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Comparison of (GTG)₅-oligonucleotide and ribosomal intergenic transcribed spacer (ITS)- PCR for molecular typing of *Klebsiella* isolates

Running title: Molecular typing of *Klebsiella* species

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Key words: *Klebsiella* spp., molecular typing, (GTG)₅-PCR, ribosomal intergenic transcribed spacer (ITS)-PCR, fingerprint analysis, multiple displacement amplification

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Abstract

Molecular typing of *Klebsiella* species has become important for monitoring dissemination of β -lactamase-producers in hospital environments. The present study was designed to evaluate poly-trinucleotide (GTG)₅- and rDNA intergenic transcribed spacer (ITS)-PCR fingerprint analysis for typing of *Klebsiella pneumoniae* and *Klebsiella oxytoca* isolates. Multiple displacement amplified DNA derived from 19 *K. pneumoniae* (some with an ESBL-phenotype), 35 *K. oxytoca* isolates, five *K. pneumoniae*, two *K. oxytoca*, three *Raoultella*, and one *Enterobacter aerogenes* type and reference strains underwent (GTG)₅ and ITS-PCR analysis. Dendrograms were constructed using cosine coefficient and the Neighbour joining method. (GTG)₅ and ITS-PCR analysis revealed that *K. pneumoniae* and *K. oxytoca* isolates, reference and type strains formed distinct cluster groups, and tentative subclusters could be established. We conclude that (GTG)₅ and ITS-PCR analysis combined with automated capillary electrophoresis provides promising tools for molecular typing of *Klebsiella* isolates.

1. Introduction

Bacteria of the genus *Klebsiella* are important Gram negative opportunistic pathogens that can lead to severe diseases such as sepsis, pneumonia, and urinary tract infections (Brisse and Verhoef, 2001; Sardan et al., 2004). β -lactamases from Gram-negative bacteria inactivate penicillins and cephalosporins by hydrolysis. So far, more than 350 β -lactamases have been identified and on the basis of their amino-acid sequences, substrate and inhibitor profiles, Gram-negative β -lactamases are divided into four classes (A to D) (Ambler et al., 1991, Shah et al., 2004). Class A enzymes which include the plasmid-encoded broad-spectrum *bla*_{TEM}- and *bla*_{SHV}-families, and class C enzymes, which include chromosomally encoded cephalosporinases, are the most frequently occurring in enterobacterial species, including *Klebsiella*. Plasmid encoded *bla*_{CTX-M} enzymes represent another important subgroup of class A β -lactamases which hydrolyse broad-spectrum β -lactam antibiotics causing an extended-spectrum β -lactamase (ESBL) phenotype, which is increasingly found in enterobacterial species, including *Klebsiella* (Haeggman et al., 2004). Moreover, it has been shown that *R. planticola* and *R. ornithinolytica* (formerly *K. planticola* and *K. ornithinolytica*) colonise or infect human beings (Walckenaer et al., 2004). Thus, molecular typing methods allowing for an unequivocal identification and molecular epidemiological typing of *Klebsiella* clinical isolates have been developed (Vogel et al., 1999; Wang et al., 2008).

Klebsiella pneumoniae and *Klebsiella oxytoca* exhibit a high degree of genetic heterogeneity as demonstrated by phenotyping and genotyping analysis (Brisse et al., 2001; Vogel et al., 1999). Methods routinely used for species identification are not able to differentiate *K. oxytoca* from other indole-positive *Klebsiella* species such as *R. ornitholytica*, *R. planticola* and *R. terrigena* (formerly *K. ornitholytica*, *K. planticola* and *K. terrigena*) (Monnet et al., 1991, Drancourt et al., 2001). However, on the basis of 16S rDNA and *rpoB* DNA sequence analyses, it has been shown that *K. oxytoca* is phylogenetically distant from

other indole-positive *Klebsiella* species. Thus, the indole-positive *Klebsiella* species other than *K. oxytoca* have been renamed as *Raoultella* species (Drancourt et al., 2001). Molecular typing methods based on degenerated primers such as arbitrarily primed polymerase chain reaction (AP-PCR) and random amplified polymorphic DNA PCR (RAPD-PCR) are commonly used to establish a phylogenetic relationship between bacteria including *Klebsiella* strains and to generate epidemiological fingerprint patterns (Vogel et al., 1999; Brisse et al., 2001; Sardan et al., 2004).

