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Development of a TaqMan Multiplex PCR Assay for Detection of Plasmid-Mediated AmpC β-Lactamase Genes

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A multiplex, real-time TaqMan assay was designed to identify clinical isolates carrying plasmid-mediated *ampC* genes. The specificity and sensitivity of this assay were 100% when testing characterized AmpC/non-AmpC-producing isolates and randomly selected clinical isolates. This is a rapid assay that can be performed in a clinical microbiology laboratory.

Antibiotic resistance is a global health crisis. There are at least two approaches to address this problem. One way is the design and use of novel therapeutic drug classes to treat infections caused by resistant pathogens. A second approach is the development and implementation of novel surveillance techniques in which to identify not only the pathogen but the resistance mechanisms employed by these organisms. Implementation of molecular-based surveillance techniques is the most immediate response to this crisis and will increase the speed and accuracy of detecting resistance, which is important for both infection control and therapeutic options in hospital and community settings.

The most common Gram-negative resistance mechanism associated with β-lactams is the production of inactivating β-lactamases, including extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpCs, and the *Klebsiella pneumoniae* carbapenemases (KPCs) (6, 12, 13, 14). *K. pneumoniae*, *Escherichia coli*, and *Salmonella* spp. are the most common organisms that produce plasmid-mediated AmpCs. Genes encoding AmpCs are derived from the chromosomal *ampC* genes of various members of the *Enterobacteriaceae* family, including *Enterobacter cloacae* and *Enterobacter asburiae*, *Citrobacter freundii*, *Morganella morganii*, *Aeromonas sobria*, *Aeromonas hydrophila*, and *Hafnia alvei* (1, 3, 4, 5, 7, 8, 9, 10, 14, 16).

Production of plasmid-mediated AmpCs in Gram-negative organisms is clinically important because of their ability to confer resistance to broad-spectrum penicillins, broad/extended-spectrum cephalosporins, monobactams, and the cephamycins (3, 4, 5, 9, 14). In addition, the presence of plasmid-mediated AmpCs can mask the phenotypic detection of ESBLs and KPC-producing organisms, which can hinder surveillance and infection control practices (8, 11, 13, 14, 17). An additional concern is that plasmid-mediated AmpCs are frequently associated with false susceptibility to the cephalosporins in routine susceptibility testing, which increases the risk of therapeutic failure (14). However, given these concerns, there are no guidelines set forth by the CLSI to help clinical microbiologists identify these types of organisms.

Modifications to a previously designed endpoint AmpC multiplex PCR has allowed us to develop a real-time multiplex PCR assay using TaqMan probes for the detection of plasmid-mediated AmpC β-lactamase genes, which allows ease of implementation into the clinical laboratory (9). The primers and TaqMan probes used for amplification were designed with Beacon Designer 7 software and presented in Table 1. BLAST analysis using sequences submitted to GenBank was used to evaluate the ability of the primer/probe combinations to anneal to target gene variants. All of the primer/probe sequences annealed with 100% specificity to the target gene variants listed in Table 1. TaqMan probes specific for each *ampC* product and ribosomal DNA were labeled 5′ with 6-carboxyfluorescein (FAM) and hexachlorofluorescein (HEX) fluorescent dyes, respectively. Fluorophores attached 3′ included black hole quencher-1 and Iowa black FQ, respectively. Real-time multiplex PCR was performed using the Rotor-Gene Q (Qiagen, Valencia, CA) system with fluorescence acquisition in the green channel to detect *ampC* amplification (FAM) and in the yellow channel to detect 16S ribosomal DNA amplification (HEX) as a control for DNA integrity.

Test or positive-control organisms were cultured as previously described (15). Total DNA was extracted from an overnight culture using the DNeasy blood and tissue kit (Qiagen). Multiplex PCR was performed using a 50-μl final reaction volume. Each PCR mixture contained a 1× final concentration of QuantiTect multiplex buffer (Qiagen); 100 μM primers CMY2-F1 4P1, CMY2-R1 4P1, ACT-F1 4P1, ACT-R1 4P1, DHA-F3 4P1, DHA-R3 4P1, MOX-F1 4P1, MOX-R1 4P1, ACC-F2 4P1, ACC-R2 4P1, 16srRNAEcKp-F1, and 16srRNAEcKp-R1; 7.5 μM CMY2-Taqprobe 4P1 and FOX1-Taqprobe 4P1; 5 μM ACT-Taqprobe 4P1; 1.25 μM DHA3-Taqprobe 4P1; 1 μM 16srRNAEcKp-probe; 0.625 μM MOX-Taqprobe 4P1 and ACC2-Taqprobe 4P1 (Table 1). Template DNA (2 μl of eluate, ~250 ng) was added to 48 μl of the master mix. The PCR conditions consisted of an initial denaturation step at 95°C for 15 min for HotStar *Taq* polymerase activation. Two-step cycling conditions followed and included 40 cycles of denaturation at 95°C for 1 min and primer/probe binding and primer extension at 55°C for 1 min. No template controls contained sterile nanopure water in place of template DNA.

