Application of PCR ribotyping and tDNA-PCR for *Klebsiella pneumoniae* identification

Ana Catarina S Lopes/+, Juliana Falcão Rodrigues, Maysa BM Clementino*, Catia AC Miranda*, Ana Paula A Nascimento*, Marcos Antônio de Morais Júnior**

Departamento de Medicina Tropical, Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Av. Prof. Morais Rego s/n, 50732-970 Recife, PE, Brasil *Departamento de Microbiologia, Instituto Nacional de Controle de Qualidade em Saúde-Fiocruz, Rio de Janeiro, RJ, Brasil **Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brasil

PCR analysis of 16S-23S internal transcribed spacer (PCR ribotyping) and tRNA intergenic spacer (tDNA-PCR) were evaluated for their effectiveness in identification of clinical strains of Klebsiella pneumoniae *and differentiation with related species. For this purpose both methods were applied to forty-three clinical isolates biochemically identified as* K. pneumoniae *subsp.* pneumoniae *isolated from patients clinical specimens attended at five hospitals in three Brazilian cities. References strains of* K. pneumoniae *subsp.* pneumoniae*,* K. pneumoniae subsp. ozaenae, K. oxytoca, K. planticola *and* Enterobacter aerogenes *were also analyzed. Both PCR methods showed specific patterns for each species. A conserved PCR ribotype pattern was observed for all clinical* K. pneumoniae *isolates, while differing from other related analyzed species. tDNA-PCR revealed five distinct patterns among the* K. pneumoniae *clinical isolates studied, demonstrating a predominant group with 90,6% of isolates presenting the same pattern of* K. pneumoniae *type strain. Both PCR-based methods were not able to differentiate* K. pneumoniae *subspecies. On the basis of the results obtained, both methods were efficient to differentiate the* Klebsiella *species analyzed, as well as* E. aerogenes*. Meanwhile tDNA-PCR revealed different tRNA arrangements in* K. pneumoniae*, suggesting intra-species heterogeneity of their genome organization, the polymorphism of the intergenic spacers between 16S and 23S rRNA genes appears to be highly conserved whithin* K. pneumoniae *clinical isolates, showing that PCR ribotyping can be an useful tool for identification of* K. pneumoniae *isolates.*

Key words: *K. pneumoniae* - PCR ribotyping - tDNA-PCR.

Klebsiella pneumoniae is a microorganism able to infect debilitated and immunocompromised patients, especially those with risk factors to acquire hospital infections. Due to the extensive spread of antibiotic resistant strains, especially of extended-spectrum beta-lactamase producing strains (Souza Lopes et al. 2005), there has been renewed interest in *Klebsiella* infections.

According to the taxonomic classification of Orskov and Orskov (1984), the genus *Klebsiella* includes *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *rhinoscleromatis*, *K. oxytoca*, *K. terrigena*, *K. planticola* and *K. ornithinolytica*. Clinically, *K. pneumoniae* is the most important species, accounting for about 95% of *Klebsiella* strains isolated from clinical specimens. *K. pneumoniae* subsp. *pneumoniae* is usually referred to as *K. pneumoniae* and the other two subspecies as *K. ozaenae* and *K. rhinoscleromatis* (Podschun & Ullmann 1998). Recent phylogenetic studies have demonstrated heterogeneity within the genus *Klebsiella* and suggested its division into two genera, with the genus *Raoultella* being proposed for species previously called *K. planticola*, *K. terrigena* and *K. ornithinolytica* (Drancourt et al. 2001). Other phylogenetic analyses using RAPD, sequencing of *gyrA* and *parC* genes and automated ribotyping, have revealed that *K. pneumoniae* isolates fell into three clusters, *K. oxytoca* isolates fell into two clusters, while *K. planticola* isolates formed a sixth cluster (Brisse & Verhoef 2001, Haeggman et al. 2004).