The poly-trinucleotide (GTG)₅ motif represents a class of conserved repetitive sequences present in bacterial genomes (Versalovic et al., 1994). In some recent studies, (GTG)₅-PCR fingerprint analysis has been used for molecular typing of *Acinetobacter baumannii* (Huys, et al., 2005), *Salmonella enterica* (Rasschaert et al., 2005), *Campylobacter concisus* (Matsheka et al., 2006), *Enterococcus faecium* (Svec et al., 2005; Jurkovic et al., 2007), *Escherichia coli* (Mohapatra et al., 2007; Mohapatra et al., 2008), *Streptococcus mutans* (Svec et al., 2008), and for the identification of lactic acid bacteria isolated from human blood cultures (Svec et al., 2007). However, so far no data are available demonstrating the potential use of (GTG)₅-fingerprint analysis as a molecular means to differentiate *Klebsiella* spp. and related *Raoultella* species.

Due to the high conservation of primary and secondary structures within species, ribosomal RNA genes (16S, 23S and 5S) are commonly used for bacterial identification and evolutionary studies (Gutell et al., 1994). Because of less selection pressure, the 16S-23S rDNA intergenic transcribed spacer (ITS) sequence is more genetically variable and species-specific than that of 16S rDNA and 23S rDNA sequences (Gurtler et al., 1996; Boyer et al., 2001). Automated ribosomal intergenic spacer analysis (ARISA) is a PCR-based technique suitable for the amplification of microbial ITS-regions. To reduce PCR biases (such as selective amplification of some templates in a mixture of DNA) during ARISA, a universal

primer-set has shown to be powerful to explore microbial diversity and to create easy-to-analyse molecular fingerprints (Cardinale et al., 2004). PCR-based fingerprint analysis methods based on ITS-sequences have also been developed for the detection and identification of *Klebsiella* species (Lopes et al., 2007; Liu et al., 2008; Wang et al., 2008).

The goal of the present study was to evaluate the use of (GTG)₅-PCR and ITS-PCR analysis in molecular typing of *Klebsiella* isolates. Moreover, the possibility that the two approaches may allow differentiation between bacterial strains with different ESBL genotypes was evaluated. We conclude that (GTG)₅ and ITS-PCR analysis combined with automated capillary electrophoresis provides a promising tool for molecular typing of *Klebsiella* isolates. However, molecular typing and bacterial strain differentiation based on ESBL genotypes was not possible.

2. Materials and methods

2.1 Susceptibility testing of *Klebsiella pneumoniae* clinical isolates

Phenotypic ESBL-screening and susceptibility testing was performed on all (approximately 800 isolates/year) *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates collected during 2001-and spring 2007 at the Department of Clinical Microbiology, University Hospital Linköping, Sweden with agar disk diffusion according to the Swedish Reference Group for Antibiotics (<http://www.srga.org>) as described previously (Monstein et al., 2007; Monstein et al., 2009; Tärnberg et al., 2009). In brief, Cefadroxil was used for the screening of cephalosporin resistance, which was followed up by testing of resistant isolates with cefotaxime and ceftazidime or direct testing with cefotaxime and ceftazidime with disk diffusion and Etest (bioMérieux Sverige AB, Askim, Sweden). All cefotaxime and/or ceftazidime resistant isolates were phenotypically screened by Etest using cefotaxime and ceftazidime with and without clavulanic acid (bioMérieux Sverige AB, Askim, Sweden). Nineteen *K. pneumoniae* and 35 *K. oxytoca* isolates were selected and stored in glycerol containing Nutrient-broth No 2 (Lab M, Bury, UK) at -70° C until analysis (Monstein et al., 2009; Tärnberg et al., 2009).

