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Multiplex PCR for Identification of Two Capsular Types in Epidemic KPC-Producing *Klebsiella pneumoniae* Sequence Type 258 Strains

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We developed a multiplex PCR assay capable of identifying two capsular polysaccharide synthesis sequence types (sequence type 258 [ST258] *cps-1* and *cps-2*) in epidemic *Klebsiella pneumoniae* ST258 strains. The assay performed with excellent sensitivity (100%) and specificity (100%) for identifying *cps* types in 60 ST258 *K. pneumoniae* sequenced isolates. The screening of 419 ST258 clonal isolates revealed a significant association between *cps* type and *K. pneumoniae* carbapenemase (KPC) variant: *cps-1* is largely associated with KPC-2, while *cps-2* is primarily associated with KPC-3.

Carbapenem-resistant *Enterobacteriaceae* (CRE), especially *Klebsiella pneumoniae* carbapenemase (KPC)-harboring *K. pneumoniae*, are rapidly emerging as significant international public health concerns (1–3). Although KPCs have been found in numerous *K. pneumoniae* clones as well as in other Gram-negative species, a significant proportion of the global KPC burden is associated with a single *K. pneumoniae* multilocus sequence type (ST), ST258 (1–3). Currently, little is known about the genomic markers of this epidemiologically successful clone, and the genetic determinants of its spread, colonization, and disease progression remain unclear. Recent comparative genomic studies revealed two ST258 sublineages with two distinct capsular polysaccharide gene (*cps*) clusters (4). Here, we describe a multiplex PCR assay used to distinguish the two *cps* regions in KPC-harboring *K. pneumoniae* ST258 strains, providing a novel molecular tool for tracking their dissemination and studying their epidemiology.

We recently sequenced the genomes of 85 ST258 *K. pneumoniae* strains and single-locus variants (SLV) (ST379, ST418, and ST512) using Illumina HiSeq and Roche 454 GS-FLX platforms; two ST258 genomes were completely closed (those of *K. pneumoniae* strains NJST258_1 and NJST258_2) (4). One epidemiologically important finding was that ST258 strains carry two distinct *cps* gene clusters. One *cps* gene cluster is highly similar to that from a KPC-producing *K. pneumoniae* strain, Kp13 (cps_{Kp13}), an ST442 clinical isolate obtained from a patient in southern Brazil in 2009 (5, 6). The second *cps* gene cluster is novel, harboring several unique genes compared to others in GenBank (ncbi.nlm.nih.gov/GenBank/). We propose to name this novel *cps* gene cluster ST258 *cps-1* and the cps_{Kp13}-like cluster ST258 *cps-2*. The capsular polysaccharide is one of the major determinants of antigenic variation observed in *K. pneumoniae* and contributes to its success as a pathogen. Our preliminary human neutrophil studies revealed little or no killing effects between *cps-1*- and *cps-2*-harboring ST258 isolates (4), supporting the notion that the capsular variation observed in ST258 strains is likely involved in the survival of the pathogen and its ability to escape the host immune system. In this study, the sequences of ST258 *cps-1* and *cps-2* from two representative strains (*K. pneumoniae* BK30660 and BK32192) were generated by *de novo* assembly of the Illumina HiSeq reads using the CLC Genomics Workbench software (version 5.5.1; CLC bio, Aarhus, Denmark).

The multiplex PCR scheme to identify and distinguish the two ST258 *cps* types comprises three primer pairs. Primer pairs I and II were designed to target the *K. pneumoniae* capsular polymerase gene ($wyz_{\text{-I}}$ and $wyz_{\text{-II}}$) in ST258 *cps-1* and *cps-2*, respectively (Fig. 1A and Table 1). The third primer pair was developed to detect the *K. pneumoniae* species-specific *Klebsiella* hemolysin gene (*khe*) (7) and provides an internal positive control to confirm DNA template quality and PCR efficiency (Table 1).

The DNA template was isolated from bacterial colonies using the boiling lysis method described elsewhere (8). Multiplex PCR was performed in 15-μl reaction volumes consisting of 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 2 mM $MgCl_2$, 250 μM each deoxynucleoside triphosphate (dNTP), 0.1 μM primer for *khe*-f and *khe*-r, 0.2 μM primer for ST258*wzy*-I-f and ST258*wzy*-I-r, and 0.4 μM primer for ST258*wzy*-II-f and ST258*wzy*-II-r in 1× PCR buffer (Applied Biosystems). The optimal cycling conditions were as follows: an initial denaturation step of 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 5 min. Amplicons were visualized after running at 120 V for 1.5 h on 1% agarose gels containing GelStar nucleic acid gel stain (Ionza Ltd., Allendale, NJ) (Fig. 1B).

