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Multiplex-PCR Assay for Identification of *Klebsiella pneumoniae* Isolates Carrying the *cps Loci* for K1 and K2 Capsule Biosynthesis

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Abstract

Multiplex-PCR assay for identification of *Klebsiella pneumoniae* isolates carrying gene clusters for biosynthesis of capsular polysaccharide (CPS) types K1 and K2 was developed. Genes *wzc* and *orf10* of the *cps* cluster were applied as K1 and K2 specific markers respectively. The assay specificity was confirmed using 147 isolates of *Klebsiella* spp. including 77 K-antigen reference strains. The multiplex-PCR assay was found simple and cost-effective tool for identification of *K. pneumoniae* clinical isolates of K1 and K2 geno-serotypes.

Key words: *Klebsiella*, *K. pneumoniae* K1, *K. pneumoniae* K2, genoserotyping, multiplex-PCR

Introduction

Klebsiella pneumoniae, an important nosocomial pathogen, causes suppurative infection, pneumonia, urinary tract infection and septicaemia in humans, especially immunosuppressed (Podschun and Ulmann, 1998) or suffering from underlying diseases like diabetes mellitus (Fung *et al*., 2002). Persons of low social status and alcoholics constitute the main patients population at risk, comprising up to 66% of those suffering from community acquired pneumonia, that is a very severe illness with a rapid onset and a high mortality rates (Podschun and Ulmann, 1998; Sahly and Podschun, 1997). Despite the discovery of other virulence factors such as fimbriae, siderophores and O-antigens, capsular antigens are considered to be ultimate determinants of *K. pneumoniae* pathogenicity (Podschun and Ulmann, 1998; Sahly and Podschun, 1997; Fang *et al*., 2004; Yu *et al.*, 2007). Clinical isolates of this species produce capsular polysaccharide (CPS) (Fang *et al*., 2004; Ørskov and Ørskov, 1984). Among 77 capsular serotypes (K-types) of *K. pneumoniae* (Ørskov and Ørskov, 1984), strains belonging to serotypes K1 and K2 are considered the most virulent to mice (Simoons-Smit *et al*., 1984) and humans (Fang *et al*., 2004; Yu *et al.*, 2007). Moreover, strains of K1 and K2 are believed to escape the opsonin-independent lectin phagocytosis (Podschun and Ulmann, 1998; Kabha *et al*., 1995). Clinical studies on 134 patients with *K. pneumoniae* liver abscess exhibited predomination of serotypes K1 (63.4%) and K2 (14.2%) (Fung *et al*., 2002).

The capsular swelling (quellung) reaction and counter-current immunoelectrophoresis are the most commonly used techniques for identification of *K. pneumoniae* serotypes K1 and K2 (Janda and Abbott, 1998). The availability and costs of the antisera, which can be produced in specialised laboratories, limit the practice of serotyping. Therefore, novel molecular-serotyping tool was recently developed (Brisse *et al*., 2004). Although, this method is capable to identify all 77 K-types of *K. pneumoniae*, it requires time consuming long-range PCR followed by the endonuclease digestion and computer aided analysis of the electrophoretic patterns. Thus, despite its indisputable advantages, molecular-serotyping is not optimal for rapid identification of K1 and K2 strains in routine diagnostic. On the other hand, recently described PCR-based assays for differentiation of the major serovars of *Listeria monocytogenes* (Doumith *et al*., 2004), *Streptococcus*

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pneumoniae (Kong and Gilbert, 2003), *Yersinia pseudotuberculosis* and *Y. pestis* (Bogdanovich *et al*., 2003) have been found a rapid and practical alternative to laborious classical serotyping. For these reasons, we aimed to develop multiplex-PCR assay for identification of *K. pneumoniae* strains genetically competent to produce K1 and K2 capsular polysaccharides.

Experimental

Materials and Methods

Bacterial strains. We examined 147 isolates (Table I) including complete set of 77 *Klebsiella* spp. K-antigen reference strains (Ørskov and Ørskov, 1984) and ten reference K1 and K2 strains described elsewhere. Prior to serotyping by the counter-current immunoelectrophoresis using K1 and K2 antisera (Statens Serum Institut, Denmark) all clinical isolates listed in Table I were biochemically identified by classical tube tests. Noncapsulated variants of *K. pneumoniae* strains K1 (n = 1) and K2 (n = 6) were designed as described previously (Kałużewski, 1968).

