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Multiplex Real-Time PCR for Detection of an Epidemic KPC-Producing *Klebsiella pneumoniae* ST258 Clone

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We describe a multiplex real-time PCR assay capable of identifying both the epidemic *Klebsiella pneumoniae* ST258 clone and bla_{KPC} carbapenemase genes in a single reaction. The assay displayed excellent sensitivity (100%) and specificity (100%) for identification of ST258 clone and bla_{KPC} in a collection of 75 *K. pneumoniae* isolates comprising 41 sequence types. Our results suggest that this assay is an effective tool for surveillance of this clone among carbapenem-resistant *K. pneumoniae* clinical isolates.

Carbapenem-resistant members of the *Enterobacteriaceae* (CRE) are rapidly emerging as a significant international public health problem (12). The most common mechanism of carbapenem resistance among *Enterobacteriaceae* in the United States is production of *Klebsiella pneumoniae* carbapenemases (KPC). Since their initial identification in 1996 (15), KPC producers have spread globally, becoming endemic in the eastern United States, Puerto Rico, Colombia, Greece, Israel, and eastern China (11, 12). Although KPCs have been found in numerous *K. pneumoniae* clones as well as in other species, most cases have been associated with a single *K. pneumoniae* clone, multilocus sequence type 258 (ST258) (1, 2, 5, 9, 12). Accordingly, rapid identification of this clone is of great importance for tracking further dissemination of KPCs, as well as informing infection prevention measures directed against this emerging public health threat. Here, we describe a multiplex real-time PCR assay which can identify both bla_{KPC} (the genetic determinant of KPC) and *K. pneumoniae* ST258 in a single reaction.

Multilocus sequence typing (MLST) of *K. pneumoniae* involves sequencing of defined regions in seven housekeeping genes, namely, *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* (6). Analysis of the *K. pneumoniae* MLST database (www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) revealed that strains typed as ST258 harbor a unique *tonB* allele (*tonB79*), which thus far is found only in ST258, along with various single-locus (ST379, ST418, ST512, ST554, and ST744) and double-locus (ST650, ST683, and ST745) variants of ST258. In this study, the group of genetically related strains possessing a *tonB79* allele are tentatively referred to as the ST258-*tonB79* cluster. The specificity of the *tonB79* allele provides a unique opportunity for developing a rapid molecular assay to identify and distinguish this emerging clonal type on the basis of two single-nucleotide polymorphisms (SNPs). The design was based on *tonB* allele sequences available on the Institut Pasteur MLST website, including all STs available prior to 2012 (851 STs in total). At nucleotide (nt) 118 of *tonB79*, there is a cytosine (C)-to-thymine (T) substitution which is present in only three *tonB* alleles (*tonB79*, *tonB16*, and *tonB138*) (Table 1). Similarly, there is an adenine (A)-to-cytosine (C) substitution at nt 297, which is shared by 76 of 180 *tonB* alleles but not the aforementioned alleles *tonB16* and *tonB138* (Table 1). Among all *K. pneumoniae* STs, only members of the ST258-*tonB79* cluster (ST258, -379, -418, -512, -554, -744, -650, -683, and -745) possess both SNPs.

Two molecular-beacon probes targeting the C118T and A297C substitutions were designed (Table 1) in order to test whether the combination of these probes (*tonB*-118T-MB and *tonB*-297C-MB) could specifically identify STs in the ST258-*tonB79* cluster. Concurrently, a separate set of primers and probes was developed in order to simultaneously detect all known bla_{KPC} gene variants (bla_{KPC-2} to bla_{KPC-13}). An internal positive-control probe was incorporated into the assay in order to confirm DNA template quality and PCR efficiency, using a conserved region from another MLST gene (*gapA*) as a target (Table 1).