The characterization of *K. pneumoniae* usually includes phenotypic markers, like biochemical profile (Podschun & Ullmann 1998), that often do not allowed to correct identification. Recently, a number of molecular based approaches to the identification of species of clinically important bacteria have been described. The sequencing of the 16S rDNA gene represents the most precise method for the identification of *Klebsiella* species (Drancourt et al. 2001). Meanwhile, this is expensive and requires a high degree of technical competency to perform (Olive & Bean 1999) and therefore not recommended for routine identification of bacteria. In contrast, PCR-based methods have been widely used for the analysis of the genetic diversity of many microorganisms (Agodi et al. 2000, Souza Lopes et al. 2005, Spacov et al. 2006). Depending on the primers and amplification conditions employed, the results allow discrimination of organisms at the genus, species or strain level. PCR ribotyping is a rapid technique that uses specific primers complementary to the 3' end of the 16S rDNA gene and to the 5' end of the 23S rDNA gene to amplify intergenic spacer regions (ISRs). The ISRs are subject to lower evolutionary pressure, and therefore show a wider genetic variation (Gürtler & Stanisich 1996). Variations

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+Corresponding author: catarina.lopes@uol.com.br
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in the length of the 16S-23S ISRs mainly depend on the number and type of tRNA genes and sequences for enzyme recognition, found within these regions (García-Martínez et al. 1999).

Another highly promising method for bacterial identification is based on the PCR length polymorphisms of the intergenic spacers between tRNA genes (tDNA-PCR). The tRNA genes are highly conserved among eubacteria and occur in multiple copies throughout the bacterial genome. These genes are generally clustered and are separated by spacers whose length and sequences are subjected to a higher degree of variations (Welsh & McClelland 1992). The tDNA-PCR technique developed by Welsh and McClelland (1991) uses consensus primers complementary to the highly conserved edges of the flanking tRNA genes and which are directed outwardly.

The aim of the present study was to evaluate for the first time the potential of PCR ribotyping and tDNA-PCR as tools for the identification of *K. pneumoniae* clinical isolates from different geographical origins in Brazil.

MATERIALS AND METHODS

Bacterial strains - All reference strains and clinical isolates analyzed in this study are listed in Table. The 43 clinical *K. pneumoniae* isolates analyzed were obtained from patients with different types of infection between March 1999 and January 2000, with 30 epidemiologically unrelated isolates collected from three public hospitals in the city of Recife, PE, 12 related isolates from a public hospital in the city of Maceió, AL, and one isolate from the city of Rio de Janeiro, Brazil. Two clinical isolates of *E. aerogenes*, from Recife, were also used for comparative analysis as this species shows a biochemical profile closely similar to *K. pneumoniae*, which may lead to identification errors.

Phenotypic characteristics - All clinical isolates were identified biochemically by the Mini API ID 32E systems (Bio Mérieux, Marcy l'Etoile, France) according to manufacturer's instructions. The isolates were stored in 15% glycerol at -70ºC and cultured in brain heart infusion broth (Difco, Detroit, MI, USA) for later molecular analysis.

TABLE

PCR ribotyping and tDNA-PCR patterns of the 45 clinical isolates of *K. pneumoniae, E. aerogenes* from Brazil, and six reference and type strains.

