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Evaluation of a Multiplex PCR Assay To Rapidly Detect *Enterobacteriaceae* with a Broad Range of β-Lactamases Directly from Perianal Swabs

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We developed and evaluated multiplexed molecular beacon probes in a real-time PCR assay to identify prominent extended-spectrum-β-lactamase, plasmid-mediated AmpC β-lactamase (pAmpC) and carbapenemase genes directly from perianal swab specimens within 6 h. We evaluated this assay on 158 perianal swabs collected from hematopoietic stem cell transplant recipients and found that this assay was highly sensitive and specific for detection of CTX-M-, pAmpC-, and KPC-producing *Enterobacteriaceae* compared to culture on chromogenic agar.

Enterobacteriaceae have accumulated an extensive array of β-lactamase genes encoding extended-spectrum-β-lactamases (ESBL), AmpC β-lactamases, and, more recently, carbapenemases. These enzymes typically render these organisms resistant to ceftriaxone and many other β-lactam agents. The repeated acquisition of resistance and the spread of these multidrug-resistant (MDR) bacteria present a global public health challenge, and infections due to these organisms are associated with inadequate empirical antimicrobial therapy and high mortality rates, particularly in immunocompromised hosts (1, 2). Patients with hematologic malignancies and hematopoietic stem cell transplant (HSCT) recipients who are colonized with ceftriaxone-resistant *Enterobacteriaceae* (CRO-R-E) in their gastrointestinal tract have a high risk of developing subsequent infection due to these organisms (1, 3, 4). Screening for gastrointestinal colonization with these bacteria may provide a guide to improved empirical antimicrobial therapy in these high-risk patient populations. Furthermore, combined with implementation of appropriate isolation precautions for colonized patients, gastrointestinal screening may limit the inpatient transmission of these organisms, particularly in an outbreak setting (3, 5).

Although stool samples are the gold standard for detecting enteric bacteria, perianal swabs are preferred for surveillance in an inpatient unit because they can be easily collected and processed. Current culture-based methods for detecting colonization with CRO-R-E typically take 2 to 3 days to yield results (6). This leads to delays in implementation of isolation precautions in colonized patients and in identification of patients that are potentially at high risk of infection caused by these pathogens. There clearly is a need for a molecular test that could be applied directly to perianal swabs and could rapidly identify within hours those patients colonized with *Enterobacteriaceae* expressing the most common β-lactamases. Furthermore, compared to culture-based diagnostic tests, a molecular test provides genetic information that can be used to better investigate potential transmission events and to guide therapeutic decisions.

In this study, we implemented multiplexed molecular beacon (MB) probes in a real-time PCR assay to identify the most prominent ESBL, plasmid-mediated AmpC β-lactamase, and carbapenemase genes. We then evaluated the performance characteristics of this test, compared to culture on selective chromogenic agar, applied directly to perianal swab specimens.

Perianal swabs were collected from HSCT recipients at New York-Presbyterian Hospital/Weill Cornell Medical Center on admission for transplantation and weekly thereafter between December 2013 and December 2015. These studies were approved by the Institutional Review Boards of Rutgers and Weill Cornell Medical College (Pro20140000185 and 1406015231, respectively). Both flocked BD Liquid Amies elution swabs (Becton, Dickinson, and Co., Sparks, MD) and nonflocked BBL CultureSwabs were used during the study. The swabs were plated directly onto HardyCHROM ESBL and carbapenem-resistant *Enterobacteriaceae* (CRE) agar plates (Hardy Diagnostics, Santa Maria, CA). Colonies identified on these plates after 24 to 48 h of incubation were subcultured onto sheep blood and MacConkey agar plates and reincubated overnight. Colonies that grew on sheep blood and MacConkey agar plates were identified, and antimicrobial susceptibility testing (AST) was performed to evaluate their phenotypic resistance patterns using a MicroScan WalkAway *plus* system (Diagnostic Division, Beckman Coulter Inc., Brea, CA).