In some cases, *K. pneumoniae* and *K. oxytoca* isolates originate from the same patient, collected at different occasions and showed an identical phenotype and similar antibiotic susceptibility profiles (Table 1)

2.2 Type and reference strains

Reference strain *K. oxytoca* K1980-K1 was kindly provided by Dr. D. Livermore, Health Protection Agency, Antibiotic Resistance Monitoring and Reference Laboratory, London, UK. Reference and type strains were purchased from the American Type Culture Collection (ATCC; <http://www.atcc.org>) or the Culture Collection University of Gothenburg (CCUG; <http://ccug.se>); *K. pneumoniae* ATCC 700603 *K. pneumoniae* CCUG 54718, *K. pneumoniae*

spp. *pneumoniae* CCUG 225^T, *K. pneumoniae* spp. *ozaenae* CCUG 15938^T, *K. pneumoniae* spp. *rhinoscleromatis* CCUG 417^T, *K. oxytoca* CCUG 15717^T, *Raoultella terrigena* CCUG 12372^T, *R. planticola* CCUG 15718, *R. ornithinolytica* CCUG 26769^T and *Enterobacter aerogenes* CCUG 1429^T. *Acinetobacter baumannii* clinical isolate No 200 was provided by the Clinical Microbiology Laboratory, University Hospital, Linköping, Sweden.

2.3 Multiple displacement amplification of bacterial DNA

To perform concurrent genotyping analysis omitting multiple bacterial culturing, sufficient amounts of bacterial DNA were produced by multiple displacement amplification as described elsewhere (Monstein et al., 2005; Monstein et al., 2009; Tärnberg M, et al., 2009). In brief, scrapings of frozen bacterial cultures (1 µl) were added to Sample buffer from an Illustra GenomiPhi V2 DNA amplification kit as recommended by the manufacturer (GE-Healthcare, Uppsala, Sweden). Upon completion of the reaction, 80 µl ultrapure water was added to each tube. The product was used as template-DNA in down-stream applications.

2.4 (GTG)₅-PCR based analysis

Initially, a PCR annealing temperature gradient assay was performed using 2 µl MDA-amplified DNA derived from *K. pneumoniae* CCUG 225^T and *K. oxytoca* CCUG 15717^T DNA, 50 pmol/reaction (GTG)₅-primer (Mohapatra et al., 2007), a final volume of 25 µl HotStarTaq Master mix (Qiagen, Hilden, Germany), MicroAmp optical 96-well reaction plates (Applied Biosystems, Stockholm, Sweden) and an Eppendorf Mastercycler gradient (Eppendorf, VWR International, Stockholm, Sweden). PCR conditions were as follows: initial denaturation step at 95° C for 15 min; 30 cycles of denaturation at 95° C for 30s, annealing temperature gradient at 40° C to 60° C for 30 s; extension at 72° C for 3 min, followed by a final extension step at 72° C for 10 min. *K. pneumoniae* and *K. oxytoca* strains were PCR

3. Results

3.1 (GTG)₅-PCR based analysis

The PCR annealing temperature gradient assay revealed an optimal annealing temperature of 51° C at an extension time of 3 min at 72° C in each cycle. *K. pneumoniae* DNA generates a more complex fingerprint pattern as compared to *K. oxytoca* DNA, allowing for molecular typing discriminating between these two strains (Fig. 1). Computerised densitometric (GTG)₅-PCR analysis revealed that the clinical isolates and reference strains could be divided into cluster I and II and outliers (cluster III) (Fig. 2) at a similarity level of 70%.