This multiplex PCR assay was initially validated using 60 ST258 clonal group *K. pneumoniae* strains that had been characterized by whole-genome sequencing (WGS) (4). The *cps* gene clusters of these 60 isolates were evaluated by *de novo* and refer-

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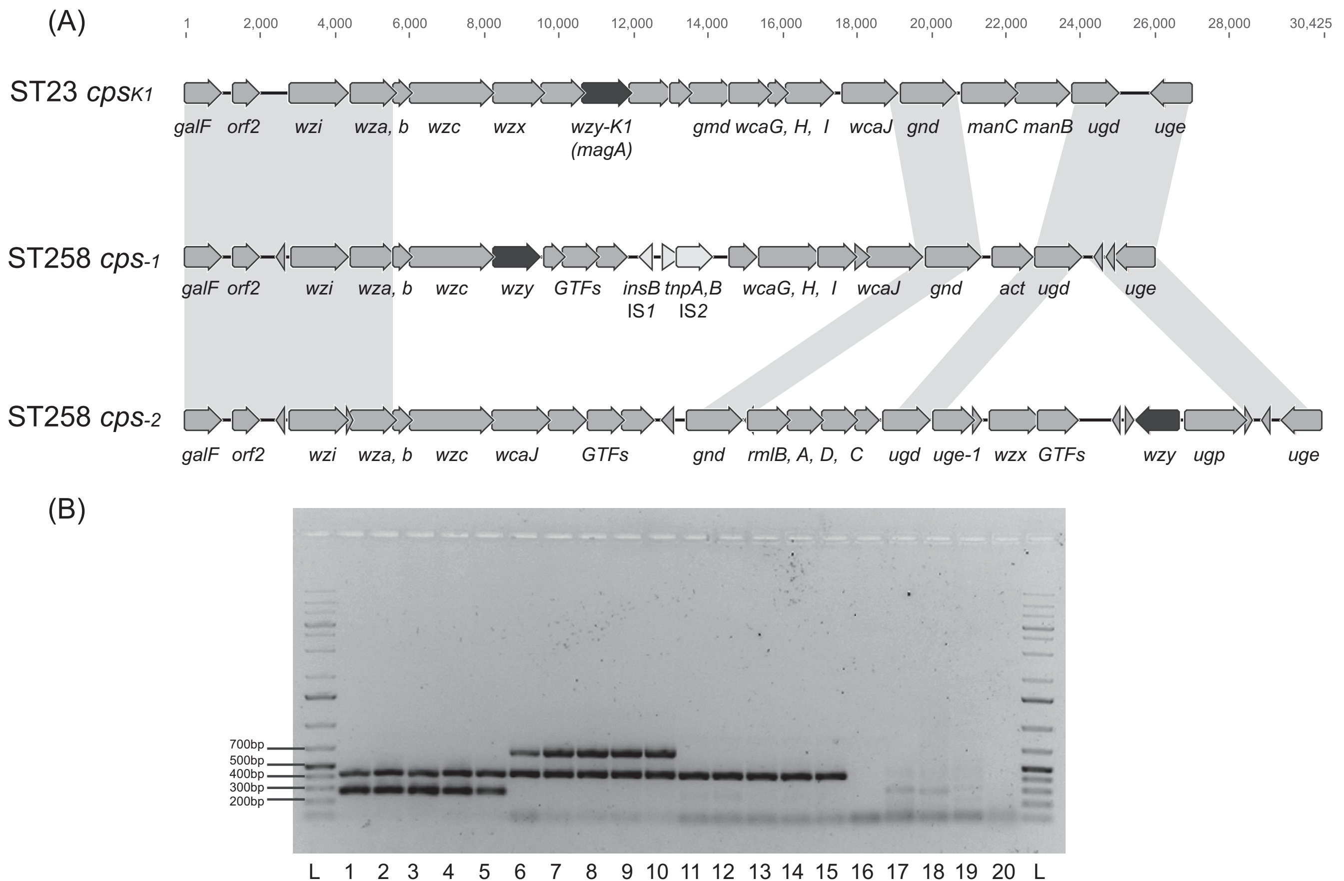

FIG 1 (A) *cps* region genetic structures of ST258 *cps-1* (GenBank accession no. KF793262), ST258 *cps-2* (GenBank accession no. KF793263), and *cpsK1* (from ST23 strain *K. pneumoniae* NTUH-K2044, GenBank accession no. AB198423). Gray shading denotes shared regions of homology of >95% nucleotide similarity. Open reading frames are portrayed by arrows, and the K-antigen polymerase gene, *wzy*, is highlighted in black. (B) Examples of ST258 *cps* multiplex PCR. Lanes L, ladder; lanes 1 to 5, five *cps-1*-carrying ST258 *K. pneumoniae* isolates; lanes 6 to 10, five *cps-2*-carrying ST258 *K. pneumoniae* isolates; lanes 11 to 15, five non-ST258 *K. pneumoniae* isolates; lanes 16 to 20, five non-*K. pneumoniae* Gram-negative isolates (*K. oxytoca*, *E. coli*, *E. cloacae*, *Citrobacter freundii*, and *P. mirabilis*).