All swabs were placed back into the Amies liquid and refrigerated at 4°C after being plated onto chromogenic agar. Each week, all swabs from the previous week from which a CRO-R-E strain was recovered on chromogenic agar were analyzed by a multiplex molecular beacon PCR assay. For each identified positive swab, we also collected and analyzed a negative-control swab from the same day that did not yield a CRO-R-E isolate. Investigators performing the molecular beacon PCR (MB-PCR) assay on swab samples were blinded to the culture results.

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TABLE 1 Primer and probe sequences for β-lactamase genes included in the MB-PCR assays

MB-PCR	Target	Primer or probe	Sequences (5′–3′)[a]	Length of target (bp)	Reference
1	KPC	KPC-F684	GGCAGTCGGAGACAAAACC	177	10
		KPC-R860	CCCTCGAGCGCGAGTCTA		
		KPC-MB	5′-QUASAR 670-cgcgatCCTATTGTGTTGGCCGTCTAatcgcg-BHQ2-3′		
	16S rRNA	BAC16S-F	TGGAGCATGTGGTTTAATTCGA	160	11
		BAC16S-R	TGCGGGACTTAACCCAACA		
		BAC16S-MB	5′-ROX-cgctggCGAGCTGACGACARCCATGCAccagcg-DABCYL-3′		
2	OXA-48	Oxa48(MB)-F	GCAAAGGAATGGCAAGAAAA	72	This study
		Oxa48(MB)-R	CACAACTACGCCCTGTGATTT		
		oxa48-MB	5′-CAL Fluor Red 610-cgcgatAGTTGGAATGCTCACTTTACTGatcgcg-BHQ2-3′		
	VIM	Vim(MB)a-F	TCTACCCGTCCAATGGTCTC	91	This study
		Vim(MB)a-R	AGAAGKGCCGCTGTGTTTTT		
		Vim-MB	5′-HEX-cgcgatTGTCCGTGATGGTGATGAGTTGatcgcg-BHQ1-3′		
	IMP	Imp(MB)-F	GGAATAGAGTGGCTTAAYTCTC	188	12
		Imp(MB)-R	CCAAACYACTASGTTATCT		
		Imp-MB	5′-QUASAR 670-cgcaggCCCACGTATGCATCTGAATTAACAAcctgcg-BHQ2-3′		
	NDM	NDM(MB)-F1	ATATCACCGTTGGGATCGAC	109	This study
		NDM(MB)-R1	TAGTGCTCAGTGTCGGCATC		
		NDM-MB	5′-FAM-cgcgaAAGGACAGCAAGGCCAAGTCGtcgcg-BHQ1-3′		
3		CTX-M(MB)-F2	TGCGGARAARCACGTYRAYGGSACRAT	116	This study
		CTX-M(MB)-R2	TAGCCGGVCCMCCVASVTGRGMAATCA		
	CTX-M-1 group	CTX-M-1-MB	5′-FAM-ccggaAGCGATAACGTGGCGATGAAtccgg-BHQ1-3′		
	CTX-M-2 group	CTX-M-2-MB	5′-HEX-cgcgtAGTATAGCGACAATACTGCCAacgcg-BHQ1-3′		
	CTX-M-9 group	CTX-M-9-MB	5′-CAL Fluor Red 610-gccgtAGCGACAATACcGCCATGAAacggc-BHQ2-3′		
		CTX-M-825-F	ATAACACCTTCCGGCTCGAT	85	This study
		CTX-M-825-R	GCTAATGGCGTGGTGGTATC		
	CTX-M-8/25 group	CTX-M-825-MB	5′-QUASAR 670-cggctTCAACACCGCGATCCCCGagccg-BHQ2-3′		
4	CMY1/MOX	CMY1MOX.MB-F	GCTGCTCAAGGAGCACAGGAT	91	This study
		CMY1MOX.MB-R	CCGRTYGGCCACMCCGTA		
		CMY1MOX-MB	5′-FAM-cgcgatAGGATGGCAAGGCCCACTATTTCatcgcg-BHQ1-3′		
	CMY2/LAT/CEF	CMY2LATCEF.MB-F	KCCGCMAAAACAGAACAACA	102	This study
		CMY2LATCEF.MB-R	GTAGATAAYSGCMACRGCCATA		
		CMY2LATCEF-MB	5′-HEX-cgcgaGGAGCAGGCTATTCCGGGtcgcg-BHQ1-3′		
	ACT/MIR	ACTMIR.MB-F	GTRCCGGATGAGGTCRMGGAT	100	This study
		ACTMIR.MB-R	TGGYRTTRGCGTAAAGACG		
		ACTMIR-MB	5′-CAL Fluor Red 610-cgcgatCTTTTATCAAAACTGGCAGCCatcgcg-BHQ2-3′		
	DHA	DHA.MB-F	ACCGCTGGGRTTATCTCACAC	95	This study
		DHA.MB-R	ACACGCGGACCGGTTTTT		
		DHA-MB1	5′-QUASAR 670-cgcgagGCAAAGCCAGTATGCGTACGGTTctcgcg-BHQ2-3′		
5	TEM (104K, 164S, 164H, 164C, 238S, 240K*)	blaTEM-F	GAGTATTCAACATTTCCGTGTCGC	856	This study
		blaTEM-R	TACCAATGCTTAATCAGTGAGGC		
		TEM104K-MB	5′-FAM-cgcgtACTTGGTTAAGTACTCACCacgcg-BHQ1-3′		
		TEM164R-MB	5′-HEX-cgcgatGCCTTGATCGTTGGGAACCGatcgcg-BHQ1-3′		
		TEM238S-MB	5′-CAL Fluor Red 610-cgcgatATCTGGAGCCAGTGAGCGTGGatcgcg-BHQ2-3′		
6	SHV (156D, 238S, 240K*)	blaSHV-F1	TGTGGTTATGCGTTATATTCGCCTGTGTAT	868	This study
		blaSHV-R	TTAGCGTTGCCAGTGCTCGATC		
		SHV156D-MB1	5′-QUASAR 670-gcgcaGCCAGATCGACGACAACGtgcgc-BHQ2-3′		
		SHV238240-MB1	5′-FAM-gcgcaGGAGCTAGCAAGCGGGGTtgcgc-BHQ1-3′		

