bla_{CTX\text{-}M}$ identified, 38 (82.6%) were $bla_{CTX\text{-}M\text{-}1}$ type, and the remaining 8 were $bla_{CTX\text{-}M\text{-}9}$ type. Genes encoding both types of enzymes were identified in a broth containing *K. pneumoniae*; however, this culture was noted to contain two different colony morphologies and may have contained two different strains of *K. pneumoniae*.

Only 2/703 (0.3%) fresh prospectively collected broths were identified as positive for bla_{KPC}. Both were identified in cultures containing *K. pneumoniae*. One of two was also positive for

TABLE 2 Resolution of monomicrobial blood culture broths with discrepant results

Target	No. of broth cultures	Results with (no. of isolates):			Initial call (no. of broth cultures)[c]	Resolved call (no. of broth cultures)[d]
		BC-GN[a]	Culture	Sequence analysis[b]		
E. coli	1	*E. coli* + *K. pneumoniae*	*K. pneumoniae*	*E. coli/Shigella* sp.	FP	TP
	1	*E. coli* + *Enterobacter* sp.	*Enterobacter* sp.	*E. coli/Shigella* sp.	FP	TP
	1	*E. coli*	*K. oxytoca*	*E. coli/Shigella* sp.	FP	TP
	1	*E. coli* + *Proteus* sp.	*P. mirabilis*	ND	FP	ND
	1	*E. coli* + *K. oxytoca*	*K. oxytoca*	*E. coli/Shigella* sp.	FP	TP
	1	*E. coli*	No growth[e]	ND	FP	ND
K. pneumoniae	24	ND	*K. pneumoniae*	*K. variicola*	FN (24)	TN (24)
	1	*Enterobacter* sp.	*K. pneumoniae*	*K. variicola*	FN	TN
	1	*K. pneumoniae*	*Enterobacter* sp.	*K. variicola*	FP	FP
	1	ND	*K. pneumoniae*	*K. pneumoniae*	FN	FN
K. oxytoca	1	*E. coli*	*K. oxytoca*	*E. coli*	FN	TN
	1	*K. pneumoniae* + *K. oxytoca*	*K. pneumoniae*	*K. pneumoniae*	FP	FP
	6	*K. oxytoca*	*R. planticola*	*Raoultella planticola* (1), *Raoultella ornithinolytica* (4), ND (1)	FP (6)	FP (5), ND (1)
	1	ND	*K. oxytoca*	*K. variicola*	FN	TN
	1	ND	*K. oxytoca*	*K. oxytoca*	FN	FN
Enterobacter spp.	2	*Enterobacter* spp. + *K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	FP (2)	FP (2)
	1	*Enterobacter* sp.	*K. pneumoniae*	*K. variicola*	FP	FP
	2	*Enterobacter* spp.	No growth	ND (2)	FP (2)	ND (2)
	1	*K. pneumonia* + *Enterobacter* sp.	*K. pneumoniae*	*K. pneumoniae*	FP	FP
	1	*K. pneumoniae*	*E. cloacae*	*K. variicola*	FN	TN
P. aeruginosa	2	ND	*P. aeruginosa*	*P. aeruginosa*[f]	FN (2)	FN (2)
	1	*P. aeruginosa*	No growth	ND	FP	ND
Proteus spp.	2	*Proteus* spp.	No growth	ND (2)	FP (2)	ND (2)
Acinetobacter sp.	1	*Acinetobacter* sp.	*Alcaligenes faecalis*	ND	FP	ND
	1	*Acinetobacter* sp.	*Cupriavidus pauculus*	*A. baumannii*[f]	FP	TP
	1	ND	*A. baumannii*	*A. baumannii*[f]	FN	FN
Citrobacter sp.	1	*Citrobacter* sp.	*Serratia marcescens*	*S. marcescens*	FP	FP

[a] "+" indicates positivity for both species by the BC-GN assay.
[b] A slash indicates that the BC-GN assay cannot differentiate between the two species.
[c] BC-GN result compared to reference culture.
[d] BC-GN result compared to bidirectional sequence analysis. ND, not determined, as specimen was unavailable for sequence analysis.
[e] No growth, Gram-negative bacilli observed in primary Gram stain of blood culture.
[f] Identification confirmed by MALDI-TOF MS.

bla_{CTX-M}. A single fresh broth containing *E. coli* was positive for bla_{NDM}. This broth was also positive for bla_{CTX-M}. None of the prospectively collected fresh broths contained bla_{VIM} or bla_{IMP}.

Identification of Gram-negative organisms in monomicrobial cultures. The positive percent agreement (PPA) of the BC-GN assay with the reference method across fresh, frozen, and simulated blood culture broths was 100% for *E. coli* (675/675), *Proteus* spp. (75/75), and *Citrobacter* species (58/58) (Table 1). Of note, one broth called positive for *E. coli* by the BC-GN assay was further identified as *Shigella* sp. by reference culture. This broth was still considered a correct identification by the BC-GN assay, because the capture probe cannot differentiate *E. coli* from *Shigella* spp. and is considered a combined *E. coli/Shigella* spp. target by the assay. Although the BC-GN assay is capable of detecting 5 different species within the genus *Proteus*, 73/75 cultures positive for *Proteus* spp. by the BC-GN assay were identified as *Proteus mirabilis*.