The ability of the seven designed primer/probe combinations to anneal to target genes was tested first using a constructed panel of 6 *E. coli* transformants housing each plasmid-mediated *ampC* target and the conditions described above. Each sigmoidal curve in Fig. 1A represents amplification of one of the 6 families of plasmid-mediated

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TABLE 1 Primers and TaqMan probe sequences used for amplification

Target(s)[a]	Primer	Sequence (5′ to 3′)	Expected amplicon size (bp)	Nucleotide positions	GenBank accession no.
MOX-1 to MOX-7, CMY1, CMY8 to CMY11	MOX-F1 4P1	AGACCCTGTTCGAGATAG	148	242–259	AF373217
	MOX-R1 4P1	ATGGTGATGCTGTCAAAG		389–372	
	MOX-Taqprobe 4P1	5′-6-FAM-CGTGAGCAAGACCCTGACTG-3′ BHQ 1		264–283	
FOX-1 to FOX-8	FOX-F1 4P1	ACATATTTCAACTATGGGGTT	148	881–898	X77455
	FOX-R1 4P1	TTGTCATCCAGCTCAAAG		1026–1009	
	FOX1-Taqprobe 4P1	5′-6-FAM-TGACCGCAGCATAGGCAC-3′ BHQ 1		1001–984	
CMY2, 4, 6, 7, 14–16, 18, 22, 25–44, 49, 53–56, 59	CMY2-F1 4P1	TCCAGCGTTATTGATATGG	147	733–751	HM565135
	CMY2-R1 4P1	CATCTCCCAGCCTAATCC		879–862	
	CMY2-Taqprobe 4P1	5′-6-FAM-ACATATCGCCAATACGCCAGT-3′ BHQ 1		856–836	
DHA-1, DHA-6, DHA-7	DHA-F3 4P1	TTATCTCACACCTTTATTACTG	139	469–490	EF078892
	DHA-R3 4P1	TATCTTTTGAGGCGGATT		607–590	
	DHA3-Taqprobe 4P1	5′-6-FAM-CCGTAAGATTCCGCATCAAGC-3′ BHQ 1		584–564	
ACT-1, ACT-2, ACT-5, ACT-8, MIR-1 to MIR-4	ACT-F1 4P1	GTGGCGGTGATTTATGAG	125	178–195	U58495
	ACT-R1 4P1	CCGGTGAAGGTTTTACTT		302–285	
	ACT-Taqprobe 4P1	5′-6-FAM-CAGCCGCACTACTTCACCT-3′ BHQ 1		199–217	
ACC-1	ACC-F2 4P1	CGCTGATGCAGAAGAATA	86	771–788	AJ133121
	ACC-R2 4P1	CGCTAACCCATAGTTATAAATG		856–835	
	ACC2-Taqprobe 4P1	5′-6-FAM-TCACTGCGACCGACATACCG-3′ BHQ 1		815–796	
16S rRNA in *E. coli*, *Klebsiella* spp., and *Salmonella* spp.	16srRNAEcKp-F1	GAGAGGATGACCAGCCACAC	55		
	16srRNAEcKp-R1	CGCCATTGTGCAATATTCC			
	16srRNAEcKp-probe	5′-HEX-TGAGACACGGTCCAGACTCCTACGG-3′ Iowa Black FQ			

[a] Plasmid-mediated *ampC* genes detected.