[a] Molecular beacon hairpin sequences are shown in lowercase letters. ROX, rhodamine; HEX, hexachlorofluorescein; FAM, fluorescein; BHQ, black hole quencher; DABCYL, 4-(4′-dimethylaminophenylazo)benzoic acid.

The swabs (with the transport medium) were enriched in 1 ml of brain heart infusion (BHI) broth and incubated at 37°C for 3 h at 150 rpm. The enrichment culture was centrifuged at 10,000 rpm for 10 min, and the pellet was resuspended in 100 μl of distilled water (dH_2O). Genomic DNA was isolated using a boiling lysis method described previously (7, 8). A panel of target-specific molecular beacon probes in a real-time PCR assay (MB-PCR) was developed (9) and used to rapidly identify genes encoding carbapenemases, ESBLs, and plasmid-mediated AmpC-type β-lactamases (Table 1). MB-PCR-1 can detect *Klebsiella pneumoniae* carbapenemase (bla_{KPC}) and bacterial 16S rRNA genes. MB-PCR-2 and MB-PCR-3 target other important carbapenemase genes (bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}-like) and CTX-M-type ESBLs (CTX-M-1, -2, -9, and -8/25 groups), respectively. MB-

TABLE 2 Culture and isolate genotyping results of 77 perianal swabs that yielded a ceftriaxone-resistant *Enterobacteriaceae* isolate when plated to ESBL and CRE selective chromogenic agar[a]

