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ASSESSMENT OF GENETIC RELATIONSHIP BETWEEN *Klebsiella pneumoniae* AND *Klebsiella oxytoca* SAMPLES ISOLATED FROM A DENTAL OFFICE

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ABSTRACT: The present study aimed to analyze the genetic similarity between genomic profiles of thirteen *Klebsiella oxytoca* and seven *Klebsiella pneumoniae* samples isolated from two different collections carried out in different places of dental offices. Random amplified polymorphic DNA (RAPD) technique and similarity coefficients (calculated by Sorensen-Dice and simple matching) were applied to determine their genetic profile of randomic DNA sequences. The majority of the isolates of *K. pneumoniae* and *K. oxytoca* presented similar coefficient values (≥ 0.80). Thus, it was possible to identify that strain dissemination occurred mainly via the hands of the surgeon-dentists and, finally, to determine the genetic similarity of the strains from dental office environments.

KEY WORDS: *Klebsiella oxytoca*, *Klebsiella pneumoniae*, RAPD, dental office.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Klebsiella pneumoniae and *Klebsiella oxytoca* are important opportunistic hospital-acquired pathogens that cause high morbidity and mortality rates among newborn infants, the elderly and immunocompromised patients. *K. oxytoca* cannot be affected by amino or carboxy penicillins; meanwhile, *K. pneumoniae* is highly resistant to broad-spectrum cephalosporins and aminoglycosides (4, 6). Outbreaks of these infectious species have been associated with a wide variety of sources and reservoirs, including lavatory basins and ultrasonographic gels (8, 18).

Hospital environments can be compared with dental offices, so that the control of emerging and persistent pathogenic microorganisms presents great importance (1). Both humidity and temperature of the oral cavity can create a wide range of habitats that present different environmental conditions and provide ideal media for microorganism colonization (8). Dental health professionals, due to their repeated exposure to these microorganisms, frequently are at high risk for developing infectious diseases (17).

Infection control efforts aim to identify the source of infection and the mode of transmission. Traditional techniques, based on phenotypic characteristics for classifying the *Klebsiella* genus, are often insufficiently sensitive for typing all strains and to discriminate among them (11). A distinctive polymorphism generated by the random amplified polymorphic DNA (RAPD) is a useful method for detecting strain differences and its ability to distinguish a wide variety of bacteria in a short time suggests that it constitutes a functional molecular epidemiological tool (2). Thus, the present study aimed to identify the transmission routes and genetic similarity of both microorganisms by the RAPD technique.

Thirteen *Klebsiella oxytoca* and seven *Klebsiella pneumoniae* samples from several sources (Table 1) were isolated and divided into two collections: the first was performed in April (Collection I) and the other in June (Collection II), both in 2006, within the Clinic of Surgery and Buco-Maxilo-Facial Traumatology, Ribeirão Preto University, São Paulo State, Brazil. The samples collected by conventional procedures employing swabs were submitted to growth in brain-heart infusion broth (BHI) (Biobrás, Brazil) directly plated on 5% sheep blood agar (Biobrás, Brazil) and MacConkey agar (Biobrás, Brazil), and were incubated under aerobic conditions for 24 hours at 37°C. The *Klebsiella oxytoca* and *Klebsiella pneumoniae* isolates were

identified by the Probac Kit® (Probac do Brasil, Brazil) and maintained as frozen stocks at –70°C in the presence of 15% glycerol. The bacterial strains were cultured in BHI for 24 hours, at 37°C, and finally centrifuged for 30 minutes at 14,000 rpm (Centrifuge 5417R®, Eppendorf AG, Germany) for pellet formation for DNA extraction.

