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Development of a multiplex PCR assay for identification of *Klebsiella pneumoniae* hypervirulent clones of capsular serotype K2

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Hypervirulent *Klebsiella pneumoniae* isolates of capsular serotype K2 (hvKP-K2) that cause community-acquired invasive infections represent several unrelated clones, which all belong to phylogenetic group Kpl. These clones can be recognized using multilocus sequence typing and genomic analyses, but no rapid method currently exists to differentiate them. In this work, a multiplex PCR assay was developed to identify three hvKP-K2 groups: (i) sequence type (ST)86; (ii) ST380 and ST679 (i.e. clonal group 380); and (iii) ST65 and ST375. A specific genetic marker, Kp50233, allowing *K. pneumoniae sensu stricto* (corresponding to phylogroup Kpl) to be distinguished from closely related species, was included in the assay. This PCR assay will be useful in better defining the epidemiology and clinical features of emerging virulent *K. pneumoniae* clones.

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INTRODUCTION

Klebsiella pneumoniae, which is mostly known as an agent of hospital-acquired infections (Podschn & Ullmann, 1998), is also emerging throughout the world as a community-acquired pathogen that can be responsible for pyogenic liver abscesses, bacteraemia, pneumonia and meningitis in previously healthy young people (Wang *et al.*, 1998; Ko *et al.*, 2002; Fang *et al.*, 2007). *K. pneumoniae* isolates causing such community-acquired invasive infections are regarded as strains of a novel, hypervirulent clinical variant, called

hypervirulent *K. pneumoniae* (hvKP) (Shon *et al.*, 2013). Defining characteristics of hvKP strains include the ability to cause serious infections in ambulatory, healthy hosts and a capability for metastatic spread (Shon *et al.*, 2013). Previous studies have shown that, among the 77 *Klebsiella* capsular polysaccharide (K) types, serotypes K1 and K2 are predominant among the hvKP strains (Fang *et al.*, 2007; Yu *et al.*, 2007). Other features of hvKP strains include the presence of a large virulence plasmid (pLVPK) encoding the aerobactin cluster and gene *rmpA*, which codes for the regulator of mucoid phenotype and which can also be chromosomally encoded (Nassif & Sansonetti, 1986; Chen *et al.*, 2004; Tang *et al.*, 2010; Hsu *et al.*, 2011). The presence of the *rmpA* gene is associated with a hypermucoviscous phenotype, which can be revealed by a positive 'string test' (Fang *et al.*, 2004).

Multilocus sequence typing (MLST) analysis of geographically diverse hvKP strains of serotype K1 (hvKP-K1) has

Abbreviations: ATCC, American Type Culture Collection; CC, clonal complex; CG, clonal group; CIP, Collection de l'Institut Pasteur; hvKP, hypervirulent *Klebsiella pneumoniae*; KCTC, Korean Collection for Type Cultures; MLST, multilocus sequence typing; NCTC, National Collection of Type Cultures; pLVPK, large virulence plasmid of *Klebsiella pneumoniae*; ST, sequence type.

One supplementary table is available in the online version of this paper.

revealed that they belong to a single clonal complex (CC), CC23-K1, which comprises sequence type (ST)23 and ST57 (Turton *et al.*, 2007; Brisse *et al.*, 2009; Siu *et al.*, 2011; Merlet *et al.*, 2012; Liao *et al.*, 2014). In contrast, hvKP strains of serotype K2 (hvKP-K2) are more diverse, belonging to several unrelated STs, including ST86, ST375 and ST380, that have been identified in Asia, Europe and the USA (Decré *et al.*, 2011; Bialek-Davenet *et al.*, 2013; Jung *et al.*, 2013; Russo & Gill, 2013; Liao *et al.*, 2014; Lin *et al.*, 2014). Based on core genome MLST, which records allelic variations at 694 loci, *K. pneumoniae* clonal groups (CGs) have been defined as clones within which isolates differ among themselves by fewer than 100 gene sequences out of 694 (Bialek-Davenet *et al.*, 2014). This approach confirmed that CC23-K1 represents a single CG and demonstrated that hvKP-K2 isolates of ST86, ST375 and ST380 correspond to three unrelated CGs named CG86, CG375 and CG380, respectively. The diversity of hvKP-K2 clones underlines the necessity to distinguish among them in order to better define their epidemiological and clinical characteristics. In addition, hvKP-K2 isolates must be distinguished from K2 isolates belonging to ST14, which is often encountered in multidrug-resistant nosocomial infections, but has low virulence (Brisse *et al.*, 2009; Giske *et al.*, 2012). Unlike hvKP-K1 isolates, which can be rapidly identified using PCR assays specific for serotype K1 and for the *allS* gene of the allantoinase cluster (Chou *et al.*, 2004; Fang *et al.*, 2007; Turton *et al.*, 2008; Brisse *et al.*, 2009), no rapid method exists to distinguish hvKP-K2 clones, even though they can collectively be recognized as K2 by K type-specific PCR at the *wzy* locus, which belongs to the capsule polysaccharide synthesis (*cps*) gene cluster (Fang *et al.*, 2007).