Molecular-beacon design and DNA template isolation were performed using a method described elsewhere (4). Real-time PCR amplification was performed using the Stratagene Mx3005P multiplex quantitative PCR system (Agilent Technologies, Santa Clara, CA). PCR was performed with 10-μl reaction mixtures consisting of 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 3 mM $MgCl_2$, a 250 μM concentration of each deoxynucleoside triphosphate (dNTP), and a 0.5 μM concentration of each primer in 1× PCR buffer (Applied Biosystems). Molecular-beacon probe concentrations were 0.4 μM for *tonB*-118T-MB and *tonB*-297C-MB and 0.2 μM for *gapA*-MB and KPC-MB. Optimal cycling conditions included an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 20 s (denaturation) and 64°C for 45 s (annealing and extension). The

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TABLE 1 Oligonucleotide primers and molecular beacon probes used in this study

Primer or probe[a]	Sequence (5′–3′)[b]	Length (bp)	Targets[c]
tonB79-118T-F	GAGCCGGAGCCAGAGGTAG	77	*tonB79*, *tonB16*, and *tonB138*
tonB79-118T-R	TTCGGCTTAGGTTCCGGTTT		
tonB-118T-MB	5′- FAM-cgcgatAAGAGGCGTCGGTGGTGAatcgcg-BHQ-1-3′		
tonB79-297C-F1	CTCGCCGTTTGAAAACAACA	87	*tonB79*, *tonB2*, *tonB4*, *tonB6*, *tonB10*, etc., but not *tonB16* or *tonB138*
tonB79-297C-R1	GGAGCAGTAACGGTGGGTTT		
tonB-297C-MB	5′- CAL Fluor Red 610-cgcgaACCTCGACCGCAGCGtcgcg- BHQ-2-3′		
gapA-F	CGAAACCGCTCGTAAACACA	140	All *K. pneumoniae gapA* alleles
gapA-R	AGGAAGCGTTGGAAACGATG		
gapA-MB	5′-HEX-cgcgatGTTCTGACTGGCCCGTCCAAAGAatcgcg-BHQ-1-3′		
KPC-F684	GGCAGTCGGAGACAAAACC	177	bla_{KPC-2} to bla_{KPC-13}
KPC-R860	CCCTCGAGCGCGAGTCTA		
KPC-MB	5′- QUASAR 670-cgcgatCCTATTGTGTTGGCCGTCTAatcgcg-BHQ-2-3′		

[a] Primers KPC-F684 and KPC-R860 were published previously (4).
[b] Molecular beacon hairpin sequences are shown in lowercase letters; single nucleotide polymorphism target positions are underlined. FAM, fluorescein; HEX, hexachlorofluorescein; BHQ, black hole quencher.
[c] Probe *tonB*-297C-MB targets 76 of 180 *tonB* alleles in the *K. pneumoniae* MLST database (www.pasteur.fr), including alleles *2*, *4*, *6*, *10*, *11*, *12*, *14*, *17*, *18*, *19*, *21*, *23*, *25*, *27*, *28*, *30*, *32*, *34*, *35*, *36*, *37*, *39*, *41*, *42*, *43*, *46*, *54*, *56*, *57*, *59*, *60*, *61*, *62*, *64*, *66*, *68*, *69*, *70*, *76*, *77*, *79*, *81*, *83*, *86*, *87*, *88*, *89*, *92*, *100*, *107*, *109*, *110*, *111*, *112*, *113*, *116*, *117*, *120*, *123*, *127*, *129*, *135*, *136*, *137*, *139*, *140*, *141*, *143*, *144*, *157*, *160*, *161*, *168*, *170*, *174*, and *177*.

entire MB-PCR can be performed in less than 1.5 h on the Mx3005P platform.