Table 1. *Klebsiella oxytoca* and *Klebsiella pneumoniae* collections

Isolates	Collection area	Collection	Identification	Surgical procedure	
				Before	After
K. pneumoniae Total = 7	Lavatory basin	I	Pi8		X
	Mask	I	M13		X
	Gloves	II	L14	X	
	Hand skin	I / II	E49, E124/E34	X	X
	Eyeglasses	II	O48		X
K. oxytoca Total = 13	Away from lavatory basin	I	Pd43	X	
	Hand skin	I / II	E3, E31/E34, E35	X	X
	Reflector	I	R21		X
	Eyeglasses	I	O33	X	
	Gloves	II	L40, L41	X	X
	Tap	II	T44	X	
	Near lavatory basin	II	Pp45, Pp106	X	X
	Lavatory basin	I	Pi199	X	

Pi: isolates from lavatory basin; Pp: isolates from near lavatory basin; Pd: isolates away from lavatory basin; L: isolates from gloves; E: isolates from hand skins of surgeon-dentists; M: isolates from masks.

The extraction of genomic DNA was carried out using the GFX® Genomic Blood DNA Purification Kit (Amersham Pharmacia Biosciences Inc., UK) with modifications and adaptations to enzymatic buffers for cell lyses. For gram-negative strains, buffers employed were proteinase K (12 mM TRIS-HCl pH 8.0; 6 mM EDTA; 0.6% SDS) (Gibco-BRL, USA); and proteinase K (100 mg/mL in 10 mM, TRIS-HCl pH8.0) (Pharmacia Biotec, USA). The quantification of the genomic DNA was analyzed by

estimating the band intensity in comparison with λ phage DNA (Promega Inc., USA) via 1% agarose gel electrophoresis. For RAPD analysis, preliminary assays were carried out to test 25 primers synthesized by Operon Technologies (USA) and Amersham Pharmacia Biosciences Inc. (UK). The primer Operon 18 (5'-CAGCACCCAC-3') was selected for the study on isolates of *Klebsiella pneumoniae* and *Klesiella oxytoca*, based on the accuracy and reproducibility of the amplified profiles. The polymerase chain reactions (PCR) had been prepared and optimized specifically for both bacterial isolates according to Pimenta-Rodrigues (13); in a 30-µL tube were added 3 µL of buffer 10x (Phoneutria Biotecnologia e Serviços Inc., Brazil), DNTPs (25 mM) and $MgCl_2$ (25 mM) (Phoneutria Biotecnologia e Serviços Inc., Brazil); 5 µL of primers (10 ng/µL); 0.6 µL of *Taq* DNA polymerase (5 U/µL) (Phoneutria Biotecnologia e Serviços Inc., Brazil) and 3.0 µL genomic DNA (5 ng/µL). Amplification was performed in a thermocycler (PTC-100® Programmable Thermal Controller, MJ Researcher Inc., USA) programmed for two cycles of 2 minutes at 94°C, 1 minute at 37°C and 2 minutes at 72°C; followed by 33 cycles of 10 seconds at 94°C, 20 seconds at 40°C and 2 minutes at 72°C, and finally an amplification step of 5 minutes at 72°C. Amplification products were resolved by electrophoresis in a 2.0% agarose gel (Gibco/Invitrogen Amarillo, USA) in TBE buffer 1x (Tris 90 mM, boric acid 90 mM, EDTA 2 mM pH 8.0). The amplification products were stained with ethidium bromide and, thus, became visible under ultraviolet light, when they were photographed (Image Master® VDS, Pharmacia Biotech, USA). A 110 bp ladder (Amersham Pharmacia Biosciences Inc., UK) was included in each PCR run. The reproducibility of amplification results was evaluated in parallel experiments by three repetitions of the PCR reactions.

Similarity coefficients (S_{ab}) were calculated based on absence and/or presence of bands. Dendrograms of S_{ab} were generated using a binary code according to NTSYS program (Numerical Taxonomy and Multivariate Analysis System, version 2.1) (14). The S_{ab} between the patterns for each pair of A and B strains was calculated by the formula $\boldsymbol{S_{ab} = 2E/(2E + a + b)}$ using the Sorensen-Dice similarity coefficient that considers the joint absence of bands, and $\boldsymbol{S_{ab} = a + d/a + b + c + d}$ utilizing the simple matching similarity coefficient that does not consider the joint absence of bands, where ***E*** is the number of common bands in A and B, ***a*** is the number of bands only in isolated A, ***b*** is the number of bands only in isolated B and ***d*** is the

number of absent bands found in A and B isolates and also based on the Unweighted Pair Group Method with Arithmetic mean clustering method (UPGMA).