Furthermore, phylogenetic analyses have shown that *K. pneumoniae* is composed of four major phylogenetic groups: KpI, which is the most common among clinical isolates, KpII-A, KpII-B and KpIII (Brisse & Verhoef, 2001; Brisse *et al.*, 2004; Fèvre *et al.*, 2005). Our recent genomic analyses revealed that all hvKP-K2 clones belong to phylogroup KpI, in other words, to *K. pneumoniae sensu stricto* (Bialek-Davenet *et al.*, 2014). Indeed, phylogroups KpII-A, KpII-B and KpIII have been recently described as novel *Klebsiella* taxa, named *K. quasipneumoniae* subspecies *quasipneumoniae*, *K. quasipneumoniae* subspecies *similipneumoniae* and *K. variicola*, respectively (Rosenblueth *et al.*, 2004; Brisse *et al.*, 2014). All of these closely related *Klebsiella* species are typically identified as *K. pneumoniae* by manual and automated identification systems used in clinical microbiology laboratories, and there is currently no molecular assay to differentiate them.

The objectives of the current work were: (i) to develop a single multiplex PCR assay enabling discrimination of the main hvKP clones of serotype K2, and (ii) to find a specific genetic marker for the identification of *K. pneumoniae sensu stricto*.

METHODS

Bacterial isolates. A total of 91 unique *K. pneumoniae* isolates of capsular serotype K2 were gathered. They were derived from

community-acquired or nosocomial infections, and from historical collections (Table S1, available in the online Supplementary Material). For comparison, 60 isolates representing non-K2 capsular serotypes ($n=30$), *K. pneumoniae* phylogroups other than KpI ($n=6$) and other *Enterobacteriaceae* species ($n=24$) were included (Table S1).

String test of hypermucoviscosity. The mucoviscosity of K2 isolates was assessed by using the 'string test', which consists of stretching bacterial colonies grown for 24 h at 37 °C on agar plates with an inoculation loop. The test was considered positive if the achieved string reached at least 5 mm (Fang *et al.*, 2004).

MLST. All *K. pneumoniae* isolates were subjected to MLST following the Institut Pasteur method (Diancourt *et al.*, 2005).

Primer design. To identify targets specific for hvKP-K2 CGs, the genome sequences of isolates SA1, T69 and BJ1-GA (Bialek-Davenet *et al.*, 2014), representative of ST86, ST375 and ST380, respectively, were compared with other *K. pneumoniae* genomes (available as of December 2012) using the Phyloprofile tool of the MicroScope annotation platform (Vallenet *et al.*, 2013), and putative hvKP-K2-specific regions were visually inspected using the Synteny Viewer. Five genes were selected: gene *nikA2*, coding for a nickel-binding transport protein, was shared only by the three hvKP-K2 genomes; three genes (*kpiA* and *kphA* coding for putative fimbrial subunits, and *ykkD* coding for a membrane transporter) were specific for ST86, ST380 and ST375, respectively; and a gene named Kp50233 was shared by all isolates of phylogenetic group KpI and absent from the genome of strains belonging to other phylogroups of *K. pneumoniae* or to *Klebsiella oxytoca*. This gene was selected in order to serve as a positive control and as a potential novel tool for rapid KpI identification. Primers (Table 1) were designed using the NCBI Primer-BLAST tool (Ye *et al.*, 2012).