Strains or clinical isolates[a]	Source of isolation	City/State	PCR ribotyping pattern	tDNA-PCR pattern
K. *pneumoniae* type strain (ATCC 13883)	-	-	R1	T1
K. *pneumoniae* reference strain (ATCC 10031)	-	-	R1	T1
K. *ozaenae* reference strain (ATCC 25926)	Blood	-	R1	T1
K. pneumoniae (K2-R, K11-R, K16-R, K19-R, K20-R, K3-C, K5-C, K6-C, K8-C, K9-C, K10-C, K11-C, K12-C, K15-C, K16-C, K17-C, K18-C)	Urine	Recife/PE	R1	T1
K. pneumoniae (K6-R, K10-R K13-R, K14-R)	Tracheal aspirate	Recife/PE	R1	T1
K. pneumoniae (K15-R)	Blood	Recife/PE	R1	T1
K. pneumoniae (K17-R)	Tracheal aspirate	Recife/PE	R1	T2a
K. pneumoniae (K18-R)	Urine	Recife/PE	R1	T2b
K. pneumoniae (K21-I)	Feces	Recife/PE	R1	T1
K. pneumoniae (K22-I)	Blood	Recife/PE	R1	T1
K. pneumoniae (K4-C)	Wound	Recife/PE	R1	T2c
K. pneumoniae (K7-C)	Urine	Recife/PE	R1	T3
K. pneumoniae (K13-C and K14-C)	Wound	Recife/PE	R1	T1
K. pneumoniae (P2204, P2205, P2206, P2207, P2208, P2215, P2216, P2217, P2218, P2219, P2220, P2221)	Not determinated	Maceió/AL	R1	T1
K. pneumoniae (P1298)	Cerebro-spinal fluid	Rio de Janeiro/RJ	R1	T1
K. planticola (*R. planticola* nov.) reference strain (ATCC 8329)	-	-	R2	T4
K. oxytoca reference strain (CCT 0182)	-	-	R3	T5
E. aerogenes type strain (ATCC 13048)	Tracheal aspirate	-	R4	T6
E. aerogenes (EA1-R)	Urine	Recife/PE	R4a	T6
E. aerogenes (EA2-R)	Tracheal aspirate	Recife/PE	R4b	T6

a: Hospital da Restauração (-R); Instituto Materno Infantil de Pernambuco (-I); Hospital das Clínicas, Universidade Federal de Pernambuco (-C); ATCC: American Type Culture Collection; CCT: Coleção de Culturas Tropicais.

DNA extraction - Genomic DNA was extracted from the isolates by the method described by Maniatis et al. (1982) and quantified by comparison with known amounts of lambda DNA digested with *Hin*d III in 1% agarose gel.

PCR ribotyping - The primers described by Kostman et al. (1992) were used for amplification of the spacers between the 16S and 23S rRNA genes. The primers (5'-TTG TAC ACA CCG CCC GTC A -3' and 5'-GGT ACC TTA GAT GTT TCA GTT C-3') were designed based on the sequences complementary to the conserved regions of the 16S and 23S rRNA genes of various bacterial species.

The reactions were prepared in a total volume of 25 µl, containing 20 ng genomic DNA, 1 U *Taq* DNA polymerase (Gibco-BRL, Life Technologies, Gaithersburg, MD, USA), 0.16 mM of each dNTP (Amersham-Pharmacia Biotech, USA), 1.5 mM $MgCl_2$, 20 pmol of each primer in 1X PCR buffer. PCR was performed in a thermocycler (Hybaid) programmed for 30 cycles, with each cycle consisting of 1 min at 94ºC, 1 min at 55ºC and 1 min at 72ºC, with a final extension step for 7 min at 72ºC. A negative control without DNA was included and repeating the PCR three times tested reproducibility of the amplifications. The amplification products were submitted to 2% agarose gel electrophoresis in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) at a constant voltage of 100 V. The gels were stained with ethidium bromide, visualized under an ultraviolet transilluminator and photographed with a Polaroid camera. A 100-bp DNA marker (Biotools, B & M Labs, Madrid, Spain) and a 100-bp DNA ladder (Invitrogen Co, Carlsbad, CA, USA) were used as molecular weight standard.

tDNA-PCR - The reaction was carried out with the outwardly directed consensus primers T3B (5'– AGG TCG CGG GTT CGA ATC C – 3') and T5A (5'- AGT CCG GTG CTC TAA CCA ACT GAC – 3') described by Welsh and McClelland (1991) and the PCR conditions have been described previously (Clementino et al. 2001). The amplified fragments detected in agarose gel were treated as discrete characters and recorded in a data matrix by scoring one for the presence and zero for the absence of a fragment. Similarity between strains was determined on the basis of the simple matching coefficient and the generated matrix was subjected to clustering by the unweighted pair group method with arithmetic means (UPGMA) (Grimont 1999). The Numerical Taxonomy System Program (NTSYS-PC, Applied Biostatistics, Inc.), version 1.30, was used in data analysis.

