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Multiplex PCR for Detection of Seven Virulence Factors and K1/K2 Capsular Serotypes of *Klebsiella pneumoniae*

Fabrice Compain,[a,b] Ana Babosan,[a,b] Sylvain Brisse,[c,d] Nathalie Genel,[a,b] Jennifer Audo,[a,b] Florent Ailloud,[c,d] Najiby Kassis-Chikhani,[a,b] Guillaume Arlet,[a,b,e] Dominique Decré[a,b,e]

Sorbonne Universités, UPMC Univ Paris 06 CR7, Centre d'Immunologie et des Maladies Infectieuses, CIMI, Team E13 (Bacteriology), Paris, France[a]; INSERM U1135, Centre d'Immunologie et des Maladies Infectieuses, CIMI, Team E13 (Bacteriology), Paris, France[b]; Institut Pasteur, Microbial Evolutionary Genomics, Paris, France[c]; CNRS, UMR3525, Paris, France[d]; AP-HP Département de Bactériologie, Hôpitaux de l'Est Parisien, APHP, Paris, France[e]

A single multiplex PCR assay targeting seven virulence factors and the *wzi* gene specific for the K1 and K2 capsular serotypes of *Klebsiella pneumoniae* was developed and tested on 65 clinical isolates, which included 45 isolates responsible for community-acquired severe human infections. The assay is useful for the surveillance of emerging highly virulent strains.

Klebsiella pneumoniae is associated with both community-acquired and nosocomial infections. It is responsible for a wide spectrum of infections, including urinary tract infections, pneumonia, bacteremia, meningitis, wound infections, and purulent abscesses, at various sites. A new virulent hypermucoviscous variant of *K. pneumoniae* has emerged. It initially manifested as the cause of community-acquired primary liver abscesses, sometimes complicated by metastatic meningitis and/or endophthalmitis, and it has been described by numerous investigators in Asian countries (1). Sporadic cases of severe infections have been reported throughout the world and are now increasingly recognized in Western countries (2–7). The reasons for this epidemiological change and global differences remain unexplained. Invasive *K. pneumoniae* isolates causing these infections exhibit a hypermucoviscous phenotype more often than is commonly observed and are frequently found to belong to serotype K1 or K2 (8). Shon, Bajwa, and Russo (1) recently defined this new type of variant as hypervirulent *K. pneumoniae* (hvKP) and reviewed the clinical data and bacteriological features associated with these isolates; briefly, these features include the ability to cause severe community-acquired infections (e.g., liver abscesses, pneumonia, and meningitis) in young healthy hosts, the ability to cause metastatic infections (e.g., endophthalmitis), and a hypermucoviscous aspect of colonies on agar plates that can be semiquantitatively appreciated by a positive string test. The hypervirulent serotype K1 clone belongs to sequence type 23 (ST23), whose presence has been demonstrated on three continents (7).

Two genes were initially associated with invasive infections, i.e., the mucoviscosity-associated gene A (*magA*) and the regulator of mucoid phenotype A (*rmpA*) (9, 10). It is now well established that *magA* is located within the gene cluster specifying *K. pneumoniae* capsular serotype K1 and encodes a particular capsular polymerase, Wzy_{KpK1} (11). The *rmpA* gene is a plasmid-borne regulator of extracellular polysaccharide synthesis and is associated with the hypermucoviscous phenotype (10, 12). Yu et al. (10) demonstrated that *rmpA*-carrying strains were associated with the hypermucoviscous phenotype and with the clinical syndrome caused by invasive strains.

Molecular epidemiology has shown the hypervirulent strains to possess a combination of iron acquisition systems, i.e., enterobactin (Ent), the prototypical catecholate siderophore; aerobactin, a hydroxamate siderophore whose receptor is encoded by *iut*A; yersiniabactin (YbtS), a phenolate-type siderophore that is structurally distinct from Ent (13), and Kfu, which mediates uptake of ferric iron and is more prevalent in hypervirulent strains (14, 15). The *allS* gene (associated with allantoin metabolism) is strongly correlated with *K. pneumoniae* isolates from liver abscesses (16). Other genes that are involved in *K. pneumoniae* virulence include fimbrial and nonfimbrial adhesion genes, such as *ycfM*, *KPN*, and *mrkD*. *mrkD* is believed to function as the type 3 fimbrial adhesin and to mediate binding to the extracellular matrix (17).