Organism(s)	No. of isolates with indicated result						
	KPC	CTX-M (ESBL)	pAmpC		ESBL+pAmpC[b]	KPC+pAmpC[c]	Other/Unknown[d]
			CMY2LATCEF	ACT/MIR			
Escherichia coli ($n = 50$)	2	41	4		2		1
Klebsiella pneumoniae ($n = 8$)	2	3	2		1		1
Enterobacter cloacae ($n = 5$)				3		1	1
Klebsiella oxytoca ($n = 3$)	2						1
Citrobacter freundii ($n = 2$)			1			1	
Enterobacter aerogenes ($n = 1$)							1
Pantoea agglomerans ($n = 1$)			0			1	
Proteus mirabilis ($n = 1$)							
Polymicrobial ($n = 6$)	1	2	1		1	1	
Total	7	46	8	3	4	4	5

[a] Abbreviations: KPC, *Klebsiella pneumoniae* carbapenemase; ESBL, extended-spectrum β-lactamase; CRE, carbapenem-resistant *Enterobacteriaceae*; pAmpC, plasmid-mediated AmpC.
[b] CTX-M plus CMY2LATCEF ($n = 3$) and CTX-M plus ACT/MIR ($n = 1$).
[c] KPC plus CMY2LATCEF ($n = 3$) and KPC plus ACT/MIR ($n = 1$).
[d] No carbapenemases, ESBLs, or plasmid-mediated AmpCs detected ($n = 4$) and KPC plus ACT/MIR plus ESBL SHV ($n = 1$).

PCR-4 was used to detect the AmpC β-lactamases. Primers and molecular beacons were designed using a method described previously (Table 1) (7, 8). A Stratagene Mx3005P multiplex quantitative PCR system (Agilent Technologies, Santa Clara, CA) was used to perform real-time PCR amplification. PCR was performed in 10-μl reaction mixtures which included 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), a 250 μM concentration of each deoxynucleoside triphosphate (dNTP), 3 mM $MgCl_2$, a 0.5 μM concentration of each primer, and 1× PCR buffer (Applied Biosystems), and the concentration of molecular beacon probes was 0.2 μM. MB-PCR-1 to MB-PCR-4 were performed under the same conditions, which included an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 20 s (denaturation) and 58°C for 45 s (annealing and extension).

We developed a linear-after-the-exponential PCR (LATE-PCR) melting curve analysis in order to improve the performance for single-mutation-based SHV- and TEM-ESBL detection. Combinations of MB-PCR-5 and MB-PCR-6 using two and three molecular beacons were used to detect the most common SHV- and TEM-type ESBLs, respectively, by targeting several nonsynonymous mutations in each enzyme (SHV156D, SHV238S and SHV240K, TEM104K, TEM164S, TEM164H, TEM238S, and TEM240K). Primers were designed using a protocol published previously (Table 1) (13, 14). PCR was performed in 15-μl reaction mixtures consisting 50 nmol/liter limiting primer (forward), 1 mmol/liter excess primer (reverse), a 200 nmol/liter concentration of each modular beacon, 0.08 U/ml Platinum *Taq* DNA polymerase (Life Technologies Invitrogen, Carlsbad, CA), 1× Platinum *Taq* reaction buffer, and 2.5 mmol/liter $MgCl_2$. The PCR amplification program included an initial denaturation at 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C 60 s, using a Stratagene Mx3005P quantitative PCR system. The temperature was subsequently decreased from 95°C to 25°C, then held at 25°C for 5 min, and then increased to 95°C in 1.0°C steps (again holding for 30 s at each step and detecting fluorescence), with three detections performed at the endpoint of each step.