Analytical sensitivity was evaluated using synthetic bla_{KPC} (bla_{KPC-2} to bla_{KPC-11}) targets and bacterial dilutions, as described in a previous study (4). Lower limits of detection based on DNA copy number were reliably detected within 40 cycles as follows: 20 copies per PCR for probes *gapA*-MB and KPC-MB and 40 copies for *tonB*-118T-MB and *tonB*-297C-MB. Limits of detection based on bacterial CFU were also estimated using DNA isolated from serial dilutions of bacterial cultures. The multiplex assay was capable of reproducibly detecting 2 CFU per PCR for *gapA*-MB and KPC-MB and 4 CFU per PCR for *tonB*-118T-MB and *tonB*-297C-MB. The overall detection limit of the MB-PCR assay was therefore approximately 4 CFU per reaction, close to the limits previously described (1 to 2 CFU) for various real-time PCR bla_{KPC} assays (7, 8, 13), thereby suggesting good analytical sensitivity.

The multiplex real-time PCR assay was initially validated using 75 *K. pneumoniae* clinical isolates comprising a wide variety of STs, selected from our strain collection at the Public Health Research Institute (PHRI) Tuberculosis Center (Table 2). The STs of all 75 isolates were determined using the protocol described on the Institut Pasteur website (6) and were found to comprise 41 distinct STs (Table 2), 20 of which were ST258. All ST258 isolates were positive for both the *tonB*-118T-MB and *tonB*-297C-MB targets by multiplex PCR, while non-ST258 isolates were either positive for a single *tonB79* target or negative for both (Table 2), demonstrating that the assay can specifically identify ST258 *tonB79* cluster members. Within the 75 isolates tested, bla_{KPC} was detected in 39 isolates from 15 different STs (Table 2), showing that KPC is not restricted to a single clone, as has been suggested previously (3). All of the bla_{KPC} genes detected in this study were confirmed using a previously described multiplex real-time PCR (4) as well as a conventional PCR method (14), with results showing 100% concordance. All of the isolates were positive for the *gapA*-MB internal control target. Overall, our assay demonstrated excellent sensitivity (100%) and specificity (100%) for the identification of both bla_{KPC} genes and the ST258-*tonB79* cluster in a collection of 75 *K. pneumoniae* isolates with different genetic backgrounds.

Following validation of the multiplex PCR assay, an additional 248 carbapenem-resistant clinical isolates from 5 hospitals in New York and New Jersey were also screened for bla_{KPC} and ST258-*tonB79* cluster inclusion (Table 3). As part of an ongoing surveillance project, a number of hospitals in our region routinely submit carbapenem-resistant *Enterobacteriaceae* to the PHRI Tuberculosis Center laboratory. During 2010-2011, a total of 248 unique carbapenem-resistant *K. pneumoniae* isolates were submitted by the clinical microbiology laboratories from the 5 hospitals. All of the isolates were resistant to at least one carbapenem, based on automated susceptibility testing (Vitek 2 or MicroScan) or Etest (bioMérieux). Data from this study indicated that 85% of these isolates belonged to the ST258-*tonB79* cluster ($n = 212$), while 94% harbored bla_{KPC} genes ($n = 234$). Twenty-two of the ST258-*tonB79* cluster isolates (10%) were randomly selected and typed by MLST. The results confirmed that all 22 isolates were ST258, in agreement with the real-time PCR results; no other ST258-*tonB79* cluster members were identified. Among KPC-producing isolates, 88% belonged to the ST258-*tonB79* cluster ($n = 206$). Overall, KPC-producing ST258-related strains accounted for 83% of this collection of carbapenem-resistant *K. pneumoniae* clinical isolates. These results suggest that although KPC has spread throughout different lineages, ST258 remains the predominant KPC-producing *K. pneumoniae* clone in the New York–New Jersey region. The distribution of ST258 among such a large hospitalized population from a sizeable population base has significant implications for the containment and therapy of this clone, especially regarding the potential to develop resistance to other antimicrobial agents, such as colistin (10).