In the present study, the interpretation of these results was: for 1.0 similarity coefficient the isolates were designated genetically indistinguishable; for 0.99 to 0.80 coefficients, they were considered closely related (highly similar but not identical, however could be considered the same strain); for 0.79 to 0.50, isolates were possibly related; and for less than 0.50, they were considered unrelated (16). The averages established in the present study were performed according to Pfaller *et al*. (9); total S_{ab} was estimated based on S_{ab} values from the entire collection.

Of the total isolates, 50% were obtained from Collection I while 50% were from Collection II. And from the total, about 30% of *K. oxytoca* and about 20% of *K. pneumoniae* were found on the hand skins of surgeon-dentists and their gloves.

In each one of the thirteen *K. oxytoca* isolates, the PCR low accuracy permitted the amplification of 5 to 19 bands, ranging, in size, from 400 bp to about 2,000 bp (Figure 1). It was found that all isolates had a major band, about 1,000 bp. The isolates were grouped with a total S_{ab} of about 0.89, so that the dendrogram analysis revealed two distinct groups (Figure 2). Group I contained ten isolates subdivided into two subgroups, Ia (nine isolates with S_{ab} higher than 0.85) and Ib (only one isolate, from a lavatory basin, with S_{ab} around 0.6). Different isolates from Subgroup Ia, similar to S_{ab} 1.0, were derived from the skin, gloves and away from the lavatory basin. Group II, presenting three isolates from near the lavatory basin, tap and skin, had two isolates with coefficient S_{ab} 1.0.