After the MB-PCR assay was applied to the swabs, the corresponding CRO-R-E species that had been identified on ESBL and CRE chromogenic agars were subjected to molecular testing (PCR/sequencing). The full lengths of the genes in MB-PCR panels were further PCR amplified using previously published primers (15–17), followed by Sanger sequencing. The results from the MB-PCR assay applied directly to the swab specimens were compared to the genotyping results from CRO-R-E isolates recovered on chromogenic agar.

A total of 158 perianal swabs (including 77 that yielded a CRO-R-E isolate by culture and 81 negative controls) collected from 106 different patients were analyzed by culture on chromogenic agar and by the MB-PCR assay. Forty-three of these swabs were flocked swabs, and 115 were nonflocked swabs. The most common CRO-R-E species identified by culture were *Escherichia coli* ($n = 50$), *Klebsiella pneumoniae* ($n = 8$), *Enterobacter cloacae* ($n = 5$), and *Klebsiella oxytoca* ($n = 3$) (Table 2). Six perianal swabs were polymicrobial, yielding two or more CRO-R-E isolates. CTX-M production was the most common mechanism of ceftriaxone resistance. However, many isolates possessed bla_{KPC} or plasmid-mediated AmpC β-lactamases (pAmpC) or combinations of these β-lactamases. We did not identify any ESBL TEMs or ESBL SHVs. In addition to being CRO resistant, 73% of CTX-M- or pAmpC-producing *Enterobacteriaceae* isolates were cefepime resistant, 83% were ceftazidime resistant, and 18% were meropenem resistant. Ten of the 12 KPC-producing *Enterobacteriaceae* isolates were meropenem resistant.

The MB-PCR assay, with a turnaround time of <6 h, identified CTX-M in 49 of 50 swabs that yielded a CTX-M-producing *Enterobacteriaceae* isolate in culture (98% sensitivity; Table 3), identified KPC in 11 of 12 swabs that yielded a KPC-producing *Enterobacteriaceae* isolate in culture (92% sensitivity), identified ACT/MIR in 5 of 6 swabs that yielded an ACT/MIR-producing *Enterobacteriaceae* isolate in culture (83% sensitivity), and identified CMY-II/LAT/CEF in all 13 swabs that yielded a CMY-II/LAT/CEF-producing *Enterobacteriaceae* isolate in culture (100% sensitivity). Importantly, the MB-PCR assay did not detect KPC in the 146 swabs that did not yield a KPC-producing *Enterobacteriaceae* isolate in culture (100% specificity; Table 3). The MB-PCR assay identified CTX-M in 5 of 103 swabs that did not yield a CTX-M-producing *Enterobacteriaceae* isolate in culture (95% specificity),

TABLE 3 Performance of the direct PCR of the perianal swab compared to the gold standard of PCR of bacteria isolated from chromogenic agar

Result from direct PCR from swab	No. of swabs with indicated result from PCR of bacteria isolated from chromogenic agar[a]							
	KPC-pos.	KPC-neg.	CTX-M-pos.	CTX-M-neg.	CMY2-LATCEF-pos.	CMY2-LATCEF-neg.	ACT/MIR-pos.	ACT/MIR-neg.
KPC-pos.[b]	11	0						
KPC-neg.	1	146						
CTX-M-pos.[c]			49	5				
CTX-M-neg.			1	103				
CMY2LATCEF-pos.[d]					13	8		
CMY2LATCEF-neg.					0	137		
ACT/MIR-pos.							5	9
ACT/MIR-neg.							1	143

[a] pos., positive; neg., negative.
[b] The sensitivity of the direct PCR from the swab to detect KPC-producing *Enterobacteriaceae* was 92% (95% confidence interval [CI]: 61% to 100%), and the specificity was 100% (95% CI: 98% to 100%).
[c] The sensitivity of the direct PCR from the swab to detect CTX-M-producing *Enterobacteriaceae* was 98% (95% CI: 89% to 100%), and the specificity was 95% (95% CI: 89% to 98%).
[d] The sensitivity of the direct PCR from the swab to detect plasmid-mediated-AmpC-producing *Enterobacteriaceae* (CMY2-LATCEF or ACT/MIR) was 95% (95% CI: 74% to 100%), and the specificity was 91% (95% CI: 86% to 95%).