ence assembly against sequences of ST258 *cps-1* and *cps-2*. Among them, 28 isolates carried *cps-1*, while the remaining 32 harbored *cps-2*. The *K. pneumoniae*-specific *khe* target was amplified for all 60 isolates, generating a 423-bp amplification product. Twenty-eight ST258 *cps-1* isolates were each positive for a 269-bp amplicon, while 32 ST258 *cps-2* isolates were all positive for a 638-bp amplicon, and there was 100% concordance with the WGS results. The assay showed excellent sensitivity (100%) and specificity (100%) for identifying *cps-1* and *cps-2* in this collection of 60 ST258 *K. pneumoniae* isolates.

This multiplex PCR was further evaluated using 120 Gram-negative and Gram-positive clinical isolates comprising a wide variety of different species and *K. pneumoniae* sequence types, selected from our strain collection at the Public Health Research Institute (PHRI) Tuberculosis Center. Among them, 55 non-ST258 *K. pneumoniae* isolates comprising 40 different STs were selected from our previous study (9). The remaining 65 isolates included the most common clinical Gram-negative and Gram-positive species: the Gram-negative bacterial isolates included *Acinetobacter* spp. ($n = 5$), *Citrobacter* spp. ($n = 4$), *Enterobacter* spp. ($n = 8$), *Escherichia coli* ($n = 10$), *Klebsiella oxytoca* ($n = 2$), *Morganella morganii* ($n = 2$), *Proteus mirabilis* ($n = 2$), *Pseudomonas* spp. ($n = 5$), *Salmonella* spp. ($n = 2$), *Serratia* spp. ($n = 2$), and *Shigella* sp. ($n = 1$); the Gram-positive isolates included *Entero-*

TABLE 1 Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)	Length (bp)	Target
wzy258-I-f	TACGGGGATTCCGGGAACAGCA	269	Gene *wzy* in ST258 *cps-1*
wzy258-I-r	ACAAAACCTCAATTGCTCTTCGGCT		
wzy258-II-f	GCACAAGAGAAATTGGATCTGACAACG	638	Gene *wzy* in ST258 *cps-2*
wzy258-II-r	ACTTCCAAATCCCATTGCAACTGCT		
khe-f	ACCATGTCCGATTTAATCACAACACGC	423	*K. pneumoniae*-specific gene *khe* (KPHS_02720 in HS11286)
khe-r	GCAGACGAACTTCCTGCTCGGT		

TABLE 2 Distribution of different *cps* types in clinical ST258 clonal *K. pneumoniae* isolates

	No. (%) of isolates with indicated KPC variant from:										
	PHRI (United States)				PHE (United Kingdom)			CLS (Canada)[a]			
ST258 *cps* type	KPC-2	KPC-3	KPC^{-b}	Total	KPC-2	KPC-3	Total	KPC-2	KPC-3	KPC-11	Total
cps-1	120 (90.9)	4 (3.0)	8 (6.1)	132	34 (100)	0 (0)	34	13 (68.4)	0 (0)	6 (31.6)	19
cps-2	18 (9.7)	162 (87.1)	6 (3.2)	186	3 (9.4)	29 (90.6)	32	0 (0)	7 (100)	0 (0)	7
Non-*cps-1* or *-2*	8 (88.9)	1 (11.1)	0 (0)	9	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0

[a] Isolates were from Greece and Israel (10).
[b] KPC^{-}, negative for bla_{KPC}.

coccus spp. ($n = 4$), *Staphylococcus* spp. ($n = 8$), *Streptococcus* spp. ($n = 8$), and *Micrococcus* spp. ($n = 2$).

All non-ST258 *K. pneumoniae* isolates were *khe* positive by multiplex PCR, while all other Gram-negative and Gram-positive isolates were negative for the *khe* target, demonstrating both its sensitivity and specificity for identifying and distinguishing *K. pneumoniae* from other organisms. Among the 55 non-ST258 *K. pneumoniae* isolates, 54 were PCR positive only for *khe*, as the ST258 *cps-1* and *cps-2* primers failed to generate PCR amplicons; one ST42 *K. pneumoniae* isolate from a hospital in New York in 2001 produced a PCR amplicon for ST258 *cps-1*. The *wyz* and *wzi* genes of this isolate were further amplified (wyz1-f, TGGCCTTA GCAATACCAACT; wzy1-r, TTTTCATTTCTATATATTTTTCA TCAATGC; wzi-f4, TAACAAGCAGCTTAGGGTAA; wzi-r, TTC TTCATAATGTCACATCAT), and the sequences of the two genes showed 100% identity compared with the ST258 *cps-1* genes in strain BK32192, strongly suggesting that this ST42 isolate harbors the same ST258 *cps-1* region. This finding indicates that ST258 *cps* regions are not limited to ST258 and may be found in other *K. pneumoniae* backgrounds.