PCR. The hvKP-K2 multiplex PCR was carried out using the primers listed in Table 1 and the Qiagen Multiplex PCR kit with the addition of Q-Solution, according to the manufacturer's instructions. Reactions were carried out in a final volume of 25 µl with 5 pmol of each primer and 1 µl boiled DNA extract. For DNA extraction, a loopful of culture was emulsified in 200 µl distilled water, heated at 95 °C for 10 min and centrifuged at 16000 g for 5 min. PCR conditions consisted of an initial activation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 63 °C for 90 s and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis in a 1% (w/v) gel made with TAE (Tris-acetate-EDTA) buffer and stained with ethidium bromide. After electrophoresis (running conditions: 100 V and 400 mA for 2 h in TAE buffer), DNA fragments were visualized using a UV transilluminator. pLVPK-derived loci *iutA* (aerobactin receptor) and *rmpA* were detected following the methodology of and using the primers designed by Tang *et al.* (2010).

RESULTS AND DISCUSSION

Clonal diversity of serotype K2 *K. pneumoniae* isolates

Based on MLST, the 91 isolates of serotype K2 were split into 19 STs. The collection comprised 38 isolates of ST14, 13 of ST86, 10 of ST65, five each of ST375 and ST380, four of ST25, two each of ST17, ST66 and ST555, and a single representative of 10 other STs. The 30 non-K2 isolates were of 24 different STs, among which ST23 was represented by five isolates.

Table 1. Primers used in the hvKP-K2 multiplex PCR

Target region (gene name)	Product size (bp)	Primer	Sequence (5'–3')	Interpretation of positive PCR
Nickel-binding transport protein (<i>nika2</i>)	653	nika2F	AATTCTCAGATGGCTCGCCC	Indicative of hvKP-K2 clones, among serotype K2 strains
Putative acyltransferase (Kp50233)	484	nika2R	CACCGCGCTATAAGCAGGTA	Marker for <i>K. pneumoniae sensu stricto</i>
		50233F	GCTCTGGGAGATAGACCGCA	
Major pilin of fimbriae (<i>kpiA</i>)	361	50233R	GCGATSGCAGACCAGATGAAT	Indicative of clone ST86, among serotype K2 strains
		kpiAF	TTAGTACTGTCTCTGGCGCAC	
Major pilin of fimbriae (<i>kphA</i>)	270	kpiAR	GTTGGTACCCCCAGCTTGAT	Indicative of clone CG380 (ST380 + ST679), among serotype K2 strains
		kphAF	TCTGACTCAGTGCATGCTG	
Membrane transporter (<i>ykkD</i>)	157	kphAR	GAAATCTTGATCGGCGCGTT	Indicative of clones ST65 and ST375, among serotype K2 strains
		ykkDF	AGGTTGCGGGTATTTCCACC	
		ykkDR	ATGGCATAGGCCACGGAAAG	

The most frequent ST was ST14. This ST included 38 isolates from patients in the Caribbean (van Westreenen *et al.*, 2003) and several European countries (Table S1). ST14 has been previously reported in the context of hospital infections (van Westreenen *et al.*, 2003; Diancourt *et al.*, 2005; Poirel *et al.*, 2011; Breurec *et al.*, 2013). To our knowledge, ST14 has never been associated with invasive infections, consistent with its low virulence in a mouse model (Brisse *et al.*, 2009), and the absence of genes *rmpA* and *iutA* and of the hypermucoviscous phenotype (see below). Therefore, ST14 can be considered a non-hypervirulent K2 group.

Remarkably, 14 of the 16 isolates (88%) from recent invasive infections (Table S1) belonged to only three STs: ST86 (six isolates), ST380 (five isolates) and ST375 (three isolates). Furthermore, one isolate from a case of liver abscess associated with sepsis belonged to ST679, which differs from ST380 by a single locus. Based on core genome MLST, this isolate appears to be genetically closely related to ST380 isolates, together forming CG380 (Bialek-Davenet *et al.*, 2014).