The BC-GN assay was falsely negative in 1 broth each containing *Enterobacter* sp. and *Acinetobacter* sp., resulting in sensitivities of 99.3% (144/145) and 98.4% (60/61), respectively. In both instances, the single false-negative result was obtained from the frozen set of specimens. The BC-GN assay failed to detect any target in the broth that was falsely negative for *Acinetobacter* species (Table 2). The identification of this isolate was confirmed to be *Acinetobacter baumannii* by both reference culture and MALDI-TOF MS. The broth identified as falsely negative for *Enterobacter* sp. was called positive for a different target (*K. pneumoniae*) by the BC-GN assay, raising the possibility of an incorrect identification by the BC-GN assay or reference culture method. Upon sequence analysis, this isolate was identified as *K. variicola*. This indicates that the BC-GN assay was correct in not calling *Enterobacter* sp. positive in this broth, raising the postdiscrepant resolution PPA to 100% for *Enterobacter* species. Two broths positive for *P. aeruginosa* by reference culture failed to be detected by the BC-GN assay,

resulting in a PPA of 98.9% (176/178). Both broths were frozen specimens, and the identity of these isolates was confirmed using MALDI-TOF MS. Three broths were initially positive for *K. oxytoca* upon reference culture but negative by the BC-GN assay. In two of three samples, the BC-GN assay was negative for all targets. The identity of one of these isolates was confirmed as *K. oxytoca* by sequence analysis; however, the other was identified as *K. variicola* and therefore was resolved as a true-negative result for the BC-GN assay. The third broth was called positive for *E. coli* by the BC-GN assay. Sequence analysis confirmed this identification, indicating an incorrect reference culture result. Taken together, these data raise the postdiscrepant resolution PPA to 98.4% (63/64) for *K. oxytoca*.

The largest number of initial false-negative results was observed in broth cultures containing *K. pneumoniae*. A total of 26 broths, including 12/115 (10.4%) fresh and 13/116 (11.2%) frozen with *K. pneumoniae* reported by reference culture, were not detected by the BC-GN assay (Table 1). This resulted in a PPA of as low as 89.6% for *K. pneumoniae* in fresh prospectively collected broths. However, 25/26 isolates obtained from these broths were identified as *K. variicola* by sequence analysis (Table 2). While phenotypically similar to *K. pneumoniae*, *K. variicola* is a distinct species (see Discussion), which is not claimed as a target by the BC-GN assay. Therefore, the postdiscrepant resolution PPA of the BC-GN assay for broths containing *K. pneumoniae* is 99.7% (364/365).

The negative percent agreement (NPA) of the BC-GN assay for all eight identification targets ranged from 99.5% to 99.9%. The *E. coli* target was detected in six broths that failed to yield *E. coli* when cultured. Of interest, 4/6 of these broths contained a different organism that was recovered by reference culture and correctly called by the BC-GN assay (Table 2). The second organism was different for each of these cultures (*K. pneumoniae*, *Enterobacter* spp., *Proteus* spp., and *K. oxytoca*), suggesting that the false-positive *E. coli* result was unlikely the result of cross-reactivity of the capture probe used for these targets. Additionally, sequence analysis of nucleic acid extracts from three of these cultures identified the presence of *E. coli* sequences. It is unclear whether this represents the presence of nonviable organisms, free nucleic acid, or contamination of these specimens with *E. coli* nucleic acid. One specimen called positive for *E. coli* by the BC-GN assay contained *K. oxytoca* by reference culture; however, sequence analysis confirmed the identification of the isolate to be *E. coli*. The single most prevalent cause of false-positive results ($n = 6$) was observed in broths called positive for *K. oxytoca* that subsequently yielded *Raoultella* spp. upon reference culture (Table 2). Sequence analysis was conducted on nucleic acid extracts from 5 of 6 broths falsely identified as positive for *K. oxytoca* by the BC-GN assay and confirmed the reference culture identification of *Raoultella* species. *K. oxytoca* and *Raoultella* spp. are closely related both phenotypically and genotypically, and the observed false-positive calls are the result of cross-reactivity of the *K. oxytoca* capture probe used by the BC-GN assay with *Raoultella* species (see package insert).

Identification of resistance markers in monomicrobial cultures. The prevalence of the 6 genetic resistance determinants detected by the BC-GN assay was low (0.0% to 0.6%) among 1,465 prospectively or retrospectively collected fresh and frozen specimens, with the exception of $bla_{\mathrm{CTX\text{-}M}}$ (5.0%). Therefore, to adequately assess the clinical performance of the BC-GN assay for the identification of these targets, we relied on the use of simulated broths (see Materials and Methods).

The PPA of the BC-GN assay was 100.0% for the identification of bla_{KPC} (53/53), bla_{VIM} (68/68), and bla_{IMP} (47/47) in prospective, retrospective, and simulated blood culture broths (Table 3). The majority of the bla_{KPC}-positive results (46/53 [87%]) were called in broths containing *K. pneumoniae*, but there were also bla_{KPC}-positive results in broths containing *K. oxytoca* ($n = 2$), *E. coli* (2), *Citrobacter* sp. ($n = 1$), *Enterobacter* sp. ($n = 1$), and *P. aeruginosa* ($n = 1$). Of interest, 8 specimens called positive for bla_{KPC} were positive for a second resistance determinant (5 $bla_{\mathrm{CTX\text{-}M}}$ and 3 bla_{VIM}), and bla_{KPC} was correctly identified in two cultures in which the BC-GN assay failed to detect the organism recovered by reference culture (one each of *K. pneumoniae* and *K. oxytoca*). Eight different bla_{IMP} types (as determined by the reference methods) were detected by the BC-GN assay in the 47 broths with positive results. Among these, $bla_{\mathrm{IMP\text{-}4}}$ was most common (14/47 [29.8%]), followed by $bla_{\mathrm{IMP\text{-}1}}$ (9/47 [19.1%]), $bla_{\mathrm{IMP\text{-}2}}$ (8/47 [17.0%]), $bla_{\mathrm{IMP\text{-}5}}$ and $bla_{\mathrm{IMP\text{-}7}}$ (4/47 [8.5% each]), $bla_{\mathrm{IMP\text{-}18}}$ (3/47 [6.4%]), $bla_{\mathrm{IMP\text{-}13}}$ and $bla_{\mathrm{IMP\text{-}27}}$ (2/47 [4.3% each]), and $bla_{\mathrm{IMP\text{-}16}}$ (1/47 [2.1%]). Detection of bla_{IMP} was most common in broths containing *P. aeruginosa* (15/47 [31.9%]); however, bla_{IMP} was detected in at least one broth containing each of the 8 bacteria identified by the BC-GN assay. Three bla_{VIM} types were identified by reference methods in 68 broths positive for this marker. These included 53 broths containing $bla_{\mathrm{VIM\text{-}1}}$, 14 containing $bla_{\mathrm{VIM\text{-}2}}$, and 1 containing $bla_{\mathrm{VIM\text{-}17}}$. The majority of these (28/68 [41.2%]) were identified in broths containing *K. pneumoniae*; however, bla_{VIM} was detected in at least one broth containing each of the 8 bacteria identified by the BC-GN assay. Eight broths positive for bla_{VIM} were positive for a second genetic resistance marker, including 4 $bla_{\mathrm{CTX\text{-}M}}$, 3 bla_{KPC}, and 1 $bla_{\mathrm{OXA\text{-}48}}$.