Most of the *K. pneumoniae* isolates, including reference strains *K. pneumoniae* CCUG 54718 and the three *K. pneumoniae* type strains, comprise cluster I. *K. pneumoniae* isolates No 184 and 205, which originate from the same patient having a *bla*_{LEN}-genotype, and *K. pneumoniae* isolate No 92 having a *bla*_{OKP}-genotype fell outside the two major clusters. A majority of the *K. oxytoca* isolates, the reference strain *K. oxytoca* K1980-K1 and type strain *K. oxytoca* CCUG 15717^T comprise cluster II which could be divided into tentative subclusters A - D. *K. oxytoca* isolate No 59, 60 (right and left nephrostomy), No 210 and *A. baumannii* isolate No 200, and the *Raoultella* type strains also fell outside the cluster I and II (Fig. 2).

K. pneumoniae and *K. oxytoca* isolates, which originate from the same patient but taken at different time points and in some cases of different origin (Table 1), clustered closed together except *K. oxytoca* isolates No 59 and 60, and *K. oxytoca* isolate No 142 which was expected to form a group together with isolate No 91, 169, 190, and 199 (Table 1; Fig. 2).

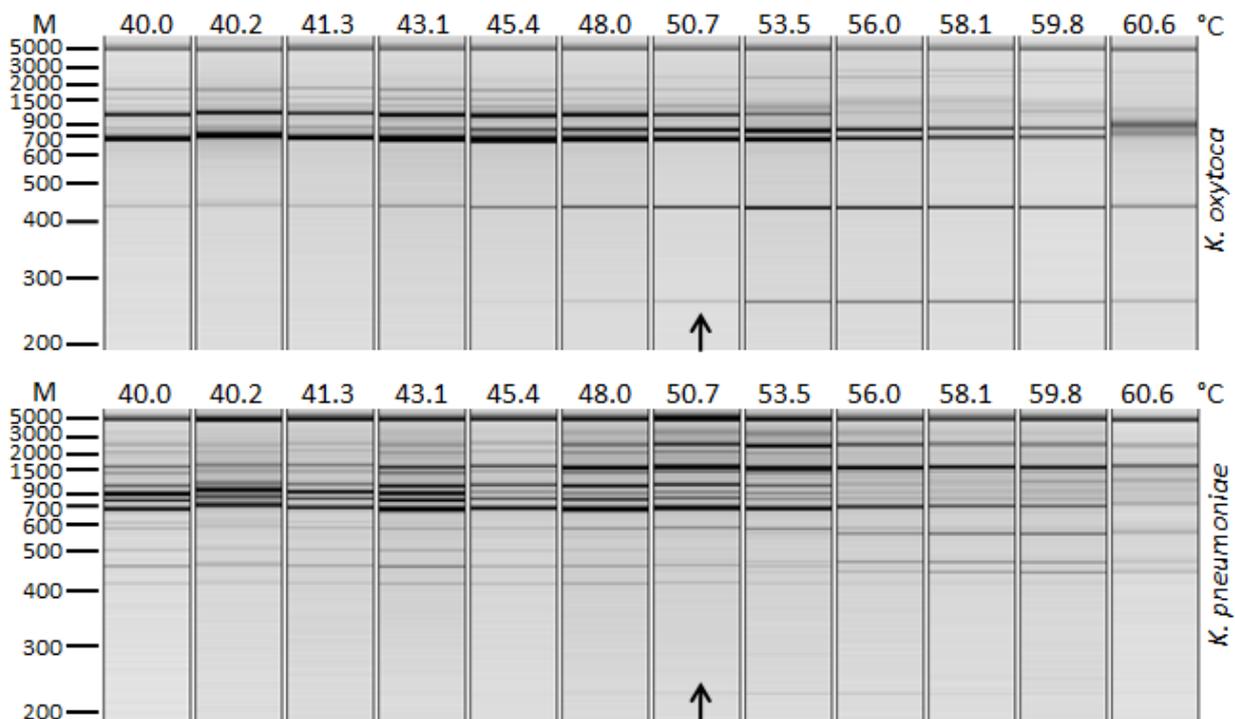


Figure 1: (GTG)₅-primer annealing temperature gradient optimisation. M represents a virtual DNA size marker (bp) used in the QIAxcel capillary electrophoresis system. Upper panel represents *K. oxytoca* CCUG 15717^T and the lower panel *K. pneumoniae* CCUG 225^T. Arrows indicate the (GTG)₅-primer annealing temperature chosen (51° C) generating *K. oxytoca* and *K. pneumoniae*-specific fingerprints.