PCR procedure. Template DNA was prepared from 0.5 ml of an overnight culture at 37°C in nutrient broth as described previously (Gierczyński *et al*., 2004) but the lysozyme treatment was omitted. Primers listed in Table II were used for amplification of fragments of *wzc*, *orf10* and *K. pneumoniae* 16S rRNA gene in multiplex-PCR. PCRs were carried out in 20 ml reaction volumes in a thermalcycler (Mastercycler, Eppendorf, Germany), with 0.75 U of the recombinant *Taq* DNA Polymerase (Fermentas, Lithuania), 1 × Mg-free PCR buffer with $(NH_4)_2SO_4$, each deoxynucleoside triphospate at a concentration of 0.2 mM, 3.0 mM $MgCl_2$, each primer at a concentration shown in Table II and 2.5 µl of the template DNA solution. A general program consisting of 35 cycles for 45 s of each denaturation at 94°C, annealing at 60°C and elongation at 72°C was used for amplification. Finally, DNA synthesis was completed at 72°C for 3 min. Prior to cycling, 5 min denaturation step at 94°C was included. The 2% gel (MP Biomedicals, Germany) in TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) was used for the multiplex-PCR products separation. Gels were run at a constant voltage of 80 V for 2 hours, stained in 2 µg/ml ethidium bromide for 10 min and photographed under UV by Gel-Scan apparatus (Kucharczyk, Poland). Each strain was analysed in triplicate.

Table I
List of tested strains and results of the multiplex-PCR assay

Strain	Number of isolates	Capsular type (serotyping)	Geno-serotype		Reference
			K1 (*wzc*)	K2 (*orf10*)	
A5054 (O1:K1)[a]	1	K1	+	–	(Ørskov and Ørskov, 1984)
A5054[b]	1	NT[e]	+	–	This study
408 (SB3182)[c]	1	K1	+	–	(Brisse *et al*., 2004)
468 (SB3186)[c]	1	K1	+	–	(Brisse *et al*., 2004)
643 (SB3188)[c]	1	K1	+	–	(Brisse *et al*., 2004)
920 (SB3192)[c]	1	K1	+	–	(Brisse *et al*., 2004)
B5055 (O1:K2)[a]	1	K2	–	+	(Ørskov and Ørskov, 1984)
B5055[b]	1	NT	–	+	This study
1584 (SB3201)[c] (C2b)[d]	1	K2	–	+	(Brisse *et al*., 2004)
777 (SB3202)[c] (C2c)[d]	1	K2	–	+	(Brisse *et al*., 2004)
34 (SB3203)[c] (C2d)[d]	1	K2	–	+	(Brisse *et al*., 2004)
778 (SB3199)[c] (C2e)[d]	1	K2	–	+	(Brisse *et al*., 2004)
B4631 (O2:K2)	1	K2	–	+	(Kauffmann, 1954)
B7380 (O2:K2)	1	K2	–	+	(Kauffmann, 1954)
K3-K82[a]	75	NT	–	–	(Ørskov and Ørskov, 1984)
Clinical isolates K1	3	K1	+	–	This study
Clinical isolates K2	10	K2	–	+	This study
Clinical isolates K2[b]	5	NT	–	+	This study
Clinical isolates	40	NT	–	–	This study
Total:	147				

[a] *Klebsiella* K-antigen reference strains excluding false serotypes K73 and K75-78 (Ørskov and Ørskov, 1984),
[b] noncapsulated variants,
[c] genomic DNA template (capsular type cited from the reference),
[d] C-patterns (subgenotypes) of *K. pneumoniae* K2 (Brisse *et al*., 2004),
[e] NT, strains nontypeable by K1 and K2 antisera.

Table II
Primers used in this study

Primer name	Target locus	GenBank accesion number	Primer sequence	Primer Position	Primer concentration
wzcf	*wzc*	AY762939	5'-GATACAGGTGTATTGTCGC-3'	8947–8966	0.4 µM
wzcr	*wzc*	AY762939	5'-GAGCTCTATATGTTGGATGC-3'	9283–9302	0.4 µM
or10f	orf10	D21242	5'-CCAGAGTTAGACCCGATATTC-3'	14205–14225	0.4 µM
or10r	orf10	D21242	5'-GAAGTCTATTACCCCTGAAG-3'	14848–14867	0.4 µM
K16Sf	16S rRNA	AF453251	5'-AGGGTGCAAGCGTTAATCGG-3'	493–512	0.2 µM
K16Sr	16S rRNA	AF453251	5'-TGTCTCACAGTTCCCGAAGG-3'	981–1000	0.2 µM

Results and Discussion

In order to select marker *loci* specific for serotypes K1 and K2 we performed comparative analysis of the *cps* gene clusters for K1 and K2 capsule biosynthesis deposited in GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers: AY762939 and D21242 respectively. For multiplex-PCR assay we selected gene *wzc* encoding tyrosine-protein kinase and the open reading frame 10 (*orf10*) encoding putative inner membrane protein for K1 and K2 serotypes respectively. Fragment of the *K. pneumoniae* 16S rRNA gene served as a positive multiplex-PCR control. The expected size of 16S rRNA gene amplicon was 508 bp while PCR-product for *wzc* (K1) and *orf10* (K2) was 356 bp and 663 bp respectively.

The multiplex-PCR assay result for K1 and K2 was judged as a positive when two bands were present – one specific for the 16 RNA gene and the other specific for *wzc* or *orf10* respectively. The presence of the16S rRNA gene amplicon alone indicted that tested DNA sample contained neither *wzc*, *orf10* and PCR inhibitors. In this case, the assay result was valid but negative for *K. pneumoniae* K1 and K2 geno-serotypes. The optimal yield of PCR products was observed for concentrations of *wzc* and *orf10* primers ranging from 0.50 to 0.25 µM and the 16S rRNA gene primers between 0.250 and 0.125 µM.