Previous multiplex PCRs have targeted a few virulence genes and six capsular serotypes (7) or targeted virulence plasmid pLVPK-derived loci, i.e., *iutA*, *rmpA*, and two resistance genes (18). Recently, a multiplex PCR assay was designed to identify three groups of *hvKP* clonal groups of capsular serotype K2 (27). The purpose of the present study was to design a reliable PCR assay for the rapid detection of the most frequently encountered virulence genes and serotypes that are associated with hvKP.

A multiplex PCR was designed using the FastPCR software. The following genes were targeted: *magA* (K1 serotype), *rmpA*, *entB*, *ybtS*, *kfu*, *iutA*, *mrkD*, *allS*, and the K2 capsular serotype-specifying *wzi* gene (19) (Table 1); the primers targeting the capsular serotype K1- and K2-specifying genes were previously described (9, 20). As *entB* is widely spread among *K. pneumoniae* strains (G. Arlet and D. Decré, personal communication) (21), we included primers for *entB* in the design of the multiplex PCR as a positive control.

Crude DNA was prepared by lysis at 100°C for 10 min of 2 colonies in 500 μl of sterile distilled water, followed by centrifugation. The supernatant was used in the PCRs.

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Address correspondence to Fabrice Compain, compain.fabrice@wanadoo.fr.
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TABLE 1 Primers used in this study

Primer name	DNA sequence (5′ to 3′)	Target gene product/function	EMBL accession no.	Amplicon size (bp)	Primer concn (pmol/μl)	Reference or source
ybtS_for	GACGGAAACAGCACGGTAAA	Siderophore	AB298504	242	0.4	This study
ybtS_rev	GAGCATAATAAGGCGAAAGA					
mrkD_for	AAGCTATCGCTGTACTTCCGGCA	Adhesin type 3 fimbriae	EU682505	340	0.1	This study
mrkD_rev	GGCGTTGGCGCTCAGATAGG					
entB_for	GTCAACTGGGCCTTTGAGCCGTC	Siderophore	CP000647	400	0.1	This study
entB_rev	TATGGGCGTAAACGCCGGTGAT					
rmpA_for	CATAAGAGTATTGGTTGACAG	Regulator of mucoid phenotype A	X17518	461	0.2	This study
rmpA_rev	CTTGCATGAGCCATCTTTCA					
K2_for	CAACCATGGTGGTCGATTAG	Capsular serotype K2 and hypermucoviscosity phenotype	EF221827	531	0.4	20
K2_rev	TGGTAGCCATATCCCTTTGG					
kfu_for	GGCCTTTGTCCAGAGCTACG	Iron transport and phosphotransferase function	AB115591	638	0.075	This study
kfu_rev	GGGTCTGGCGCAGAGTATGC					
allS_for	CATTACGCACCTTTGTCAGC	Allantoin metabolism	AB115590	764	0.1	This study
allS_rev	GAATGTGTCGGCGATCAGCTT					
iutA_for	GGGAAAGGCTTCTCTGCCAT	Siderophore	AY378100	920	0.1	This study
iutA_rev	TTATTCGCCACCACGCTCTT					
magA_for	GGTGCTCTTTACATCATTGC	Capsular serotype K1 and hypermucoviscosity phenotype	AY762939	1,283	0.3	9
magA_rev	GCAATGGCCATTTGCGTTAG					

Multiplex PCR was carried out in a 25-μl volume using the Qiagen multiplex PCR kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The final reaction mixture contained a 1× PCR mixture (which consists of preoptimized concentrations of hot start DNA polymerase, $MgCl_2$, dinucleoside triphosphate [dNTP], and PCR buffer), various primer concentrations (Table 1), and 1 μl of the crude DNA extract. The PCR conditions were as follows: initial activation at 95°C for 15 min, followed by 30 cycles at 94°C for 30 s, 60°C for 90 s, and 72°C for 60 s, and a final extension at 72°C for 10 min. The amplicons were separated at 100 V for 2 h in a 2% (wt/vol) agarose gel containing ethidium bromide. The functionality and specificity of all primer pairs were tested in single reactions before their combined use in the multiplex PCR assay. In order to confirm primer specificity, bidirectional sequencing of the PCR amplicons was performed, and the sequences determined were compared with those in GenBank. Multilocus sequence typing (MLST) analysis was performed using the international *K. pneumoniae* MLST typing scheme (http://bigsdb.web.pasteur.fr/), as previously described (5, 22).