3.2 ITS-PCR based analysis

ITS-PCR based analysis allowed for molecular typing of *Klebsiella* strains at the species level except for strain *K. pneumoniae* isolate No 92 having a *bla*_{OKP}-genotype which clusters closer to *K. oxytoca*. The *K. pneumoniae* isolates and reference strains comprise cluster I including *K. pneumoniae* isolates No 184 and 205 having a *bla*_{LEN}-genotype (Fig. 3). Similarly, *K. oxytoca* isolates and reference strains comprise cluster II, including *K. pneumoniae* isolate No 92 having a *bla*_{OKP}-genotype. Apparently, ITS-PCR generated fingerprint patterns do not allow for a separation of *K. oxytoca* isolates at the subspecies level as shown for isolates No. 91, 142, 169, 190 and 199 (Table 1; Fig. 3). In contrast, *K. pneumoniae* isolates No 33 and 43, isolates No 137, 138 and 143, and isolates No 179 and 185 clustered pair-wise within cluster I, respectively (Fig. 3). *Raoultella* species, *A. baumannii*,

and *E. aerogenes* are outliers (cluster III), distinct from the *K. pneumoniae* and *K. oxytoca* cluster I and II (Fig. 3).

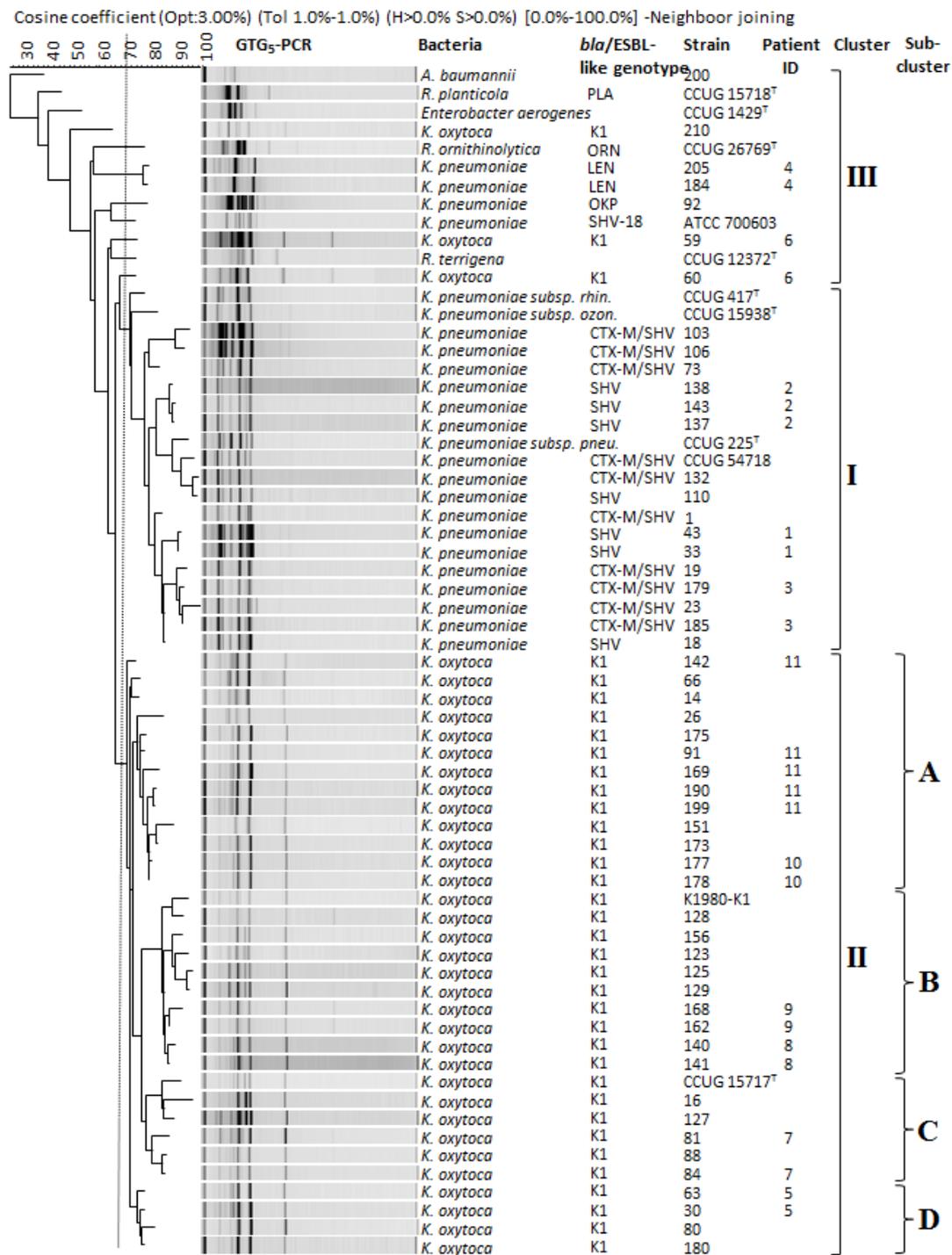


Figure 2: (GTG)₅-PCR dendrogram derived from 19 *K. pneumoniae*, 35 *K. oxytoca* clinical isolates, *Klebsiella* spp., *Enterobacter aerogenes*, and *Raoultella* spp. strains. Dashed line indicates 70% similarity level; I-II indicate two major cluster, and III an outlier group. A to D represent tentative subcluster at a similarity level of 70%. Bacterial phenotypes, ESBL genotypes (CTX-M, K1, SHV, LEN and OKP) of the clinical isolates were established in previous studies (Monstein et al., 2009; Tärnberg et al., 2009). ESBL-genotypes in *R. planticola* (PLA) and *R. ornithinolytica* (ORN) type strains have been described earlier (Walckenaer et al., 2004). Patient ID 1 – 11: multiple samples taken from 11 patients at different occasions (Table 1).

Cosine coefficient (Opt:3.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [4.6%-72.2%] –Neighbor joining