RESULTS

PCR ribotyping - Electrophoretic analysis of the amplified products of the 16S-23S ISRs of *K. pneumoniae* ATCC 13883 and ATCC 10031 and *K. ozaenae* ATCC 25926 reference strains revealed identical patterns consisting of six bands with estimated sizes of 570, 720, 800, 890, 950 and 1000 bp (Fig. 1A). This pattern differed from those individually obtained for the reference strains of *K. oxytoca* CCT 0182, *K. planticola* ATCC 8329 and *E. aerogenes* ATCC 13048.

The 43 clinical *K. pneumoniae* isolates from Recife, Maceió and Rio de Janeiro showed the same amplification pattern as that observed for the reference strains (Table). These results demonstrate that the primers described by Kostman *et al*. (1992) produced the same band pattern for all *K. pneumoniae* tested, both clinical isolates and reference strains (Fig. 1A). With respect to the

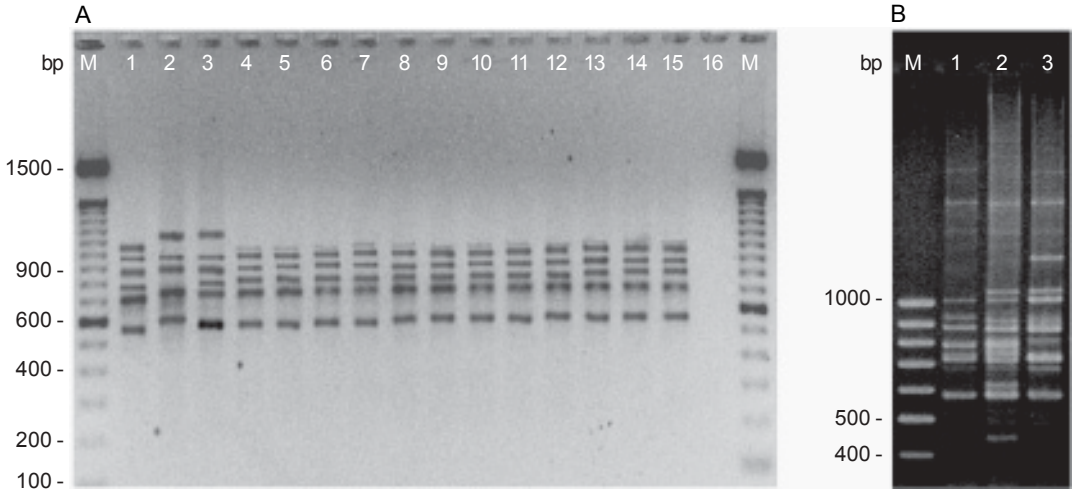

Fig. 1: PCR ribotyping patterns of *Klebsiella* and *E. aerogenes* strains. A: lanes - 1: *E. aerogenes* ATCC 13048; 2: *K. planticola* ATCC 8329; 3: *K. oxytoca* CTT 0182; 4: *K. pneumoniae* ATCC 13883; 5: *K. pneumoniae* ATCC 10031; 6: *K. ozaenae* ATCC 25926; 7 – 15: *K. pneumoniae* strains from Recife (K20-R, K21-I, K22-I, K3-C, K4-C, K5-C, K6-C, K7-C and K8-C, respectively); 16: negative control with no added template DNA. B: lanes - 1: *E. aerogenes* ATCC 13048; 2 – 3: *E. aerogenes* (EA1-R and EA2-R). M: molecular size marker (100 bp).

two clinical *E. aerogenes* isolates, both showed distinct amplification patterns that differed from those of the type strain (Fig. 1B). The electrophoretic patterns observed by PCR ribotyping were constant and reproducible, with identical profiles being obtained both after the three repetitions of the same sample and when using different batch of the same isolate.

tDNA-PCR - Identical tDNA-PCR patterns were obtained for *K. pneumoniae* ATCC 13883 and ATCC 10031 reference strains, with seven DNA fragments of 140, 200, 270, 290, 390, 510 and 580 bp being detected. This pattern was identical to *K. ozaenae* ATCC 25926 reference strain, but differed from those observed for *K. oxytoca* CCT 0182, *K. planticola* ATCC 8329 and *E. aerogenes* ATCC 13048 strains (Fig. 2A). It should be noted that both PCR-based methods used in the present study were not able to differentiate *K. pneumoniae* subspecies.