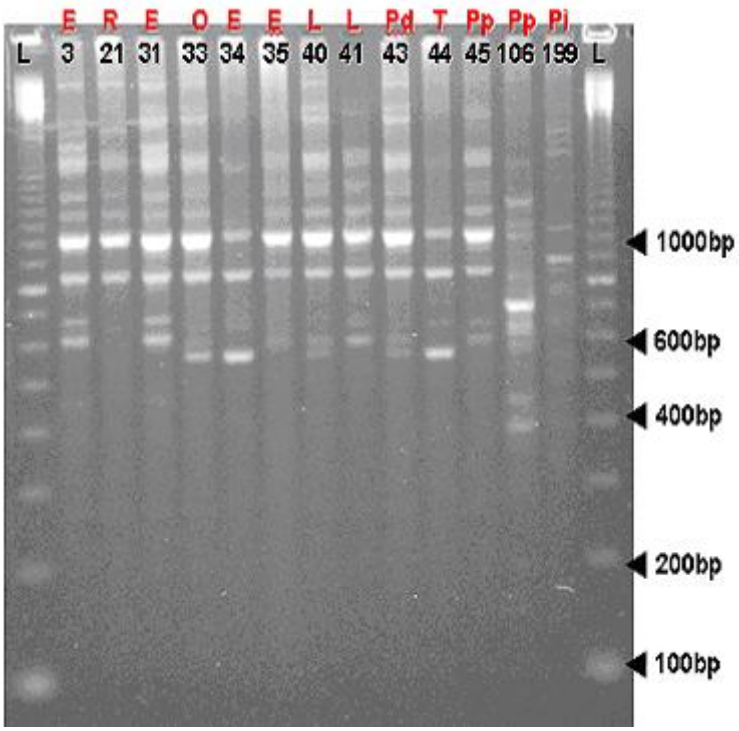
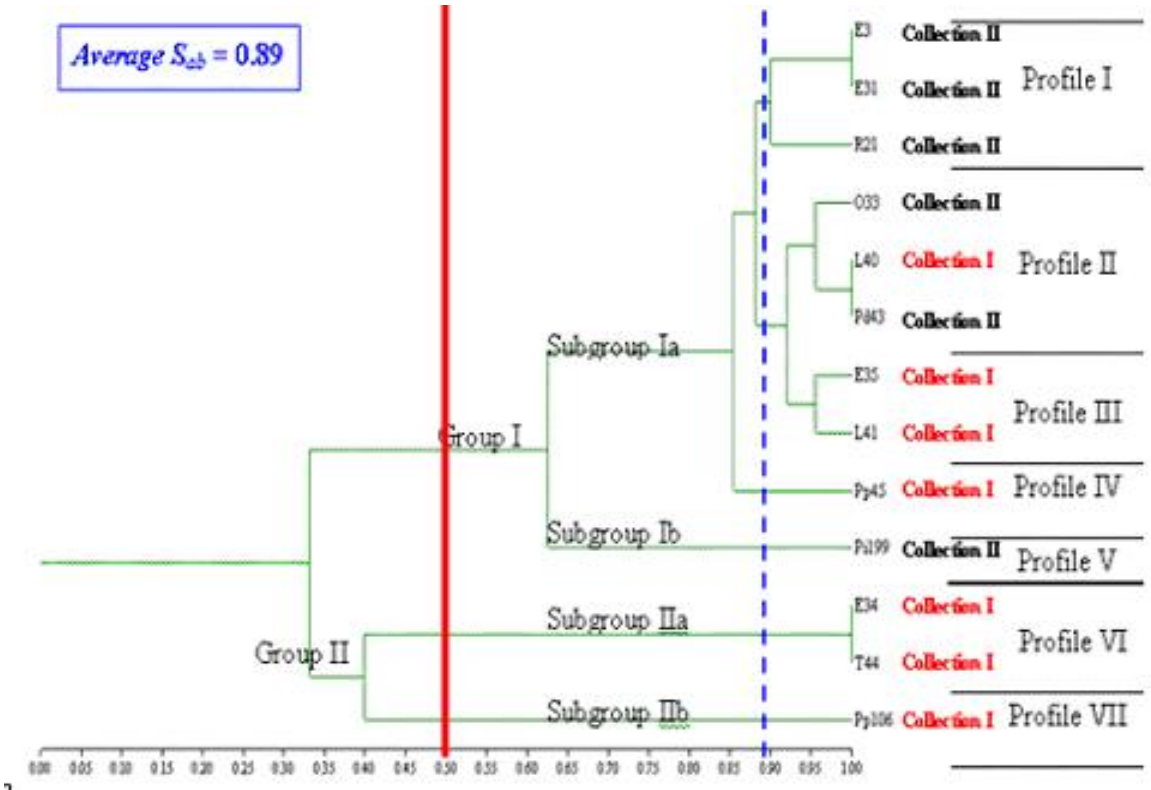


Figure 1. Gel electrophoresis and dendrogram of the RAPD profiles obtained from *Klebsiella oxytoca* isolates with primer Operon 18.

L*: ladder 100 bp; Pi: isolates from lavatory basin; Pp: isolates from near lavatory basin; Pd: isolates away from lavatory basin; L: isolates from gloves; O: isolates from eyeglasses; T: isolates from tap; E: isolates from hand skins of surgeon-dentists; R: isolates from reflector.



