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Comparison of $(GTG)_5$-oligonucleotide and ribosomal intergenic transcribed spacer (ITS)-PCR for molecular typing of *Klebsiella* isolates

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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 and Verhoef, 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 and Verhoef, 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 baumanii* (Huys, et al., 2005),

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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 and Stanisich, 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 K. pneumoniae clinical isolates

Phenotypic ESBL-screening and susceptibility testing was performed on all (approximately 800 isolates/year) *K. pneumoniae* and *K.* 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 (bioMerieux 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 (bioMerieux 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. *A. baumanii* 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;

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

Strain	Patient ID	Isolate No	*bla*-genotype	Origin	Collection year	$(GTG)_5$ cluster location (Fig. 2)
K. pneumoniae	1	33	SHV	Urine	2004-March 12	I
		43	SHV	Wound secrete	2004-May 28	
K. pneumoniae	2	137	SHV	Urine	2006-February 23	I
		138	SHV	Rectum	2006-February 23	
		143	SHV	Rectum	2006-February 28	
K. pneumoniae	3	179	CTX-M/SHV	Urine	2006-October 5	I
		185	CTX-M/SHV	Urine	2006-October 23	
K. pneumoniae	4	184	LEN	Wound secrete	2006-October 21	Outlier (III)
		205	LEN	Wound secrete	2007-February 8	
K. oxytoca	5	30	K1	Urine	2004-February 6	II-D
		63	K1	Urine	2004-October 18	
K. oxytoca	6	59	K1	Nephrostomy right	2004-October 4	Outlier (III)
		60	K1	Nephrostomy left	2004-October 4	
K. oxytoca	7	81	K1	Urine	2005-March 2	II-C
		84	K1	Urine	2005-March 10	
K. oxytoca	8	140	K1	Urine	2006-February 23	II-B
		141	K1	Blood	2006-February 23	
K. oxytoca	9	162	K1	Wound secrete	2006-July 27	II-B
		168	K1	Wound secrete	2006-August 15	
K. oxytoca	10	177	K1	Wound secrete	2006-October 2	II-A
		178	K1	Wound secrete	2006-October 2	
K. oxytoca	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