Five different amplification profiles (T1, T2a, T2b, T2c and T3) were identified by tDNA-PCR in the 43 clinical *K. pneumoniae* isolates from Brazil (Fig. 2C, Table), with 39 (90,6%) showing banding patterns similar to *K. pneumoniae* reference strains ATCC 13883 and ATCC 10031 and *K. ozaenae* ATCC 25926 (pattern T1) (Fig. 2B). Three isolates from Recife fell into pattern T2 and were divided into three subtypes. The isolate K7-C fell within pattern T3 (Fig. 2C). All *K. pneumoniae* isolates analyzed showed patterns distinct from those detected in *K. oxytoca, K. planticola* and *E. aerogenes* (Fig. 2A). The two clinical *E. aerogenes* isolates ana-

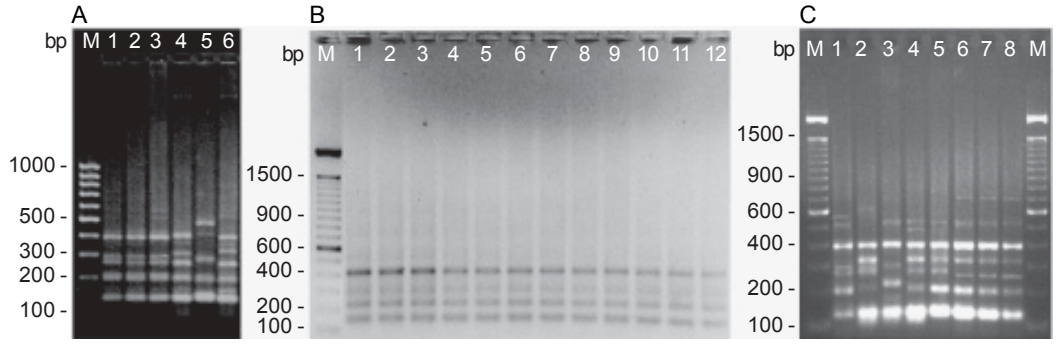

Fig. 2: tDNA-PCR patterns of *Klebsiella* and *E. aerogenes* strains. A: lanes - 1: *K. pneumoniae* ATCC 13883; 2: *K. pneumoniae* ATCC 10031; 3: *K. ozaenae* ATCC 25926; 4: *K. oxytoca* CTT 0182; 5: *K. planticola* ATCC 8329; 6: *E. aerogenes* ATCC 13048. B: lanes - 1: *K. pneumoniae* ATCC 13883, 2: *K. pneumoniae* ATCC 10031; 3: *K. ozaenae* ATCC 25926; 4 – 12: clinical isolates of *K. pneumoniae* K2-R, K6-R, K11-R, K13-R, K14-R, K15-R, K16-R, K19-R and K20-R (pattern T1). C: lanes - 1: *K. pneumoniae* ATCC 13883; 2 – 5: *K. pneumoniae* strains showing amplification patterns T2c (K4-C), T3 (K7-C), T2a (K17-R) and T2b (K18-R); 6: *E. aerogenes* ATCC 13048; 7 – 8: clinical isolates of *E. aerogenes* EA1-R and EA2-R. M: molecular size marker (100 bp).