The PPA of the BC-GN assay for identifying $bla_{\mathrm{CTX\text{-}M}}$ was 98.9% (181/183) overall. Both false-negative results were in broths containing *E. coli* and $bla_{\mathrm{CTX\text{-}M\text{-}1}}$ type enzymes by reference methods. Among the positive results, $bla_{\mathrm{CTX\text{-}M\text{-}1}}$ type was the most prevalent (145/181 [80.1%]), followed by $bla_{\mathrm{CTX\text{-}M\text{-}9}}$ type (26/181 [14.4%]). Among the 108 simulated cultures containing $bla_{\mathrm{CTX\text{-}M}}$, 88 (81.5%) contained a second genetic marker of resistance, again demonstrating the ability of the BC-GN assay to correctly identify multiple resistance markers in a single broth culture. The $bla_{\mathrm{CTX\text{-}M}}$ resistance determinant was detected primarily in cultures containing *E. coli*; however, the marker was also prevalent in broths containing *Klebsiella* spp. and *Enterobacter* species. The $bla_{\mathrm{CTX\text{-}M}}$ marker was not detected in any broth containing *P. aeruginosa*, *Citrobacter* spp., or *Acinetobacter* spp., so detection of this resistance determinant in these genera was not established. Of note, $bla_{\mathrm{CTX\text{-}M}}$ was correctly identified in two broths containing *Morganella* spp., which is not a target identified by the BC-GN assay. While potentially useful, the detection of resistance determinants in the absence of a genus or species identification by the BC-GN assay is not a part of the FDA-cleared *in vitro* diagnostic (IVD) assay.

The BC-GN assay correctly identified bla_{NDM} in 51/53 broths containing bla_{NDM} by reference methods, for a PPA of 96.2%. The two false-negative results were in broths containing *E. coli* and *K. pneumoniae* strains, both of which also harbored $bla_{\mathrm{CTX\text{-}M\text{-}1}}$. Among the 51 positive results, 26 (51.0%) were found in cultures containing *K. pneumoniae*, 13 (25.5%) in broths containing *E. coli*, 2 (3.9%) each in broths containing *Citrobacter* spp. and *Morgan-*

TABLE 3 Identification of genetic markers of resistance in monomicrobial cultures using BC-GN assay

Target	Specimen type	No. with result[a]:				Total no.	% (95% CI) for[b]:	
		TP	FP	TN	FN		PPA	NPA
bla$_{CTX-M}$	Fresh	45	0	657	1[c]	703	97.8 (87–100)	100.0 (99–100)
	Frozen	28	0	733	1[c]	762	96.6 (80–100)	100.0 (99–100)
	Simulated	108	0	226	0	334	100.0 (96–100)	100.0 (98–100)
	Total	181	0	1,616	2	1,799	98.9 (96–100)	100.0 (99–100)
bla$_{KPC}$	Fresh	2	0	701	0	703	100.0 (20–100)	100.0 (99–100)
	Frozen	1	0	761	0	762	100.0 (5–100)	100.0 (99–100)
	Simulated	50	0	284	0	334	100.0 (91–100)	100.0 (98–100)
	Total	53	0	1,746	0	1,799	100.0 (92–100)	100.0 (99–100)
bla$_{NDM}$	Fresh	1	0	702	0	703	100.0 (5–100)	100.0 (99–100)
	Frozen	0	0	762	0	762	ND	100.0 (99–100)
	Simulated	50	0	282	2[d]	334	96.2 (86–100)	100.0 (98–100)
	Total	51	0	1,746	2	1,799	96.2 (86–100)	100.0 (99–100)
bla$_{OXA}$	Fresh	6	0	696	1[e]	703	85.7 (42–99)	100.0 (99–100)
	Frozen	3	0	757	2[e]	762	60.0 (17–93)	100.0 (99–100)
	Simulated	57	1[f]	275	1[e]	334	98.3 (90–100)	99.6 (98–100)
	Total	66	1	1,728	4	1,799	94.3 (85–98)	99.9 (99–100)
bla$_{VIM}$	Fresh	0	0	703	0	703	ND	100.0 (99–100)
	Frozen	0	0	762	0	762	ND	100.0 (99–100)
	Simulated	68	0	266	0	334	100.0 (93–100)	100.0 (98–100)
	Total	68	0	1,731	0	1,799	100.0 (93–100)	100.0 (99–100)
bla$_{IMP}$	Fresh	0	0	703	0	703	ND	100.0 (99–100)
	Frozen	0	0	762	0	762	ND	100.0 (99–100)
	Simulated	47	0	287	0	334	100.0 (91–100)	100.0 (98–100)
	Total	47	0	1,752	0	1,799	100.0 (91–100)	100.0% (99–100)

[a] TP, true positive, FP, false positive, TN, true negative, FN, false negative.
[b] PPA, positive percent agreement; NPA, negative percent agreement; 95% CI, 95% confidence interval; ND, not detected.
[c] Confirmed to be *bla*$_{CTX-M-1}$ by sequence analysis.
[d] One of two confirmed to be *bla*$_{NDM}$ by sequence analysis. One was not available for discrepant analysis.
[e] Confirmed to be *bla*$_{OXA-23}$ by sequence analysis.
[f] Negative for *bla*$_{OXA}$ by sequence analysis.

ella spp., and 1 (2.0%) in a broth containing *Acinetobacter* species. The majority (35/51 [68.6%]) of the broths positive for *bla*$_{NDM}$ were also positive for *bla*$_{CTX-M}$. There were no positive *bla*$_{NDM}$ results in broths containing *Proteus* spp., *K*, *oxytoca*, *P. aeruginosa*, or *Proteus* species.