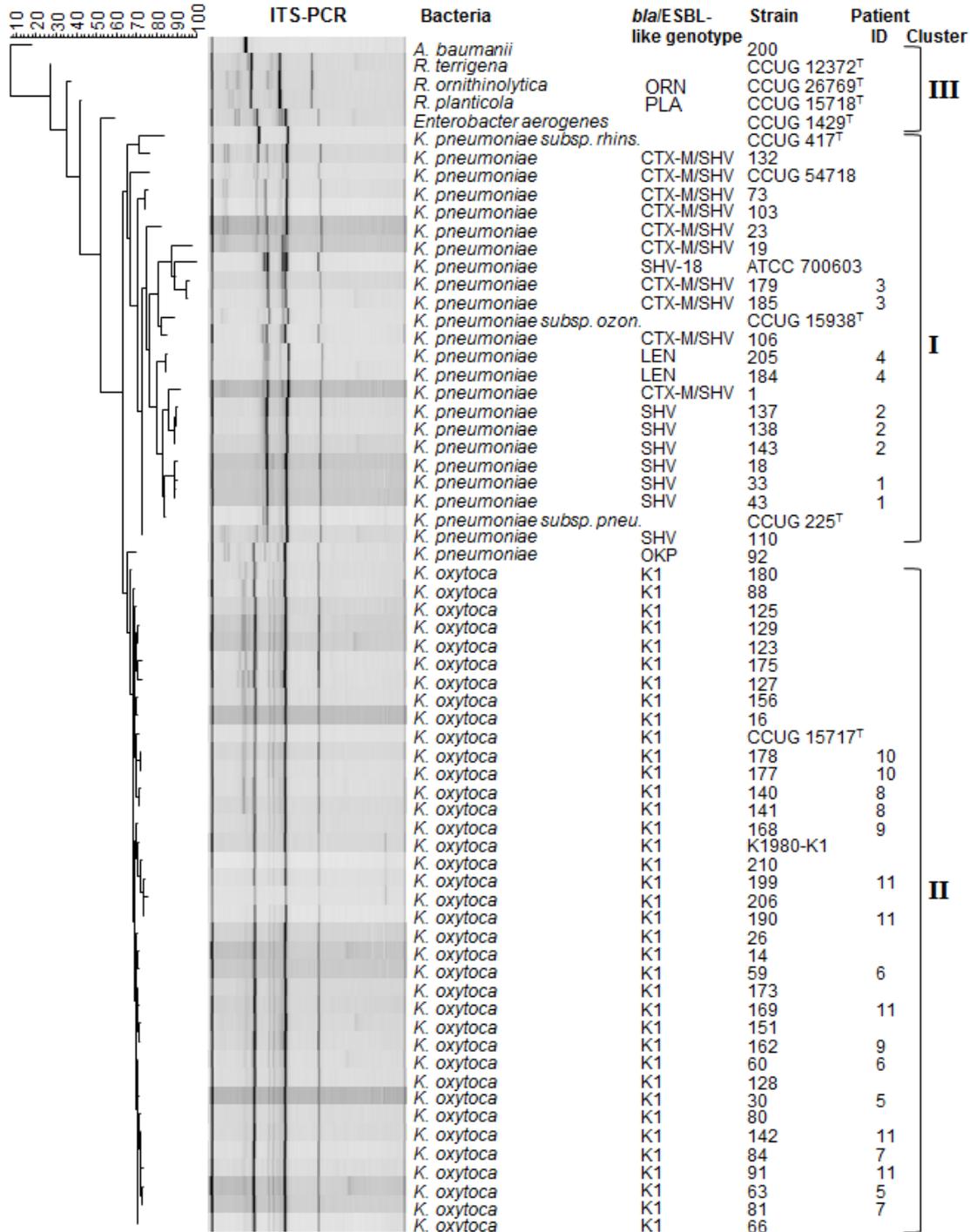


Figure 3: ITS-PCR dendrogram derived from 19 *K. pneumoniae*, 35 *K. oxytoca* clinical isolates, *Klebsiella* spp., *Enterobacter aerogenes*, and *Raoultella* spp. reference strains. I-II indicates two major cluster representing *K. pneumoniae* and *K. oxytoca* strains, and III represents outliers at a similarity level of 60 %, respectively. Bacterial phenotypes, ESBL genotypes (CTX-M, K1, SHV, LEN and OKP) of the clinical isolates were established in previous studies (Monstein et al., 2009; Tärnberg et al., 2009). ESBL-genotypes in *R. planticola* (PLA) and *R. ornithinolytica* (ORN) type strains have been described earlier (Walckenaer et al., 2004). Patient ID 1 – 11: multiple samples taken from 11 patients at different occasions (Table 1).

DISCUSSION

Biochemical tests including API-20E may not always be adequate phenotypical methods for typing *Klebsiella* species in clinical microbiology laboratories since several species share similar biochemical profiles (Brisse et al., 2001; Hansen, et al., 2004; Alves et al., 2006). Although many of the currently used molecular typing methods are PCR-based assays, the amount of isolated bacterial DNA may be a limiting factor for multiple molecular typing assays from the same DNA sample. Therefore, using MDA-amplified bacterial DNA derived from few bacterial cells may help to accomplish bacterial genotyping at reasonable costs and time. This is in accordance with similar studies where it was shown that MDA-amplified DNA derived from few bacterial cells and human biopsy specimens provided a reliable and representative source for multiple molecular typing analysis (Monstein et al., 2005; Grothouse et al., 2006; Ryberg et al., 2008; Tärnberg et al., 2009).