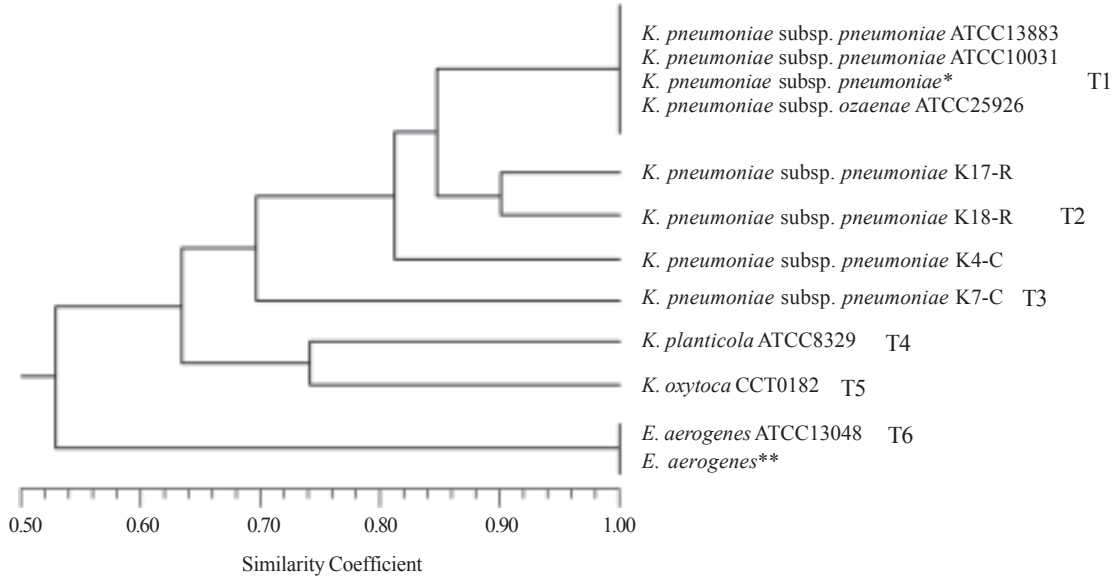

Fig. 3: dendrogram of tDNA-PCR patterns relatedness of clinical and reference strains of *Klebsiella* species and *E. aerogenes* generated with Simple Matching (SM) coefficient and the UPGMA clustering method. *: 39 clinical isolates of *K. pneumoniae* subsp. *pneumoniae* with band pattern T1; **: 2 clinical isolates of *E. aerogenes*; T1 to T6: tDNA-PCR patterns.

lyzed showed the same pattern as the type strain but a distinct pattern compared to the other species (Fig. 2C).

The dendrogram obtained from the clustering analysis of the tDNA-PCR fingerprinting is reported in Figure 3. *K. ozaenae* ATCC 25926, *K. pneumoniae* ATCC 13883 and ATCC 10031 reference strains and 39 *K. pneumoniae* clinical isolates clustering at 100% similarity and were grouped in pattern T1. The *K. pneumoniae* clinical isolates K17-R, K18-R and K4-C (pattern T2) which showed distinct band patterns from *K. pneumoniae* reference strains, clustering at 84%, 84% and 80%, respectively, with *K. pneumoniae* ATCC 13883. The isolate K7-C (pattern T3) showed 69% of similarity with ATCC 13883. The dendrogram also showed that *K. planticola* and *K. oxytoca* were grouped in different patterns, with a similarity coefficient of 74%. The species *K. planticola* appeared more closely related to *K. oxytoca* than to *K. pneumoniae*.

DISCUSSION

The identification of *K. pneumoniae* has been based on phenotypic methods such as biochemical profile analysis, which is time-consuming and often inconclusive since related species, like *K. pneumoniae* and *E. aerogenes*, frequently present similar biochemical patterns. Currently, the molecular methods that can be used to identify *K. pneumoniae* are sequencing of *rpoB*, *gyrA* and *parC* genes and 16S rRNA region (Brisse & Verhoef 2001, Drancourt et al. 2001). These methods are more complex and expensive than PCR based-methods.

To investigate the performance of PCR ribotyping and tDNA-PCR for identification of *K. pneumoniae* clinical isolates, these PCR methods were evaluated. No polymorphism was detected in the 16S and 23S ISRs among *K. pneumoniae* isolates from different geographical origins within Brazil, demonstrating that this species presents a specific PCR ribotyping pattern with the primers described by Kostman et al. (1992). Its amplification pattern differed from related species analyzed, showing that this technique is efficient for the molecular identification of *K. pneumoniae* isolates. In other reports, PCR ribotyping also did not show polymorphism of amplification patterns for different isolates of *Pseudomonas aeruginosa* (Agodi et al. 2000), *Bacillus cereus* (Daffonchio et al. 1998) and for various species of the genus *Salmonella* (Lagatolla et al. 1996), indicating that this method is efficient for the identification of these bacteria. On the other hand, isolates of *Enterobacter cloacae* (Clementino et al. 2001), and *Staphylococcus aureus* (Pereira et al. 2002), exhibit a high degree of polymorphism, and PCR ribotyping has therefore been indicated as a tool for the epidemiological study of these species.