Among the 8 genetic resistance determinants identified by the BC-GN assay, the detection of *bla*$_{OXA}$ was the least sensitive, at 94.3% (66/70). The 4 false-negative results were distributed across fresh ($n = 1$), frozen ($n = 2$), and simulated ($n = 1$) specimen types. All were confirmed to be *bla*$_{OXA-23}$ type by discrepant sequence analysis. Among the positive results, *bla*$_{OXA-48}$ type was most common (46/66 [69.7%]), with the remaining positive broths containing the *bla*$_{OXA-23}$, *bla*$_{OXA-40}$, or *bla*$_{OXA-58}$ types. A second resistance determinant was correctly identified in 60.6% (40/66) of the broths positive for *bla*$_{OXA}$. Broths positive for *bla*$_{OXA}$ contained *Acinetobacter* spp. ($n = 20$), *Citrobacter* sp. ($n = 1$), *Klebsiella* spp. ($n = 23$), *Enterobacter* spp. ($n = 3$), *Shewanella* sp. ($n = 1$), *Serratia* sp. ($n = 1$), or *Morganella* species ($n = 2$).

Taken together, these data demonstrate the ability of the BC-GN assay to correctly identify six genetic resistance determinants, irrespective of the bacterium being present in the specimen or the presence of multiple resistance determinants in a single specimen. It is important to note that while the BC-GN assay is capable of detecting several alleles of the *bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{OXA}$, *bla*$_{KPC}$, and *bla*$_{NDM}$ types, it does not differentiate among the various subtypes.

Polymicrobial cultures. A total of 48 broths analyzed by the BC-GN assay contained multiple organisms by reference culture. This included 26/729 (3.6%) fresh specimens, 19/781 (2.4%) frozen specimens, and 3/337 (0.9%) simulated cultures. Eight of these cultures contained Gram-negative organisms that are not part of the BC-GN panel, including *S. maltophilia*, *Serratia* spp., *Achromobacter* spp., *Sphingomonas* spp., *Delftia* spp., and *Pantoea* species. The BC-GN result was correctly reported as not detected for all targets in these cultures. Of the remaining 40 broths, 22 contained two organisms that were both targets of the BC-GN assay, 13 contained two organisms, of which only one was a target of the BC-GN assay, and 5 contained three organisms (Table 4).

The BC-GN assay correctly identified at least one organism in 21/22 (95.4%) of broths and correctly identified all organisms in 12/22 (54.5%) broths. Among broths with false-negative results, *P. aeruginosa* was not detected in 2 broths containing *K. pneumoniae* and *P. aeruginosa*. The BC-GN assay failed to detect *K. oxytoca* in 2/3 broths containing *E. coli* and *K. oxytoca* and failed to detect *K. pneumoniae* in 2/4 broths containing *E. coli* and *K. pneumoniae* (Table 4). The BC-GN assay also failed to detect *P. aerugi-*

TABLE 4 Identification of Gram-negative organisms in polymicrobial cultures using BC-GN assay