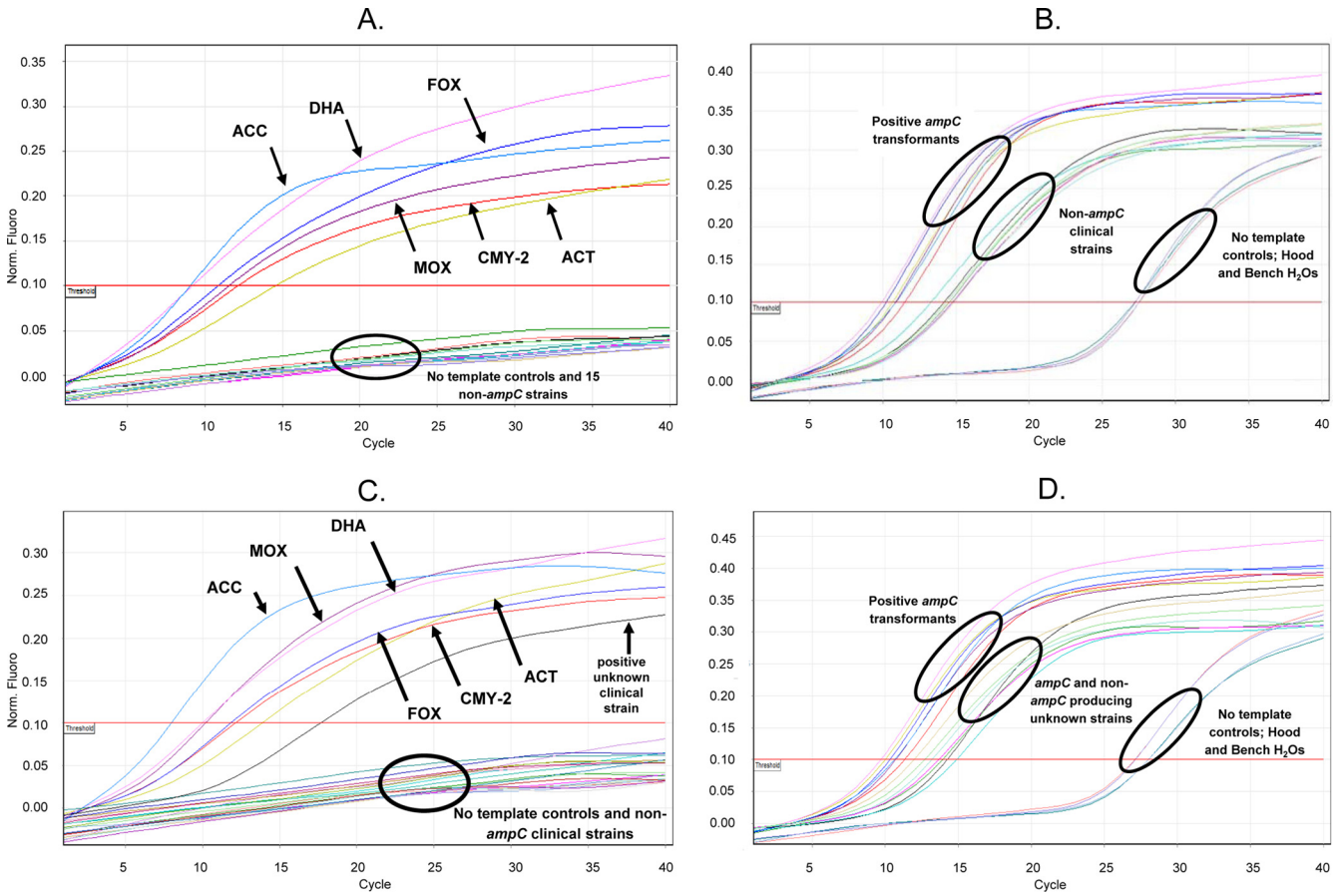

FIG 1 Evaluation of *ampC* TaqMan multiplex PCR assay. (A and B) Detection and FAM fluorescence of AmpC transformant controls (A) and HEX fluorescence of the 16S ribosomal internal control (B) for AmpC transformants and non-AmpC-producing clinical strains tested in panel A. (C and D) FAM fluorescence of positive AmpC transformant controls and an unknown clinical isolate from CUMC (C) and HEX fluorescence of the 16S ribosomal internal control for those strains tested in panel C (D). Arrows indicate the specific *ampC* transformant control or unknown clinical isolate detected; circles indicate the group of strains tested or no template controls.