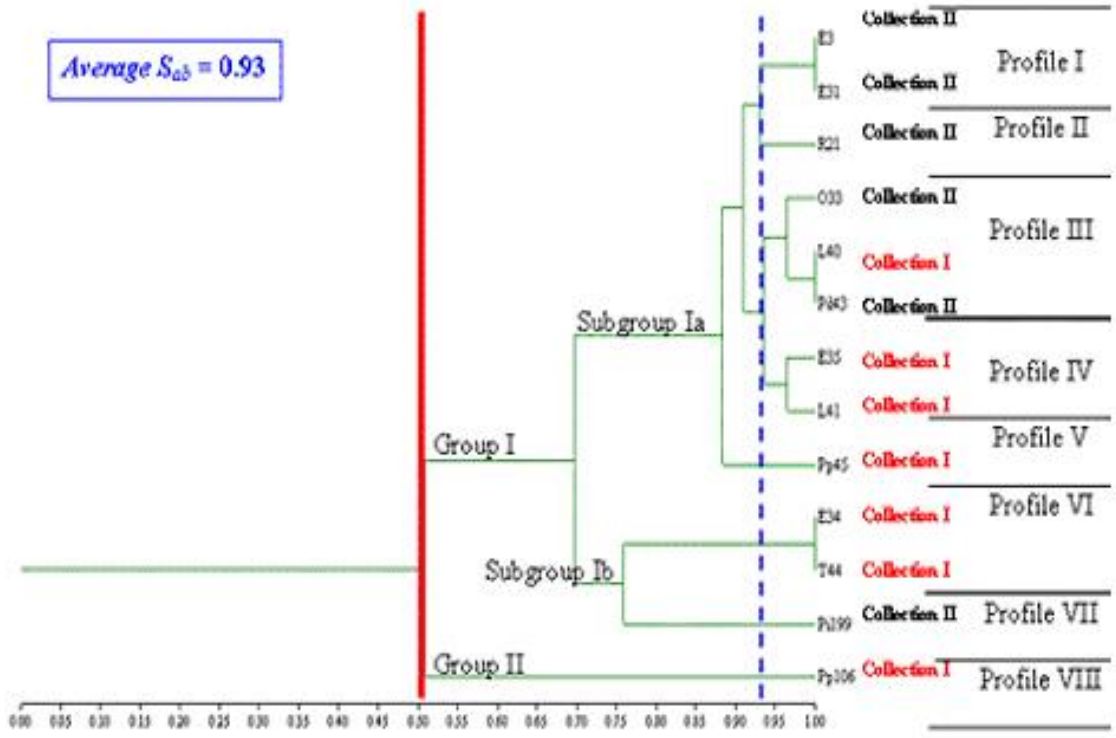
	E3	R21	E31	O33	E34	E35	L40	L41	Pd43	T44	Pp45	Pp106	Pi199
E3		0.9	1	0.9	0.2	0.9	0.86	0.86	0.86	0.28	0.86	0.31	0.63
R21	0.9		0.9	0.9	0.33	0.9	0.85	0.85	0.85	0.33	0.85	0.28	0.7
E31	1	0.9		0.9	0.28	0.9	0.86	0.86	0.86	0.33	0.86	0.31	0.63
O33	0.9	0.9	0.9		0.12	0.9	0.95	0.86	0.95	0.42	0.78	0.31	0.63
E34	0.2	0.33	0.28	0.12		0.28	0.4	0.26	0.4	1	0.26	0.4	0.36
E35	0.9	0.9	0.9	0.9	0.28		0.95	0.95	0.95	0.28	0.86	0.26	0.63
L40	0.86	0.85	0.86	0.95	0.4	0.95		0.91	1	0.4	0.83	0.33	0.6
L41	0.86	0.85	0.86	0.86	0.26	0.95	0.91		0.91	0.26	0.91	0.33	0.6
Pd43	0.86	0.85	0.86	0.95	0.4	0.95	1	0.91		0.4	0.83	0.33	0.6
T44	0.28	0.33	0.33	0.42	1	0.28	0.4	0.26	0.4		0.26	0.4	0.36
Pp45	0.86	0.85	0.86	0.78	0.26	0.86	0.83	0.91	0.83	0.26		0.41	0.6
Pp106	0.31	0.28	0.31	0.31	0.4	0.26	0.33	0.33	0.33	0.4	0.41		0.3
Pi199	0.63	0.7	0.63	0.63	0.36	0.63	0.6	0.6	0.6	0.36	0.6	0.3	

Figure 2. Dendrogram based on S_{ab} coefficients, showing the genetic similarity, based on RAPD-PCR standards, of *Klebsiella oxytoca* with primer Operon 18 using the Sorensen-Dice coefficient. S_{ab} average indicated by dotted line.

Pi: isolates from lavatory basin; Pp: isolates from near lavatory basin; Pd: isolates away from lavatory basin; L: isolates from gloves; O: isolates from eyeglasses; T: isolates from tap; E: isolates from hand skins of surgeon-dentists; R: isolates from the reflector.