The multiplex PCR yielded DNA fragment of about 500 bp for all tested strains, whereas additional fragments about 350 bp and 650 bp were detected for strains of capsular type K1 and K2 respectively (Fig. 1). No bands were observed for DNA-free negative control (data not shown). Specificity of the PCR products was confirmed by DNA nucleotide sequencing performed as described previously (Gierczyński *et al*., 2004). Notably, *orf10* amplicons were obtained for strains of *K. pneumoniae* K2 belonging to different subgenotypes (C-patterns) (Brisse *et al*., 2004). This finding proved usefulness of the developed multiplex-PCR assay for identification of genetically diverse strains of capsular type K2 (Table I). Moreover, developed assay correctly identified rarely occurring strains O2:K2. The *wzc* and *orf10* were also detected in the noncapsulated variants of strain A5054 and B5055 respectively. Consequently, *orf10* was also traced in noncapsulated derivatives of clinical K2 isolates. This is in agreement to previous findings (Brisse *et al*., 2004), that molecular serotyping was capable to determine a potential serotype of capsule-deficient isolates. Except the 500 bp band, no PCR-products were generated for *Klebsiella* spp. K-antigen reference strains

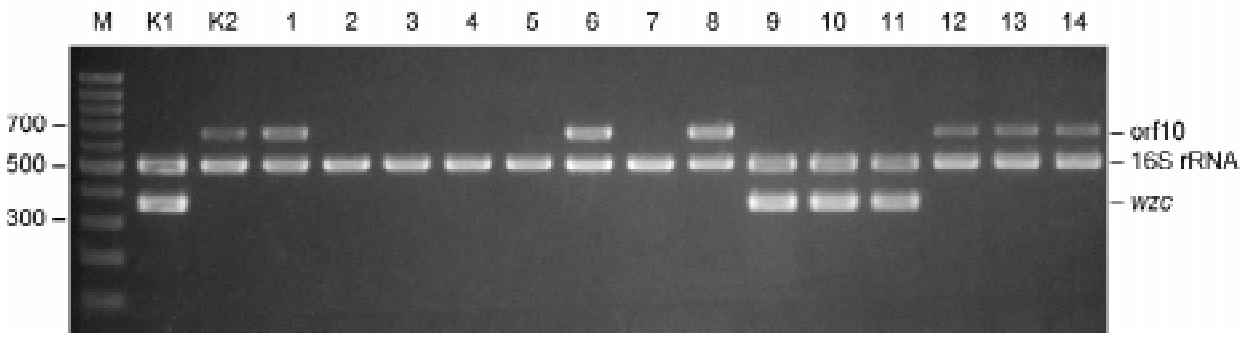

Fig. 1. Result of the multiplex PCR for identification of *K. pneumoniae* capsular genoserotypes K1 and K2
M – DNA size ladder 100 bp step (GeneRuler 100 bp, Fermetas, Lithuania). Lines: K1 – A5054 (K1 reference strain), K2 – B5055 (K2 reference strain), 1 – B5055 (noncapsulated), 2 – F5052 (K6), 3 – 889/50 (K20), 4 – 636/52 (K58), 5 – 438 (K66), 6 – B4631 (O2:K2), 7 – Kp90 (non-K1 and non-K2 clinical isolate), 8 – Kp57 (clinical isolate K2), 9 – A5054 (noncapsulated), 10 – 408 (K1), 11 – Kp229 (clinical isolate K1), 12 – 778 (K2), 13 – 1584 (K2), 14 – 34 (K2).

tested other than A5054 or B5055, as well clinical isolates nontypeable by K1 and K2 antisera (Table I). It is noteworthy, strain K58 that was reported to cross-react with K1 in serotyping did not yield *wzc* specific amplicon. The lack of such cross-reactions may be an advantage of developed assay when compared with a classical serotyping.

Taken together, obtained results show that developed multiplex-PCR assay is potentially useful tool for identification of K1 and K2 serotypes of *K. pneumoniae*. Moreover, developed assay is capable to determine whether a capsule defective strain is K1 or K2 derivative. Thus, the assay detects K1 and K2 geno-serotypes of *K. pneumoniae* in fact. However, due to reported horizontal transfer of the *cps* cluster to strains representing other species of *Enterobacteriaceae* (Nelson and Selander, 1994; Rahn *et al*., 1999) the multiplex-PCR assay may not be used instead the classical biochemical tests for *K. pneumoniae* identification (Janda and Abbott, 1998). We recommend this assay as a relatively inexpensive and robust tool for screening for *K. pneumoniae* K1 and K2 genoserotypes. However, to diversify capsule producing and capsule deficient isolates classical serotyping with K1 and K2 antisera is recommended. The multiplex-PCR may help to reduce total cost and workload of *K. pneumoniae* K1 and K2 capsular types identification in epidemiological surveys and routine diagnostic.

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