TABLE 2 Previously characterized strains containing a plasmid-mediated AmpC β-lactamase

Strain no.	Organism	AmpC type
Vitek901664	*K. pneumoniae*	FOX
HVAMC39	*K. pneumoniae*	ACT-1
01HNH5	*K. pneumoniae*	ACT-1
V110977	*K. pneumoniae*	CMY-2-like[a]
Ecoli226	*E. coli*	CMY-2-like[a]
V110963	*E. coli*	CMY-2-like[a]
Misc 345	*E. coli*	CMY-2
01CSHS31	*E. coli*	CMY-2-like[a]
Ecoli264	*E. coli*	CMY-2-like[a]
UMJMH14	*K. pneumoniae*	DHA-1
Misc340	*K. pneumoniae*	FOX-1
CCF52	*K. pneumoniae*	FOX-5
MHM2	*K. pneumoniae*	FOX-5
NSLIJ26	*K. pneumoniae*	FOX-5
UL3	*K. pneumoniae*	FOX-5
UMJMH21	*K. pneumoniae*	FOX-5
UMM4	*K. pneumoniae*	FOX-5
UN47	*K. pneumoniae*	FOX-5
01CMH13	*K. pneumoniae*	FOX-5
01VUMM451	*K. pneumoniae*	DHA-1-like[a]
Sal100	*Salmonella* spp.	CMY-2
Sal358	*Salmonella* spp.	CMY-2
Sal362	*Salmonella* spp.	CMY-2
Sal365	*Salmonella* spp.	CMY-2
Sal377	*Salmonella* spp.	CMY-2
Misc 304	*K. pneumoniae*	MIR-1

[a] Not sequenced.

ampC genes detected by fluorescence of FAM. To confirm that a specific primer pair amplified only one target of the predicted size, a confirmatory agarose gel was used to visualize the multiplex PCR products (data not shown). The internal control (55 bp) amplified in all isolates regardless of the presence of an *ampC* gene (Fig. 1B and D). The specificity of the AmpC primer/probe pairs and internal control for all transformants tested was 100%. The designed primer/probe combinations for this real-time assay did not cross-hybridize with the chromosomal *ampC* gene in the tested *E. coli* transformants (1, 2, 3, 9, 15). This assay did detect primer dimer formation in the no-template controls of which fluorescence was observed at 26 to 28 cycles (Fig. 1B and D).

Previously characterized strains listed in Table 2 carrying plasmid-mediated *ampC* genes were tested and positively identified using the optimized multiplex real-time PCR assay. Five AmpC-producing *Salmonella* strains were also tested in this assay (data not shown). An additional panel of 109 *ampC*-negative but KPC ($n = 14$)-, CTX-M ($n = 52$)-, and TEM/SHV ($n = 43$)-producing strains plus five *ampC*-negative *Salmonella* strains were evaluated. No FAM fluorescence was detected in the *ampC*-negative strains. However, the HEX-labeled probe for ribosomal gene detection was positive for PCR amplification in all the strains tested, indicating that the DNA extracted was of good quality and capable of being amplified and detected (Fig. 1B and D). The specificity and sensitivity of this assay when evaluating the characterized non-AmpC- and AmpC-producing isolates were 100%. These data were validated using the endpoint AmpC multiplex PCR described as the gold standard for plasmid-mediated *ampC* detection (9, 15). Using the endpoint assay, the AmpC-producing strains amplified the expected *ampC* gene, whereas the strains producing other β-lactamases were negative.

A total of 120 clinical isolates comprised of *K. pneumoniae*, *Klebsiella oxytoca*, and *E. coli* isolates were randomly collected from the clinical laboratory at Creighton University Medical Center (CUMC) and evaluated for susceptibility using disc diffusion and tested for the presence of plasmid-mediated *ampC* genes using this real-time assay (see Table S1 in the supplemental material). Of the 120 unknown isolates, 4% (5/120) were identified as plasmid-mediated AmpC producers. Three of five positive AmpC-producing isolates exhibited resistance to the third-generation cephalosporins but remained susceptible to cefepime. The remaining two *ampC*-positive isolates displayed susceptibility to the cephalosporins. A representation of a positive clinical isolate is shown in Fig. 1C. These studies were also validated using the endpoint AmpC multiplex assay to confirm the presence or absence of an *ampC* gene.

Currently, there is no recommendation by the CLSI for the detection of plasmid-mediated AmpC-producing strains. A recently written review by George Jacoby indicates the gold standard for detecting these types of isolates is the endpoint *ampC* multiplex developed by our laboratory in 2002 (9). Data from the literature indicates a clinical responsibility to identify patients infected by organisms that produce a plasmid-mediated AmpC (13, 14). The real-time TaqMan multiplex PCR assay described in this report provides a fast and easy-to-use tool to screen for plasmid-mediated AmpC genes: a resistance mechanism that can be difficult to discern phenotypically and can result in a poor clinical outcome when undetected.

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