Parameter	Results for:		
	Reference culture	BC-GN	Final call[a]
Two organisms (1 and 2), both on BC-GN panel	(1) *K. pneumoniae*, (2) *E. asburiae*	(1) *K. pneumoniae*, (2) *Enterobacter* sp.	(1) TP, (2) TP
	(1) *K. pneumoniae*, (2) *A. lwoffii*	(1) *K. pneumoniae*, (2) *Acinetobacter* sp.	(1) TP, (2) TP
	(1) *K. pneumoniae*, (2) *P. aeruginosa*	(1) *K. pneumoniae*, (2) ND[c]	(1) TP, (2) FN[b]
	(1) *K. pneumoniae*, (2) *P. aeruginosa*	(1) *K. pneumoniae*, (2) ND	(1) TP, (2) FN[b]
	(1) *K. oxytoca*, (2) *E. cloacae*	(1) *K. oxytoca*, (2) ND	(1) TP, (2) FN[d]
	(1) *E. coli*, (2) *K. oxytoca*	(1) *E. coli*, (2) *K. oxytoca*	(1) TP, (2) TP
	(1) *E. coli*, (2) *K. oxytoca*	(1) *E. coli*, (2) ND	(1) TP, (2) FN[e]
	(1) *E. coli*, (2) *K. oxytoca*	(1) *E. coli*, (2) ND	(1) TP, (2) FN[e]
	(1) *E. coli*, (2) *P. mirabilis*	(1) *E. coli*, (2) *Proteus* sp.	(1) TP, (2) TP
	(1) *E. coli*, (2) *P. mirabilis*	(1) *E. coli*, (2) *Proteus* sp.	(1) TP, (2) TP
	(1) *E. coli*, (2) *K. pneumoniae*	(1) *E. coli*, (2) ND	(1) TP, (2) FN[f]
	(1) *E. coli*, (2) *K. pneumoniae*	(1) *E. coli*, (2) ND	(1) TP, (2) FN[f]
	(1) *E. coli*, (2) *K. pneumoniae*	(1) *E. coli*, (2) *K. pneumoniae*	(1) TP, (2) TP
	(1) *E. coli*, (2) *K. pneumoniae*	(1) *E. coli*, (2) *K. pneumoniae*	(1) TP, (2) TP
	(1) *E. coli*, (2) *P. aeruginosa*	(1) *E. coli*, (2) ND	(1) TP, (2) FN[b]
	(1) *E. coli*, (2) *A. baumannii*	(1) *E. coli*, (2) *Acinetobacter* sp.	(1) TP, (2) TP
	(1) *E. coli*, (2) *E. aerogenes*	(1) *E. coli*, (2) *Enterobacter* sp.	(1) TP, (2) TP
	(1) *E. coli*, (2) *E. cloacae*	(1) ND, (2) ND	(1) FN, (2) FN[g]
	(1) *E. cloacae*, (2) *K. oxytoca*	(1) *Enterobacter* sp., (2) *K. oxytoca*	(1) TP, (2) TP
	(1) *E. cloacae*, (2) *K. pneumoniae*	(1) *Enterobacter* sp., (2) ND	(1) TP, (2) FN[g]
	(1) *E. cloacae*, (2) *K. pneumoniae*	(1) *Enterobacter* sp., (2) *K. pneumoniae*	(1) TP, (2) TP
	(1) *E. cloacae*, (2) *A. baumannii*	(1) *Acinetobacter* sp., (2) *Enterobacter* sp.	(1) TP, (2) TP
Two organisms (1 and 2), with one of two on BC-GN panel			
	(1) *E. coli*, (2) *E. faecium*	(1) *E. coli*, (2) ND	(1) TP, (2) TN
	(1) *E. coli*, (2) *E. faecalis*	(1) *E. coli*, (2) ND	(1) TP, (2) TN
	(1) *E. coli*, (2) *Aeromonas* sp.	(1) *E. coli*, (2) ND	(1) TP, (2) TN
	(1) *E. coli*, (2) *Aeromonas* sp.	(1) *E. coli*, (2) ND	(1) TP, (2) TN
	(1) *K. oxytoca*, (2) *S. marcescens*	(1) *K. oxytoca*, (2) ND	(1) TP, (2) TN
	(1) *K. oxytoca*, (2) *E. hermannii*	(1) *K. oxytoca*, (2) *Citrobacter* sp.	(1) TP, (2) FP[g]
	(1) *P. aeruginosa*, (2) *Kocuria* sp.	(1) *P. aeruginosa*, (2) ND	(1) TP, (2) TN
	(1) *P. aeruginosa*, (2) *Sphingomonas* sp.	(1) *P. aeruginosa*, (2) ND	(1) TP, (2) TN
	(1) *E. cloacae*, (2) *Morganella* sp.	(1) *Enterobacter* sp., (2) ND	(1) TP, (2) TN
	(1) *E. cloacae*, (2) *E. faecium*	(1) *Enterobacter* sp., (2) ND	(1) TP, (2) TN
	(1) *E. cloacae*, (2) *Hafnia* sp.	(1) *Enterobacter* sp., (2) ND	(1) TP, (2) TN
	(1) *A. baumannii*, (2) *S. maltophilia*	(1) *Acinetobacter* sp., (2) ND	(1) TP, (2) TN
	(1) *Acinetobacter radioresistens*, (2) CoNS[h]	(1) *Acinetobacter* sp., (2) ND	(1) TP, (2) TN
Three organisms			
	(1) *E. coli*, (2) *K. pneumoniae*, (3) *A. baumannii*	(1) *E. coli*, (2) *K. pneumoniae*, (3) *A. baumannii*	(1) TP, (2) TP, (3) TP
	(1) *E. coli*, (2) *P. mirabilis*, (3) *Sphingomonas* sp.	(1) *E. coli*, (2) *P. mirabilis*, (3) ND	(1) TP, (2) TP, (3) TN
	(1) *K. pneumoniae*, (2) *K. oxytoca*, (3) *Enterobacter* sp.	(1) *K. pneumoniae*, (2) *K. oxytoca*, (3) ND	(1) TP, (2) TP, (3) FN[i]
	(1) *K. oxytoca*, (2) *Pseudomonas stutzeri*, (3) *S. maltophilia*	(1) *K. oxytoca*, (2) ND, (3) ND	(1) TP, (2) TN, (3) TN
	(1) *K. oxytoca*, (2) *P. stutzeri*, (3) *S. maltophilia*	(1) *K. oxytoca*, (2) ND, (3) ND	(1) TP, (2) TN, (3) TN

[a] Final call versus reference culture result. TP, true positive; TN, true negative; FP, false positive; FN, false negative.
[b] *P. aeruginosa* confirmed by sequence analysis.
[c] ND, not detected.
[d] Identification of the organisms in this specimen could not be resolved by sequence analysis.
[e] *K. oxytoca* confirmed by sequence analysis.
[f] *K. pneumoniae* confirmed by sequence analysis.
[g] Specimens (15131, 17155, and 17111) were not available for sequence analysis.
[h] CoNS, coagulase-negative *Staphylococcus* species.
[i] Sequence analysis failed to identify *K. oxytoca* in the specimen.

nosa, *Enterobacter cloacae*, and *K. pneumoniae* in additional cultures positive for another target. A single culture was falsely negative for both targets recovered by reference culture (*E. coli* and *E. cloacae*).

In all 13 cultures in which one of two organisms was a target of the BC-GN assay, the target organism was correctly identified, for a PPA of 100% (Table 4). In one culture containing *K. oxytoca* and *Escherichia hermannii*, the BC-GN assay reported *K. oxytoca* and *Citrobacter* species. In one of two broths containing 3 organisms present on the BC-GN panel, all three targets were correctly identified (*E. coli*, *K. pneumonia*e, and *A. baumannii*).