It is interesting to note that ST86 comprised the high-virulence reference strain CG43, isolated in Taiwan (Chang *et al.*, 1996), as well as strain hvKP1 from the USA (Russo & Gill, 2013). ST86 also comprised strain CIP (Collection de l'Institut Pasteur) 52.204 [equivalent to ATCC (American Type Culture Collection) 7380], deposited in 1952, and strain IPEUC-340 isolated in 1975, which is our oldest ST86 isolate coming from a documented severe infection. ST375 comprised the clavulanic acid assay reference strain NCTC (National Collection of Type Cultures) 11228 (equivalent to CIP 103733), which was deposited as early as 1911 (Winslow *et al.*, 1919). We also noted that strain KCTC (Korean Collection for Type Cultures) 2242 belongs to ST375, as deduced from its genome sequence (Shin *et al.*, 2012). KCTC 2242 is a reference strain for 2,3-butanediol production research (Shin *et al.*, 2012) and was isolated

before 1964 (Gibson & Gibson, 1964). The fact that strain KCTC 2242 is genetically related to hvKP-K2 isolates might raise safety issues (Shrivastav *et al.*, 2013). Finally, ST66 comprised the K2 reference strain B5055 (equivalent to CIP 52.145), which was isolated in Java before 1935 (Ørskov & Ørskov, 1984) and from which the *rmpA* gene was initially described (Nassif *et al.*, 1989; Lery *et al.*, 2014). These observations underline the importance of several K2 clones for *K. pneumoniae* biology and demonstrate their existence long before the recognized emergence of hypervirulent infections in the 1980s.

Mucoviscosity and virulence gene content of serotype K2 *K. pneumoniae* isolates

When considering the 24 isolates from the three hvKP-K2 clones ST86, ST375 and CG380, 20 (83.3%) of them were hypermucoviscous (Table S1). This phenotype was also found for eight out of 10 isolates of ST65 and for both ST66 isolates. In contrast, only five out of the 55 isolates of other STs (9.1%) were hypermucoviscous (χ^2 test, $P < 0.0001$). These results demonstrate a strong association of hypermucoviscosity with the clonal background of K2 isolates.

The presence of the *iutA* and *rmpA* genes was detected in all isolates belonging to CG380, ST65 and ST66, as well as in 12 out of 13 (92.3%) ST86 isolates and four out of five (80%) ST375 isolates (Table S1). In contrast, they were detected in only three (5.5%) out of the 55 isolates representing other STs ($P < 0.0001$), and were totally absent from ST14-K2. As expected, a very strong association between a positive string test and *rmpA* detection was observed. The results demonstrate that, among K2 isolates, only those belonging to a few clones harbour the *rmpA* and *iutA* genes, two markers of high virulence, and thus

probably contain a virulence plasmid similar to pLVPK (Chen *et al.*, 2004). Interestingly, the two *rmpA*-negative isolates found among the hvKP-K2 clones were the ST375 strain NCTC 11228 and the ST86 strain CIP 52.204. It is possible that these strains represent sublineages that pre-date the acquisition of the virulence plasmid by their clone. Alternately, the virulence plasmid could have been lost during laboratory passage of these old strains.

Validation of the KpI-specific PCR assay

Gene Kp50233 was present in the 121 *K. pneumoniae* isolates belonging to phylogenetic group KpI, whereas it was absent from all isolates representing other *K. pneumoniae* phylogroups (i.e. KpII-A, KpII-B and KpIII) (Brisse & Verhoef, 2001; Fèvre *et al.*, 2005), as well as from two new phylogroups (unpublished data) and other *Enterobacteriaceae* species. Therefore, the sensitivity and specificity of this assay were both 100% in our setting. Kp50233 PCR consequently represents a useful novel assay with which to distinguish *K. pneumoniae sensu stricto* (i.e. phylogenetic group KpI; Brisse & Verhoef, 2001) from closely related species (Rosenblueth *et al.*, 2004; Brisse *et al.*, 2014).

Validation of the hvKP-K2 multiplex PCR assay

The hvKP-K2 multiplex PCR showed a total of nine different profiles, numbered from 0 to 8 (Fig. 1). Profile 0 corresponded to a completely negative PCR, whereas for profile 1 the 484 bp band of gene Kp50233 was present alone. In profiles 2, 3 and 4, gene Kp50233 and *nikA2* were detected in addition to gene *kpiA*, *kphA* or *ykkD*, respectively. The next four profiles corresponded to the detection of gene Kp50233 together with one of the following genes: *nikA2* (profile 5), *kpiA* (profile 6), *kphA* (profile 7) or *ykkD* (profile 8).