identified CMY-II/LAT/CEF in 8 of 137 swabs that did not yield a CMY-II/LAT/CEF-producing *Enterobacteriaceae* isolate in culture (94% specificity), and identified ACT/MIR in 9 of 143 swabs that did not yield an ACT/MIR-producing *Enterobacteriaceae* isolate in culture (94% specificity). The sensitivities of the MB-PCR assay for detection of CTX-M (100% versus 97%)-, KPC (100% versus 90%)-, and CMY-II/LAT/CEF (100% versus 100%)-producing *Enterobacteriaceae* isolates in flocked and nonflocked swabs were similar. Some of the genes included in the assay (e.g., bla_{NDM} and bla_{DHA}) were not present in the isolates; therefore, we could not evaluate the sensitivities and specificities of this assay for detection of these genes.

We demonstrated that a multiplex MB-PCR assay with a turnaround time of <6 h is a sensitive and specific tool for detection of *Enterobacteriaceae* with a broad range of β-lactamases directly from perianal swabs. Previous studies focused on using multiplex PCR platforms to directly detect carbapenemases from perianal swabs (18–21). Our assay demonstrated excellent sensitivity for the detection of not only carbapenemase genes but also other predominant resistance genes responsible for CRO-R-E, including CTX-M, and plasmid-mediated AmpC β-lactamases. Furthermore, the assay had >90% specificity for detection of all resistance mechanisms. The false positivity for the MB-PCR might have been due to the fact that real-time PCR-based tests are more sensitive than culture-based methods and that a small number of bacteria harboring these β-lactamase genes may have been present in the swab samples but were missed by the cultures. This might have been a particular issue in this analysis, as some studies have suggested that direct plating onto chromogenic agar without broth enrichment may have suboptimal sensitivity for detecting CRE (22, 23). Furthermore, it is possible that the MB-PCR assay detected AmpC β-lactamase genes that were located on chromosomes of colonizing strains of *Enterobacter* and *Citrobacter* spp. but that these genes were not sufficiently expressed to confer cephalosporin resistance and growth on selective chromogenic agar.

Use of this assay in real time, or if batched and run daily, would allow same-day detection of colonization with CRO-resistant *Enterobacteriaceae*, as opposed to waiting for 2 to 3 days using culture-based methods. Importantly, up to 20 samples can be run at one time with this assay, making it convenient for clinical laboratories to batch and efficiently test samples daily. Furthermore, unlike culture-based tests, this assay provides rapid information on the mechanisms of resistance. An additional feature of this multiplex MB-PCR assay is its flexibility with respect to adding and/or modifying genetic targets, thus adjusting to evolving changes and differences in the local epidemiology. For example, a MB-PCR assay with targets for CTX-M, KPC, and the ACT/MIR and CMY-II/LAT/CEF AmpC β-lactamases would suffice for detecting MDR-E colonization on the HSCT unit at the study institution. However, for other areas of the world, detection of NDM-, VIM-, or OXA-48-type β-lactamases might be more important.

In summary, our findings demonstrate that a MB-PCR assay can be applied directly to perianal swabs to rapidly and accurately detect colonization with *Enterobacteriaceae* with a broad range of β-lactamases. By more rapidly identifying patients colonized with CRO-R-E, use of this assay in an active surveillance program, in lieu of culture-based detection methods, could allow earlier implementation of isolation precautions in colonized patients, which could potentially lead to decreased nosocomial transmission of these organisms. Furthermore, this assay could potentially be used to guide empirical therapy in patients at high risk of CRO-R-E bacteremia, especially patients with hematologic malignancies and HSCT recipients.

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