DISCUSSION

The ability to rapidly identify Gram-negative organisms from subculture or directly from positive blood culture broths has proven beneficial to patient outcomes, antimicrobial stewardship, and overall cost of care for patients with these infections (15, 19, 20). For example, the use of MALDI-TOF MS to rapidly identify Gram-negative organisms from subculture contributed to a 51-h reduction in the time to optimal therapy and a 17% reduction in 30-day mortality in patients with Gram-negative BSI (15). Importantly, 61.4% of the antibiotic modifications were based on organism identification alone. In another study, direct analysis of positive blood culture broths using MALDI-TOF MS combined with a rapid susceptibility testing method reduced the length of hospital stay by 2.6 days and reduced the total cost of care by $19,000 (19). An early evaluation of the BC-GN assay estimated that the detection of bla_{KPC} directly from positive blood cultures could have reduced the time to most appropriate therapy by approximately 14 h in 56% of patients infected with *K. pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (20). When combined with an active antimicrobial stewardship intervention, these outcome studies demonstrate the potential value of rapid identification of organisms and genetic resistance markers among blood cultures containing Gram-negative bacteria.

A number of studies have already evaluated the BC-GN assay and demonstrated a sensitivity and specificity of 81.0% to 100% and 98.0% to 100%, respectively, for identifying individual bacterial and resistance markers (20, 27–30). However, these studies were limited by a small sample size (31 to 125 cultures) (20, 26, 29), a large proportion of simulated specimens (63% to 74% of evaluated cultures) (29, 30), or a potential lack of strain diversity due to the single center-based design of the study (20, 27, 30). The present study describes the most comprehensive evaluation of the BC-GN assay to date. In addition to the large total number of specimens evaluated ($n = 1{,}847$), seven types of blood culture media representing the three primary manufacturers of blood culture systems were included. The NPA for specimens tested using all blood culture medium types included in this study was >99.8%, and no statistical difference in NPA was found between any of the blood culture types included in this study ($P = 0.736$, Fisher's exact test). The difference in PPA between specimens tested using the various blood culture media ranged from 94.3% (Bactec Standard/10 Aerobic/F) to 100% (BacT/Alert SA). This difference was statically significant ($P = 0.030$, Fisher's exact test); however, it is important to note that BacT/Alert SA accounted for only 20 (1.1%) of the blood cultures tested, while Bactec Standard/10 Aerobic/F accounted for 292 (15.8%) of the cultures. The low number of total cultures tested using BacT/Alert SA might have impacted the calculation of statistical significance between these two medium types. The sample size, diversity of culture media, and inclusion of 12 geographically distinct clinical centers within the United States are specific strengths of this study.

The overall PPA of the BC-GN assay for eight bacterial identification targets was 97.9% (1,590/1,623). The PPA of the BC-GN assay was highest in fresh prospectively tested cultures, including 100% PPA for 6/8 genus or species identification targets on the panel. The majority of the false-negative results (6/7, excluding *K. pneumoniae/K. variicola*, as discussed below) were obtained when testing cultures that had been frozen prior to analysis. The instability of the nucleic acid targets resulting from freezing and thawing of these samples may have contributed to the false-negative results, although this hypothesis was not tested.

The BC-GN assay contains capture probe targets that enabled an identification of the organism present in approximately 90% of the prospectively collected blood cultures containing Gram-negative bacilli. Among targets not present on the BC-GN assay, the most commonly encountered was *K. variicola* (12/703 [1.7%] of prospective cultures). *K. variicola* is phenotypically similar to *K. pneumoniae* and is easily misidentified as *K. pneumoniae* when using automated phenotypic identification methods (31) and MALDI-TOF MS (our unpublished data). This may lead to perceived false-negative BC-GN results in cultures containing *K. variicola* that are reported as not detected by the BC-GN assay. Among prospectively collected cultures phenotypically identified as *K. pneumoniae*, 10.4% (range, 0% to 25% among clinical centers in this study) were subsequently identified as *K. variicola* by sequence analysis. Although initially associated with environmental sources and plant material (32), *K. variicola* may comprise up to 10% of all *Klebsiella* species isolates obtained from clinical specimens (31, 33, 34). One report details a fatal case of sepsis, which was initially attributed to *K. pneumoniae* but was later resolved as *K. variicola* (31). Literature exploring the relative virulence or susceptibility patterns of *K. variicola* is sparse, but a larger proportion of isolates of *K. variicola* may be susceptible to piperacillin and cephalosporins than *K. pneumoniae* (34). Given these data, laboratories that identify *K. pneumoniae* in blood cultures resulted as not detected by the BC-GN assay may be advised to confirm the identity with additional sequence analysis or definitive biochemical tests prior to reporting.

In addition to *K. variicola*, a potential weakness of the BC-GN assay is the omission of some other relatively common Gram-negative pathogens from the panel, including *Serratia* spp., *S. maltophilia*, and non-*aeruginosa Pseudomonas*, which combined made up 4.4% (31/703) of the positive cultures. The BC-GN assay also lacks targets for detecting *Neisseria meningitidis* and *Haemophilus influenzae*. While the prevalence of these organisms is relatively rare (neither was isolated in 703 prospectively collected cultures in this study), their presence in blood culture is indicative of serious life-threatening or invasive infection.

A specific advantage of the BC-GN assay versus other rapid identification methods (e.g., MALDI-TOF MS and peptide nucleic acid-FISH [PNA-FISH]) is the ability to identify 6 genetic markers associated with resistance to various classes of β-lactam antibiotics. Gram-negative bacteria harboring extended-spectrum β-lactamases (ESBLs) or carbapenemases are of concern because of the limited therapeutic options available to effectively treat infections with these organisms (35). Specifically, bacteria harboring carbapenemases are resistant to many, if not all, classes of β-lactams and are often resistant to several other classes of

antibiotics. This contributes to an increase in morbidity and mortality in patients infected with these organisms (6). While the prevalence of carbapenemases in prospectively collected cultures was low in this study (KPC, 2/703 [0.3%]; NDM, 1/703 [0.1%]), larger surveillance efforts have indicated a steady rise in the prevalence of KPC both in the United States and abroad since its initial characterization in 1996 (36–38). Carbapenem-resistant *Enterobacteriaceae* (CRE) currently account for >9,000 serious infections per year in the United States and have been reported in 44 states, including New York, Pennsylvania, and New Jersey, where isolates producing bla_{KPC} are considered endemic (36, 37, 39). The increasing prevalence and a lack of effective therapeutic options to treat infections caused by CRE have made early detection and implementation of infection control measures a key aspect in the management of these patients (6, 40).