All isolates belonging to *K. pneumoniae* phylogroups other than KpI, or to other *Enterobacteriaceae* species, were consistently of profile 0, whereas among KpI isolates, when excluding the three hvKP-K2 clones ST86, ST375 and CG380, the majority of isolates (83.5%) had profile 1. This was notably the case for the 38 K2 isolates belonging to ST14 and for the five ST23-K1 isolates (Fig. 2, Table S1).

In contrast, 12 out of the 13 ST86 isolates (92.3%) presented profile 2, which was also the case for a non-K2 isolate belonging to ST3 (strain ATCC 13883^T). The specificity of profile 2 for detecting ST86 isolates was 100% among K2 isolates and 99.1% among KpI isolates. In one ST86 isolate, the *kpiA* gene was lacking (profile 5).

Profile 3 was found in the five ST380 isolates, as well as in the unique isolate of ST679. Therefore, profile 3 seems to be characteristic of CG380, which was detected with a sensitivity of 100% in our dataset. The specificity of profile 3 was 100% for K2 isolates and 99.1% for all KpI isolates, as it was also found in a non-K2 ST35 isolate.

The five isolates corresponding to the last hvKP-K2 clone considered, ST375, were distributed into profiles 4 ($n=2$) and 8 ($n=3$). Interestingly, these two profiles were also shared by 90 and 10% of ST65 isolates, respectively, consistent with the fact that ST65 and ST375 differ by a single MLST locus and are related, based on core genome MLST (Bialek-Davenet *et al.*, 2014). ST65 was recently shown to represent a major group of K2 isolates in Asia, together with ST86 (Liao *et al.*, 2014; Lin *et al.*, 2014). ST65 isolates are virulent in the mouse model, and carry the virulence markers *iutA* and *rmpA* (Brisse *et al.*, 2009; Liao *et al.*, 2014; Lin *et al.*, 2014). Six ST65 isolates were retrieved from lemurs in a French zoo, in which they caused fatal infections (Richard, 1989). Finally, profiles 6 and 7 were represented by two isolates (ST37 and ST556) and one isolate (ST559), respectively.

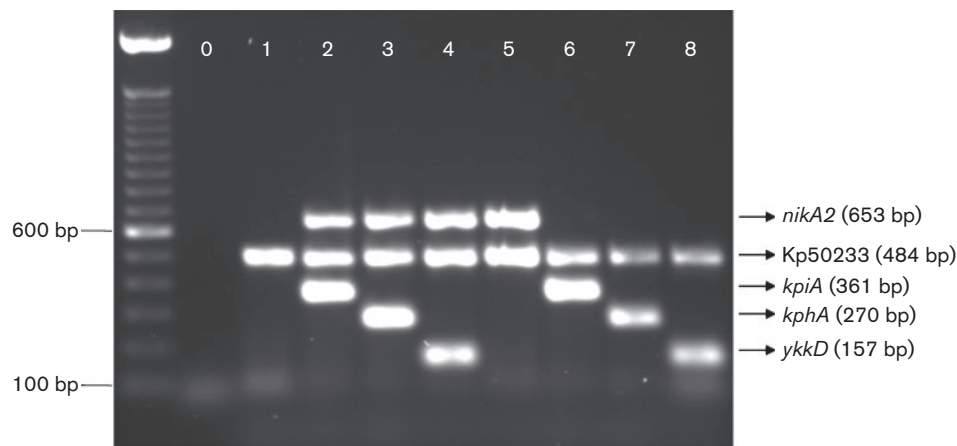


Fig. 1. Nine different profiles obtained with the hvKP-K2 multiplex PCR. The first lane corresponds to the 100 bp DNA Ladder (Invitrogen) and is followed by profiles 0–8. The gene names and amplicon sizes (in parentheses) are indicated on the right.