In the optimization assays, we used strains recently reported to cause severe and fatal infections (2). The nine primer pairs used in this study did not interfere with each other, and the PCR conditions allowed a highly reproducible synthesis of amplicons of the predicted size with the DNA of all isolates (Fig. 1). The intrarun reproducibility was examined by testing two hvKP strains of our collection (*K. pneumoniae* strains SA1 and SA2; see Table S1 in the supplemental material) 10 times (data not shown). Strain SA2 was added as an interrun control in each PCR (Fig. 1).

The multiplex PCR was applied to 65 *K. pneumoniae* isolates, which included 45 clinical isolates collected from several French hospitals between 2004 and 2014 and that were identified as hvKP, i.e., (i) were responsible for severe invasive community-acquired infections, including pyogenic liver abscesses, pneumonia, meningitis, and/or bacteremia, and (ii) tested positive on a string test, with the exception of one isolate recovered from a liver abscess (*K.*

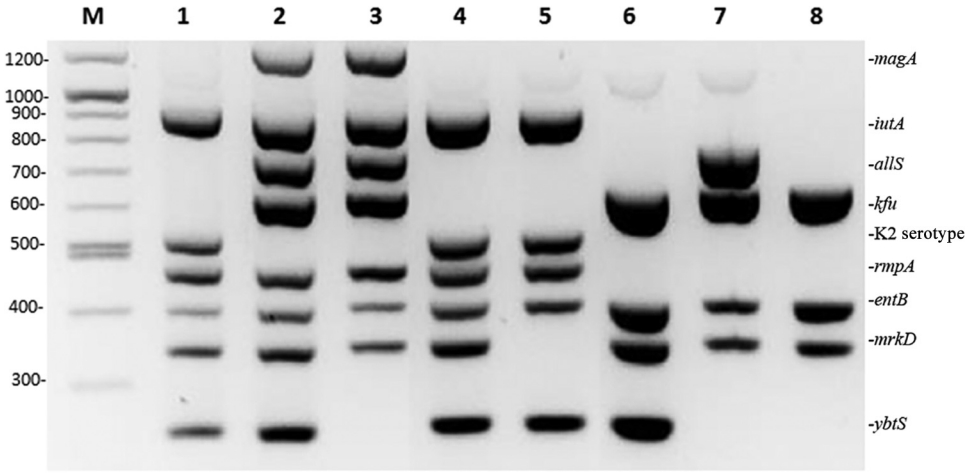

FIG 1 Virulence gene amplification assays for *K. pneumoniae*. The PCR products were separated on a 2% agarose gel. M, molecular size marker (in bp, measurements on left). Lane 1, SA1; lane 2, SA2; lane 3, SA55; lane 4, SA18; lane 5, H9; lane 6, SA4; lane 7, T3; lane 8, SA11.

TABLE 2 Results of multiplex PCR targeting virulence genes and K1/K2 capsular serotypes in 65 strains of *K. pneumoniae* according to their sequence type

Capsular serotype (no.)	MLST (no.)[a]	No. of hvKP strains[b]	No. of strains with virulence gene:						
			ybtS	*mrkD*	*entB*	*rmpA*	*kfu*	*allS*	*iutA*
K2 (36) K1 (15)	ST23 (15)	15	14	15	15	15	15	15	15
	ST13 (1)	0	1	1	1	0	1	0	0
	ST14 (2)	0	1	2	2	0	2	0	0
	ST25 (2)	1	1	2	2	1	0	1	1
	ST65 (4)	4	1	4	4	4	0	0	4
	ST86 (12)	12	9	12	12	12	0	0	12
	ST133 (1)	0	0	1	1	0	0	0	0
	ST244 (1)	0	1	1	1	0	1	0	0
	ST375 (3)	3	2	2	3	3	1	0	3
	ST380 (9)	9	9	9	9	9	8	0	9
	ST556 (1)	0	0	1	1	0	0	0	0
Non-K1/K2 (14)	ST12 (1)	0	0	1	1	0	0	0	0
	ST15 (1)	0	1	1	1	0	1	0	0
	ST35 (1)	0	0	1	1	0	1	0	0
	ST36 (1)	0	0	1	1	0	0	0	0
	ST37 (2)	0	0	2	2	0	0	0	0
	ST45 (1)	0	1	1	1	0	0	0	0
	ST60 (1)	1	1	1	1	1	1	0	0
	ST101 (2)	0	0	2	2	0	1	0	0
	ST416 (1)	0	0	1	1	0	0	0	0
	ST443 (1)	0	0	1	1	0	0	0	0
	ST528 (1)	0	0	1	1	0	0	0	0
	ST1604 (1)	0	0	1	1	0	1	1	0

[a] MLST, multilocus sequence typing.
[b] hvKP, hypervirulent *K. pneumoniae*.

pneumoniae SA67) (Table 2; see also Table S1 in the supplemental material). The results were compared with those obtained for 20 non-hvKP strains, including one multidrug-resistant strain responsible for a fatal nosocomial infection (*K. pneumoniae* KpS13) (23).