Following validation of the multiplex PCR assay, an additional 327 *K. pneumoniae* ST258 clonal strains from 10 hospitals in New York and New Jersey were also screened by this multiplex PCR method. As part of an ongoing surveillance project, hospitals in New York and New Jersey routinely submit carbapenem-resistant and -susceptible *Enterobacteriaceae* to our laboratory for genotyping. The *K. pneumoniae* ST258 clonal type and bla_{KPC} variants of all strains were characterized by two multiplex real-time PCR methods previously developed in our lab (8, 9). Among them, 146 carried bla_{KPC-2} (44.6%), while 167 harbored bla_{KPC-3} (51.1%), and the remaining 14 *K. pneumoniae* strains were negative for bla_{KPC} (4.3%) (Table 2). Among these 327 isolates, 132 (40.4%) carried *cps-1*, while 186 harbored *cps-2*. Nine ST258 isolates could not be assigned to either *cps-1* or *cps-2*, strongly suggesting that they harbor either a different *cps* region or that the *cps* region is deleted in these strains. There was a significant association between KPC variant and *cps* type. In this study, 91% of the ST258 *cps-1* isolates carried bla_{KPC-2}, which is significantly higher than the proportion of isolates with bla_{KPC-3} (3.0%) or lacking KPC (6.1%) ($P < 0.01$ for each versus bla_{KPC-2}). Conversely, 87.1% of the ST258 *cps-2* isolates carried bla_{KPC-3}, which is significantly higher than the proportion of isolates with bla_{KPC-2} (9.7%) ($P < 0.01$) or lacking KPC (3.2%) ($P < 0.01$).

Further evaluation of the assay was undertaken at the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (AMRHAI) of Public Health England (PHE) and Calgary Laboratory Services (CLS) in Canada. The PHE collection includes 92 bla_{KPC}-harboring *Enterobacteriaceae* isolates, with 56 ST258 and 10 ST512 *K. pneumoniae* isolates. The *K. pneumoniae*-specific target *khe* was amplified from 89 *K. pneumoniae* strains but not from single isolates of *E. coli*, *K. oxytoca*, or *Enterobacter cloacae* in the test panel. Among the 66 ST258/ST512 isolates, the *cps-1* target was identified in 34 isolates, all of which expressed KPC-2, whereas 29 of 32 isolates expressing KPC-3 were positive for *cps-2*. Ten ST512 isolates, which are SLVs of ST258, expressed KPC-3 and were positive for *cps-2*. Twenty-three non-ST258 *K. pneumoniae* isolates and the three other *Enterobacteriaceae* isolates were all PCR negative for both *cps-1* and *cps-2*. The CLS collection includes 26 ST258 *K. pneumoniae* isolates from Greece ($n = 18$) and Israel ($n = 8$) obtained from the Study for Monitoring Antimicrobial Resistance Trends (SMART) worldwide surveillance program (10). Thirteen isolates harboring KPC-2 or KPC-11 (bla_{KPC-11} is a single nucleotide variant to bla_{KPC-2}) were all positive for *cps-1*, whereas the remaining seven KPC-3-bearing isolates were all positive for *cps-2*, which is consistent with the association described above (Table 2).

In summary, we have developed and validated in three centers a novel multiplex PCR which can rapidly identify the two dominant *cps* types in epidemiological *K. pneumoniae* ST258 isolates. A screening study from New Jersey and New York hospitals and analyses at the PHE AMRHAI reference unit and Canada CLS confirmed the robustness of the assay and revealed the wide spread of *cps-1*- and *cps-2*-harboring ST258 isolates in geographically distinct regions. The significance of the strong association between KPC-2 and KPC-3 variants with *cps-1* and *cps-2*, respectively, among ST258 *K. pneumoniae* isolates and our finding that *cps-1* resides in an ST42 strain are currently being investigated to better understand the molecular epidemiology and evolution of these spreading carbapenem-resistant strains.

Nucleotide sequence accession numbers. The complete nucleotide sequences of ST258 *cps-1* (from strain BK32192) and *cps-2* (from strain BK30660) were deposited as GenBank accession no. KF793262 and KF793263, respectively.

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B.N.K. discloses that he holds two patents that focus on using DNA sequencing to identify bacterial pathogens.

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