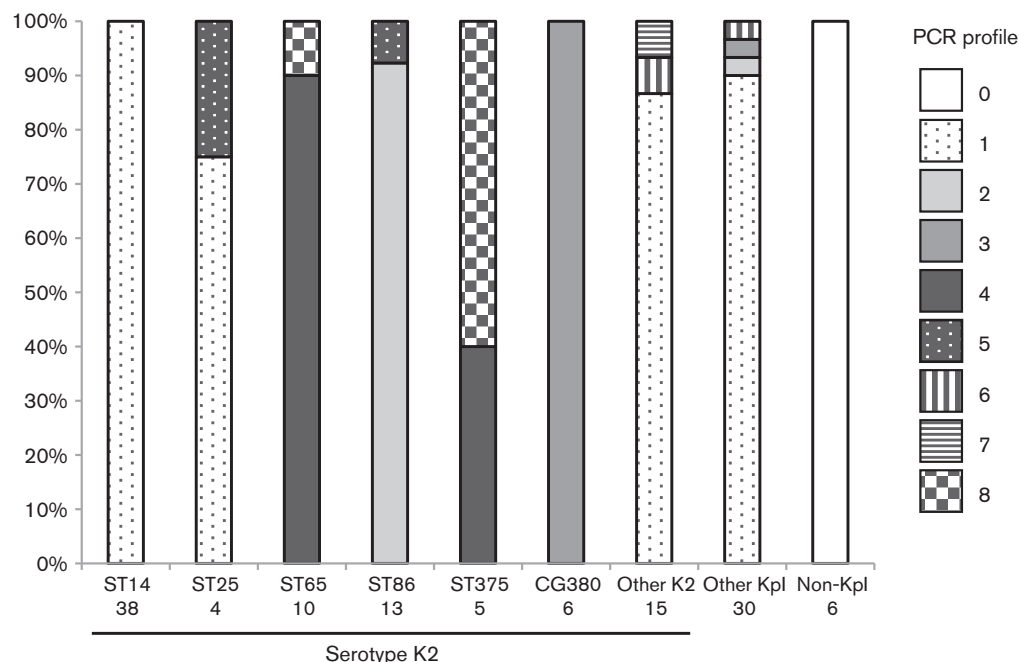


Fig. 2. Results, in percentages, of the hvKP-K2 multiplex PCR for different groups of *K. pneumoniae* isolates. The shading pattern corresponding to each profile is given on the right. The number of isolates included in each group is indicated below the group name.

Conclusions

Recent work has revealed that *K. pneumoniae* isolates of serotype K2 from invasive infections belong to several unrelated CGs (Brisse *et al.*, 2009; Decré *et al.*, 2011; Merlet *et al.*, 2012; Liao *et al.*, 2014; Lin *et al.*, 2014; Bialek-Davenet *et al.*, 2014). These clones can be recognized by MLST or whole-genome sequencing, but until now no simple assay was available to identify them. The multiplex PCR assay developed here will be useful to identify the main hvKP-K2 groups that comprise isolates of: (i) ST86, (ii) ST380 and ST679 (i.e. CG380), and (iii) ST65 and ST375. As the target genes used in this multiplex assay can also be present in non-K2 isolates, it is recommended to first determine the serotype, and then to use the hvKP-K2 assay for isolates of serotype K2. Serotype K2 isolates can be identified conveniently using K2 PCR (Fang *et al.*, 2007) or sequencing of the *wzi* (Brisse *et al.*, 2013) or *wzc* (Pan *et al.*, 2013) genes of the capsular polysaccharide gene cluster. Among isolates initially identified as serotype K2, profiles 2 and 3 presumably identify ST86 and CG380, respectively, whereas profiles 4 and 8 direct towards either ST375 or ST65. The novel PCR assay described here should represent a useful tool to characterize K2 isolates from invasive infections and to better define the epidemiology and clinical features of virulent *K. pneumoniae* clones. In addition, a multiplex PCR allowing the detection of K1 and K2 serotypes and seven virulence factors was developed recently and should be useful to compare the virulence profiles of hvKP strains (Compain *et al.*, 2014). This work

also shows that the Kp50233 gene, used here as an internal positive control for amplification, is highly specific for phylogroup KpI, and therefore represents a useful tool to identify *K. pneumoniae sensu stricto* and to differentiate it from the recently described closely related species *K. variicola* (KpIII) (Rosenblueth *et al.*, 2004) and *K. quasipneumoniae* (KpII-A and KpII-B) (Brisse *et al.*, 2014).

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