By means of (GTG)₅ and ITS-PCR analysis we were able to separate *Klebsiella* isolates and reference strains at the species level (Fig. 2). Moreover, these methods appear to have the potential to separate *K. pneumoniae* and *K. oxytoca* isolates into specific clusters. (GTG)₅-PCR analysis has not been evaluated previously for molecular typing of *Klebsiella* species and, therefore, a comparison of the present results with the literature was not possible. While (GTG)₅-PCR analysis appears to be a promising tool for molecular typing compared to ITS-PCR analysis, neither of the two approaches seem to be able to differentiate bacterial strains with different ESBL genotypes within one bacterial species.

Our study shows that *K. pneumoniae* and *K. oxytoca*, which are the most frequently isolated pathogenic *Klebsiella* species, can be discriminated confidently by ITS-PCR analysis using universal ITS-primers (Cardinale et al., 2004). However, molecular typing at the subspecies level appears not to be possible with this method (Fig. 3).

From a practical point of view, many laboratory workers are concerned about the use of ethidium-bromide stained agarose gels, which is a health-risk factor, and also a time consuming method. In agreement with previous studies from our laboratory (Ryberg et al., 2008; Monstein et al., 2010) we show that the use of automated capillary electrophoresis (CE), which is a rapid technique, successfully replaced ethidium-bromide stained agarose gel electrophoresis in (GTG)₅- and ITS-PCR analysis. The agarose gels can be directly substituted since analysis of fragment-length variations using the automated CE-system described does not require fluorescently-labelled primers in PCR amplification assays (www.qiagen.com).

In conclusion, (GTG)₅ and ITS-PCR analysis combined with automated capillary electrophoresis provides a promising tool for molecular typing of *Klebsiella* isolates. Further studies are required to assess the potential use of (GTG)₅ and ITS-PCR analysis as a tool in epidemiological typing of *Klebsiella* isolates.

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Table 1. *Klebsiella* clinical isolates taken at different time-points from the same patient

Strain	Patient ID	Isolate No.	<i>bla</i> -genotype	Origin	Collection year	(GTG) ₅ cluster location (Fig. 2)
<i>K. pneumoniae</i>	1	33	SHV	urine	2004-March 12	I
		43	SHV	wound secrete	2004-May 28	
<i>K. pneumoniae</i>	2	137	SHV	urine	2006-February 23	I
		138	SHV	rectum	2006-February 23	
		143	SHV	rectum	2006-February 28	
<i>K. pneumoniae</i>	3	179	CTX-M/SHV	urine	2006-October 5	I
		185	CTX-M/SHV	urine	2006-October 23	
<i>K. pneumoniae</i>	4	184	LEN	wound secrete	2006-October 21	outlier (III)
		205	LEN	wound secrete	2007-February 8	
<i>K. oxytoca</i>	5	30	K1	urine	2004-February 6	II-D
		63	K1	urine	2004-October 18	
<i>K. oxytoca</i>	6	59	K1	nephrostomy right	2004-October 4	outlier (III)
		60	K1	nephrostomy left	2004-October 4	
<i>K. oxytoca</i>	7	81	K1	urine	2005-March 2	II-C
		84	K1	urine	2005-March 10	
<i>K. oxytoca</i>	8	140	K1	urine	2006-February 23	II-B
		141	K1	blood	2006-February 23	
<i>K. oxytoca</i>	9	162	K1	wound secrete	2006-July 27	II-B
		168	K1	wound secrete	2006-August 15	
<i>K. oxytoca</i>	10	177	K1	wound secrete	2006-October 2	II-A
		178	K1	wound secrete	2006-October 2	
<i>K. oxytoca</i>	11	91	K1	urine	2005-April 8	II-A
		142	K1	urine	2006-February 27	II-A
		169	K1	urine	2006-August 22	II-A
		190	K1	urine	2006-November 22	II-A
		199	K1	urine	2007-January 7	II-A