Current phenotypic methods for detecting CRE, including the modified Hodge test (MHT), have a reported sensitivity of 93% to 100% for class A (e.g., bla_{KPC}) and class D (e.g., bla_{OXA}) carbapenemases; however, subjective interpretation of the test and low-level carbapenemase activity of other β-lactamases result in specificity of only 39% to 76% (41, 42). Further, the sensitivity of the MHT may be as little as 27% to 50% for detecting class B bla_{NDM} enzymes (42, 43). The Carba NP assay is a newer phenotypic test based on detection of *in vitro* hydrolysis of imipenem. This method appears to be as sensitive as the MHT for detecting strains harboring bla_{KPC} and also demonstrates 94% to 100% sensitivity for detecting bla_{NDM} while maintaining >99% specificity (44, 45). Unfortunately, the Carba NP assay demonstrated reduced sensitivity for other carbapenemases, including only 60.6% sensitivity for detecting class D bla_{OXA-48} (44, 46). In addition, both MHT and the Carba NP assay require the isolation of organisms from positive blood culture broths prior to phenotypic testing. Combined with assay setup and incubation, the total time to result for these methods can be 48 to 72 h. The molecular detection of bla_{KPC}, bla_{NDM}, and bla_{OXA} using the BC-GN assay is advantageous in that the PPA (94.3% to 100%) and NPA (99.9% to 100%) for each of these targets was high, and results are not based on a subjective interpretation of phenotypic characteristics. Additionally, the ability to detect these resistance markers directly from positive blood cultures within 2 h greatly reduces the time to reporting of positive results compared with that with phenotypic methods. This has the potential to positively impact both the selection of appropriate antimicrobial therapy and implementation of infection control measures for patients testing positive for one of these targets (20).

In addition to identifying genes encoding carbapenemase enzymes, the BC-GN assay also identifies bla_{CTX-M}, the most prevalent ESBL worldwide (47). ESBL enzymes, including bla_{SHV}, bla_{TEM}, and bla_{CTX-M}, are broad-spectrum enzymes capable of hydrolyzing penicillins, cephalosporins, and aztreonam but remain susceptible to carbapenems. ESBL activity in bla_{SHV} and bla_{TEM} arises from point mutations in narrow spectrum "parent" enzymes, which makes the differentiation of narrow- versus expanded-spectrum β-lactamases difficult without full-sequence analysis (48, 49). In contrast, all bla_{CTX-M} enzymes have native ESBL activity (49), which allows comparatively easy molecular identification of these ESBLs using nucleic acid probes. Accurate identification of isolates harboring ESBLs is of clinical importance because of the poor correlation between *in vitro* susceptibility and the clinical efficacy of cephalosporins for the treatment of infections caused by ESBL-producing bacteria (50, 51).

Phenotypic screening methods to identify strains carrying ESBLs were recommended by the Clinical and Laboratory Standards Institute (CLSI); however, the presence of AmpC enzymes in members of the *Enterobacteriaceae* family can lead to false-positive screen results or obscure the activity of ESBL enzymes during confirmatory disk diffusion tests (52–54). In response to these limitations, the CLSI elected to lower the susceptibility breakpoints for several cephalosporins in an attempt to eliminate the need for phenotypic ESBL tests (55). Despite the lowered breakpoints, strains harboring bla_{CTX-M} ESBLs may still test as susceptible to ceftazidime, cefepime, and aztreonam (56). Specifically, 14% to 45% of *E. coli* isolates and 85% to 96% of *P. mirabilis* isolates harboring bla_{CTX-M} tested as susceptible to these antibiotics based on the lower breakpoints (56). This potential undercalling of resistance was especially pronounced in isolates harboring CTX-M-9 enzymes (56). Limitations in ESBL detection exist for both phenotypic and genetic approaches, and no one-size-fits-all approach has emerged. The rapid detection of key prevalent resistance factors that are detected using the BC-GN system provide a front-line approach in the early identification of emerging resistance, which is likely to positively impact patient care. The BC-GN assay demonstrated 98.9% PPA and 100% NPA for identification of bla_{CTX-M} and is inclusive of >100 individual types in the subgroups CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (see package insert). Combined, the rapid time to result and accuracy of detecting bla_{CTX-M} has the potential to aid in earlier modification of empirical therapy and reduce the time to implementation of infection control practices.

Despite the potential benefits of molecular detection of cephalosporinases and carbapenemases, it is important to recognize that resistance may be mediated by alternative mechanisms, including other classes of β-lactamases, active drug efflux pumps, alternative or modified drug targets, and point mutations that reduce membrane permeability. These mechanisms are especially common in some species of *Enterobacteriaceae* (*Serratia* spp., *P. vulgaris*, *Citrobacter* spp., and *Enterobacter* spp.) and genera outside the family *Enterobacteriaceae*, including *P. aeruginosa* and *Acinetobacter* species. These factors resulted in a negative predictive value of only 78.6% for carbapenem resistance in *P. aeruginosa* in one evaluation of the BC-GN assay (20). Therefore, the absence of detection of any of the specific genetic markers by the BC-GN assay cannot be interpreted as an indication of a susceptible isolate. Laboratories, in conjunction with pharmacy and antimicrobial stewardship teams, may decide to tailor reporting structures within their individual institutions. This may include the addition of interpretive statements with each positive report that aid in guiding antimicrobial therapy choices. For example, a positive report of CTX-M may include a statement indicating the detection of an ESBL and advise against the use of penicillin, cephalosporin, or aztreonam antibiotics until full phenotypic susceptibility results are available. All cultures should include a statement acknowledging that a result of not detected for antimicrobial resistance markers does not indicate susceptibility.