Fifteen isolates were of capsular serotype K1 and belonged to ST23, with most of them (11/15) recovered from primary liver abscesses and 4 from pulmonary samples. The *rmpA*, *kfu*, *mrkD*, and i*utA* genes were detected in all 15 isolates. Only one was negative for *ybtS*. The gene cluster involved in allantoin metabolism, including *allS*, was present in all serotype K1 isolates, in agreement with previous reports (5, 16, 24). Only one non-K1/K2 isolate (from ST60) was recovered from a pyogenic liver abscess, a prevalence much lower than the 30% of the non-K1/K2 strains causing liver abscesses, as reported previously (24). A high genotypic diversity was found among the 36 isolates of capsular serotype K2, which were distributed among 10 STs, i.e., ST86 ($n = 12$), ST380 ($n = 9$), ST375 ($n = 3$), ST65 ($n = 4$), ST25 ($n = 2$), ST14 ($n = 2$), ST13 ($n = 1$), ST133 ($n = 1$), ST244 ($n = 1$), and ST556 ($n = 1$). Almost all isolates (64/65) were found to possess *mrkD*, a gene detected in 35/36 K2 isolates. In contrast, the presence of other virulence genes differed considerably among the STs. The *allS* gene was present in only two non-K1 isolates (from ST25 and a ST1604); *rmpA* and the aerobactin receptor-encoding gene *iutA* were detected in all isolates belonging to ST86, ST380, ST375, and ST65 but never in isolates belonging to ST13, ST14, ST133, ST244, and ST556. The *kfu* gene of the iron acquisition system was never detected in ST86 isolates and was detected in 8/9 ST380 isolates. The yersiniabactin-encoding gene *ybtS* was present in all nine ST380 isolates and was predominant in ST86 (9/12) and ST23 (14/15) isolates. The results of the multiplex PCR assay are highly concordant with the distribution of virulence factors reported in a recent analysis of the genomic content of *K. pneumoniae* clonal groups (28).

Except for the presence of *entB* and *mrkD*, the 14 non-K1/K2 isolates displayed poor virulence gene profiles: none possessed *iutA*, while *kfu* and *ybtS* were observed in five and three isolates, respectively, and *allS* and *rmpA* were observed in one isolate each.

Most hvKP isolates belonged to capsular serotypes K1 or K2 and, remarkably, contained both *rmpA* and *iutA*. Moreover, they belonged to a limited set of STs (i.e., ST23, ST25, ST60, ST65, ST86, ST375, and ST380), of which only ST25 included both hvKP and non-hvKP isolates. Our observations confirm that ST23 (K1 serotype) and ST86 and ST65 (K2 serotype) represent important clones causing severe community-acquired infections in humans (2, 25). In this study, ST86 was associated with 12 cases of severe infection, including seven fatal cases. In a previous genetic study of K2 strains, only one ST86 strain (*K. pneumoniae* CIP52.204) was not part of the two major K2 clones (5). ST65 and ST25 belong to the same clonal complex. ST375 is a single-locus variant of ST65, and both were composed of hvKP isolates. ST380, which was associated with fatal infection in seven cases, might correspond to a recently emerging virulent clone (2, 26).

In conclusion, the multiplex PCR described in this study allows the rapid, reproducible, and sensitive detection of virulence genes carried by hvKP isolates. In addition, the method is less time-consuming than MLST determination and is suitable for screening virulent clones. This PCR will be useful for comparing the

virulence profiles of large collections of *K. pneumoniae* organisms, including hvKP strains. It would be of particular interest to characterize particular groups of *K. pneumoniae* (e.g., hvKP strains and multiresistant strains.) according to the source of isolation or the presence or absence of virulence factors in order to assess their correlation with clinical and epidemiological data. This multiplex PCR is anticipated to be valuable in epidemiological surveys of invasive infections due to *K. pneumoniae*.

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