The tDNA-PCR is useful for identifying microorganisms at species level. Most bacteria analyzed by this technique showed a single species-specific pattern (De Gheldre et al. 1999, Baele et al. 2000, Clementino et al. 2001). Therefore, some authors have emphasized the importance of tDNA-PCR as a tool for the identification of bacterial species. On the other hand, different profiles were detected by tDNA-PCR for *S. aureus*, *Staphylococcus haemolyticus* (Maes et al. 1997), *Bacillus licheniformis* (Daffonchio et al. 1998) and *P. aeruginosa* (Spacov et al. 2006). In the present report tDNA-PCR allowed the discrimination of the clinical *K. pneumoniae* isolates from Brazil into three major patterns, with a predominance of the group that showed the same profile as the *K. pneumoniae* reference strains. The division of *K. pneumoniae* into three patterns suggests that this species consists of at least three different lineages as determined by tDNA-PCR, in agreement with recent reports in Europe (Brisse & Verhoef 2001, Haeggman et al. 2004). Also based on tDNA-PCR analyses, *K. pneumoniae* subsp. *ozaenae* appeared closely related to *K. pneumoniae* subsp. *pneumoniae*. Moreover, whithin *K. pneumoniae* subsp. *pneumoniae*, the tDNA patterns observed here do not appear to correspond to the three taxonomic subspecies of *K. pneumoniae*. This was supported by tDNA-PCR analyses, as the reference strain for *K. pneumoniae* subsp. *ozaenae* revealed band patterns typical for pattern T1, like the majority of *K. pneumoniae* subsp. *pneumoniae* clinical isolates studied here. Futhermore, this was not due a misidentification of the four isolates that fell into patterns T2 and T3 since their biochemical characterization, by the Mini API ID 32E systems, revealed a profile typical for *K. pneumoniae* subsp. *pneumoniae*.

In the present study both tDNA-PCR and PCR ribotyping were efficient for the differentiation among *K. pneumoniae*, *K. oxytoca*, *K. planticola* and *E. aerogenes* reference strains, but could not differentiate *K. pneumoniae* subspecies. Brisse and Verhoef (2001) showed that de *gyrA* and *parC* sequence analysis also could not differentiate *K. pneumoniae* subspecies, but automated ribotyping with *Mlu*I could distinguish their three subspecies. On the other hand, the low variability in the 16S rRNA locus had been noted sometimes as an impediment in using 16S rRNA gene sequencing to discriminate *Klebsiella* at the genus and/or species level (Boye & Hansen 2003). The discriminative power of PCR ribotyping and tDNA-PCR for *Klebsiella* species were at the first time evaluated by this work, using both reference and wild strains. Although a PCR fingerprint will generally have less information than comparing the DNA sequence of a specific region in each organism, the reference strains of the four *Klebsiella* species tested showed clearly different ribotyping and tDNA-PCR patterns, demonstrating that these two methods are reliable for differentiation of the species within the genus *Klebsiella*.

The identification method may has high discrimination power and good reproducibility, the complexity of the method and interpretation of results as well as the costs involved in setting up and using the method may be beyond the capabilities of the laboratory. The PCR based-methods used in this work showed ease of implement and interpretation, low cost and good reproducibility and discrimination power. Additionally, they use universal primers that can type others bacteria species.

While tDNA-PCR demonstrated a genetic diversity between *K. pneumoniae* strains resulted of point mutations or rearrangements detected by scoring band presence versus absence in banding patterns in the DNA am-

plification procedures, PCR ribotyping showed a conserved and reprodutible PCR ribotype pattern suggesting being a suitable alternative method to conventional biochemical identification procedures for reliable identification of this microrganism.

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