Polymicrobial cultures compose 6 to 12% of all positive blood cultures and present a particular challenge for any technology utilized for direct analysis of these specimens (18, 22, 24, 57). The BC-GN assay correctly identified at least one organism present in >95% of the polymicrobial cultures tested; however, a correct

identification of all organisms present was achieved in only 54% of these specimens. This shortcoming is not unique to the BC-GN assay and has been reported for other molecular assays and for direct analysis of blood culture broths using MALDI-TOF MS. Specifically, the Verigene BC-GP and FilmArray BCID assays are reported to be only 20.0% to 81.3% and 83.3% to 90.0% accurate, respectively, for identifying all targets present in polymicrobial cultures (18, 22, 24, 29, 30, 57). In comparison, MALDI-TOF MS is incapable of correctly identifying multiple targets in a mixed culture and identified one organism in only 64.3% to 70.0% of the polymicrobial specimens (16, 57). Because of this limitation, it is critical to perform a primary Gram stain and routine culture on all positive broths to confirm the presence of bacteria and identify cultures containing multiple organisms.

The main limitation of this study is the lack of phenotypic confirmation of resistance associated with molecular detection of each of the 6 resistance markers detected by the BC-GN assay. Though bla_{CTX-M} enzymes typically display broad-spectrum ESBL activity, point mutations in the coding region of the gene or promoter sequence may result in a phenotypically susceptible strain. Specifically, one author (20) has described the presence of a $bla_{CTX-M-9}$ gene in a strain detected by the BC-GN assay, which was phenotypically susceptible and negative upon a double disk test for ESBL (20). Likewise, strains harboring bla_{KPC}, bla_{NDM}, bla_{VIM}, and bla_{IMP} will be predictably resistant to nearly all classes of β-lactam antibiotics; however, the carbapenemase activity associated with various types of bla_{OXA} enzymes is variable (37). This may lead to discrepancies between the presence of genotypic markers and phenotypic susceptibility results. The BC-GN assay does not differentiate between the various types of bla_{OXA} enzymes; however, the assay was designed to be inclusive of 35 bla_{OXA} types associated with high-level carbapenemase activity (see package insert for complete list). We and others have specifically demonstrated the ability of the BC-GN assay to identify bla_{OXA-48}, bla_{OXA-23}, bla_{OXA-40}, and bla_{OXA-58} in prospective or simulated cultures (30). Still, without phenotypic characterization, it was not possible to correlate the reported NPA of the BC-GN assay (based on comparison to nucleic acid sequence analysis) with phenotypic susceptibility or resistance in the strains tested in our study. Despite these limitations, a positive result for any of the resistance markers may aid in early infection control measures, while the poor NPV of a not detected result for predicting phenotypic susceptibility underscores the necessity to culture and conduct routine phenotypic susceptibility testing for all isolates found in positive blood cultures (20, 30).

A second limitation of this study was the low prevalence of bla_{VIM}, bla_{IMP}, and bla_{OXA} in prospectively collected clinical specimens. These resistance genes remain rare within the United States, as indicated by global epidemiology and as supported by the findings of the current study (37). However, bacteria containing these genes are more prevalent in some eastern European countries, China, and Japan (37). We addressed this shortcoming by including a large set of retrospectively collected blood cultures ($n = 781$) or simulated specimens constructed using characterized clinical isolates ($n = 337$) (see Materials and Methods). The PPA of the BC-GN assay for detecting these 3 types of resistance genes in simulated cultures was 100% for bla_{VIM} and bla_{IMP} and 98.3% for bla_{OXA}. These data demonstrate a high PPA and negative predictive value (NPV); however, because of the high pretest probability of a positive result in the simulated cultures, the NPA may be an overestimate of actual clinical performance. This potential flaw was addressed by data from prospective cultures, in which the NPA was >99.9%.

In conclusion, the BC-GN assay demonstrated a combined 97.9% PPA and 99.7% NPA for bacterial identification targets in monomicrobial cultures compared to the reference phenotypic identification methods. The PPA and NPA for identifying 6 genetic markers of resistance were 98.3% and 99.9%, respectively. The BC-GN assay includes sample-to-result processing, followed by automated reading of the detection microarray and can be completed within 2 h of culture positivity with approximately 5 min of hands-on time. These characteristics, and the designation as a Clinical Laboratory Improvement Amendments (CLIA) moderate-complexity assay, facilitate implementation of the BC-GN assay in clinical laboratories. The rapid turnaround time combined with an active antimicrobial utilization team has the potential to result in improved infection control, earlier selection of appropriate antibiotic therapy, and a reduction in the total cost of care for patients with Gram-negative bloodstream infections.

ACKNOWLEDGMENTS

All authors and sites contributed to Gram-negative data in support of the Nanosphere Verigene FDA submission on BC-GN. B.W.B., N.A.L., B.K.L., R.T., and N.D.H. have served as consultants to Nanosphere and have received honoraria. P.G., N.D., R.T., and G.T.H. have received speaking honoraria from Nanosphere. K.C.C. has received honoraria from Nanosphere for CME presentations. N.D.H. serves as a consultant for Cubist Pharmaceuticals and has received grant support from Cubist/Merck and Strek, Inc. B.K.L. has received research support from Nanosphere, BioFire, and Cubist Pharmaceuticals, served as a consultant to Actavis. N.K.T. and C.R.P. have received research funding from Nanosphere.

BC-GN test materials, media, plastics, and financial support for this study were provided by Nanosphere. Nucleic sequence analysis was supported by Nanosphere. Raw data from each contributing site were reported to Nanosphere, and a compiled raw data set was provided to the authors. Nanosphere did not aid in data analysis or preparation of the manuscript.

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