In summary, we have described a novel multiplex real-time

TABLE 2 Multiplex real-time PCR results for 75 *K. pneumoniae* isolates with different sequence types

ST	Allelic profile[a]	*n*	PCR result with:			No. of KPC-MB-positive isolates[b]
			tonB-118T-MB	*tonB*-297C-MB	*gapA*-MB	
258	3-3-1-1-1-1-79	20	+	+	+	17
37	2-9-2-1-13-1-16	2	+	−	+	1
556	2-1-1-26-12-15-138	1	+	−	+	0
8	4-1-1-1-1-5-6	1	−	+	+	0
13	2-3-1-1-10-1-19	1	−	+	+	0
16	2-1-2-1-4-4-4	5	−	+	+	5
17	2-1-1-1-4-4-4	3	−	+	+	2
20	2-3-1-1-4-4-4	2	−	+	+	0
23	2-1-1-1-9-4-12	1	−	+	+	0
29	2-3-2-2-6-4-4	2	−	+	+	0
39	2-1-2-4-9-1-14	1	−	+	+	1
45	2-1-1-6-7-1-12	1	−	+	+	0
54	2-1-1-13-16-15-4	2	−	+	+	0
70	2-6-17-1-20-10-25	1	−	+	+	0
86	9-4-2-1-1-1-27	1	−	+	+	0
101	2-6-1-5-4-1-6	1	−	+	+	1
111	2-1-5-1-17-4-42	1	−	+	+	0
133	12-1-1-2-5-1-36	1	−	+	+	0
134	3-1-2-1-1-1-4	1	−	+	+	0
193	2-3-2-2-48-4-4	1	−	+	+	1
268	2-1-2-1-7-1-81	1	−	+	+	0
309	2-9-2-1-13-1-10	1	−	+	+	0
326	1-1-1-1-1-1-64	1	−	+	+	0
327	2-1-1-1-10-1-19	1	−	+	+	0
340	3-3-1-1-1-1-18	1	−	+	+	0
359	10-1-2-1-9-27-6	1	−	+	+	0
380	2-1-1-1-1-4-19	1	−	+	+	0
422	2-3-1-1-4-4-111	1	−	+	+	0
429	2-1-2-1-9-1-116	1	−	+	+	1
515	2-1-1-1-1-1-4	1	−	+	+	0
517	10-1-2-1-9-1-4	1	−	+	+	1
678	2-3-1-1-109-56-18	1	−	+	+	0
14	1-6-1-1-1-1-1	1	−	−	+	1
15	1-1-1-1-1-1-1	2	−	−	+	1
42	2-6-1-3-8-1-15	1	−	−	+	1
65	2-1-2-1-10-4-13	1	−	−	+	0
234	2-1-2-1-7-1-24	4	−	−	+	3
299	2-10-1-1-56-24-31	2	−	−	+	2
307	4-1-2-52-1-1-7	1	−	−	+	0
378	2-1-1-1-7-4-65	1	−	−	+	0
454	16-24-21-27-29-22-105	1	−	−	+	1

[a] The *tonB* allele in each ST is underlined.
[b] Number of KPC-positive isolates found within each sequence type.

PCR assay that can rapidly identify the ST258-*tonB79* cluster while simultaneously detecting bla_{KPC} genes in *K. pneumoniae* isolates. The assay is rapid and simple to perform, with significant sensitivity and specificity, and provides a useful tool for screening of *K. pneumoniae* isolates and surveillance of the epidemic ST258 clone in both community and health care settings.

TABLE 3 Distribution of KPC-producing ST258-*tonB79* cluster strains in 248 clinical *K. pneumoniae* isolates

Hospital	Location[a]	No.	No. (%) of isolates positive for:		
			ST258-*tonB79* cluster	KPC	ST258-*tonB79* and KPC
A	Northern NJ	43	34 (79.1)	42 (97.7)	34 (79.1)
B	Central NJ	33	28 (84.8)	31 (93.9)	26 (76.5)
C	NYC	80	66 (82.5)	73 (91.3)	63 (78.8)
D	NYC	31	24 (77.4)	27 (87.1)	23 (74.2)
E	NY	61	60 (98.4)	61 (100.0)	60 (98.4)

[a] NJ, New Jersey; NYC, New York City; NY, New York.

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