Results obtained from *Klebsiella oxytoca* samples by simple matching coefficient corroborated the division into groups I and II with S_{ab} total average around 0.93 (Figure 3). Group I comprised twelve isolates subdivided into two subgroups, Ia (nine isolates with S_{ab} varying between 0.86 and 1.0) and Ib (three isolates from the

lavatory basin, tap and skin, including two isolates with 1.0 S_{ab} coefficient). Group II had only one isolate from the collection near the lavatory basin, with S_{ab} around 0.58. For *Klebsiella pneumoniae* the PCR reaction permitted the amplification of 9 to 16 bands ranging in size from 350 bp to 2,000 bp (Figure 4). The resultant dendrogram of the seven bacterial isolates, by the RAPD analysis, presented total S_{ab} around 0.79. All the isolates were aggregated into a single group and subdivided into two subgroups (Figure 5). Subgroup Ia presented two isolates (from hand skins of dentists and lavatory basin) that presented similarity coefficient around 0.90. Subgroup Ib was subdivided into two other subgroups: Ib1 and Ib2. In Ib2 only one isolate – from hand skins of dentists – was similar to the Subgroup Ib1, with S_{ab} around 0.67. The Subgroup Ib1 was further divided into Ib1A and Ib1B; one isolate of Ib1B was collected from eyeglasses and presented similarity coefficient near 0.70. Subgroup Ib1A consisted of two isolates with S_{ab} 1.0, originating from masks and gloves, and an additional isolate with similarity coefficient around 0.87 (on dentists' hands). The results from the simple matching coefficient, with S_{ab} total average 0.82, were similar to the Sorensen-Dice coefficient, although the difference was coefficient values that were higher for simple matching (Figure 6).



	E3	R21	E31	O33	E34	E35	L40	L41	Pd43	T44	Pp45	Pp106	Pi199
E3		0.93	1	0.93	065	0.93	0.89	0.89	0.89	0.65	0.89	0.48	0.75
R21	0.93		0.93	0.93	0.72	0.93	0.89	0.89	0.89	0.72	0.89	0.48	0.82
E31	1	0.93		0.93	0.65	0.93	0.89	0.89	0.89	0.65	0.89	0.48	0.75
O33	0.93	0.93	0.93		0.72	0.93	0.96	0.89	0.96	0.72	0.82	0.48	0.75
E34	0.65	0.72	0.65	0.72		0.65	0.68	0.62	0.68	1	0.62	0.68	0.75
E35	0.93	0.93	0.93	0.93	0.65		0.96	0.96	0.96	0.65	0.89	0.41	0.75
L40	0.89	0.89	0.89	0.96	0.68	0.96		0.93	1	0.68	0.86	0.44	0.72
L41	0.89	0.89	0.89	0.89	0.62	0.96	0.93		0.93	0.62	0.93	0.44	0.72
Pd43	0.89	0.89	0.89	0.96	0.68	0.96	1	0.93		0.68	0.86	0.44	0.72
T44	0.65	0.72	0.65	0.72	1	0.65	0.68	0.62	0.68		0.62	0.68	0.75
Pp45	0.89	0.89	0.89	0.82	0.62	0.89	0.86	0.93	0.86	0.62		0.51	0.72
Pp106	0.48	0.48	0.48	0.48	0.68	0.41	0.44	0.44	0.44	0.68	0.51		0.51
Pi199	0.75	0.82	0.75	0.75	0.75	0.75	0.72	0.72	0.72	0.75	0.72	051	

Figure 3**.** Dendrogram based on S_{ab} coefficients, showing the genetic similarity developed from RAPD-PCR standards of *Klebsiella oxytoca* with primer Operon 18 using the simple-matching coefficient. S_{ab} average indicated by dotted line.

Pi: isolates from lavatory basin; Pp: isolates from near lavatory basin; Pd: isolates away from lavatory basin; L: isolates from gloves; O: isolates from eyeglasses; T: isolates from tap; E: isolates from hand skins of surgeon-dentists; R: isolates from the reflector.

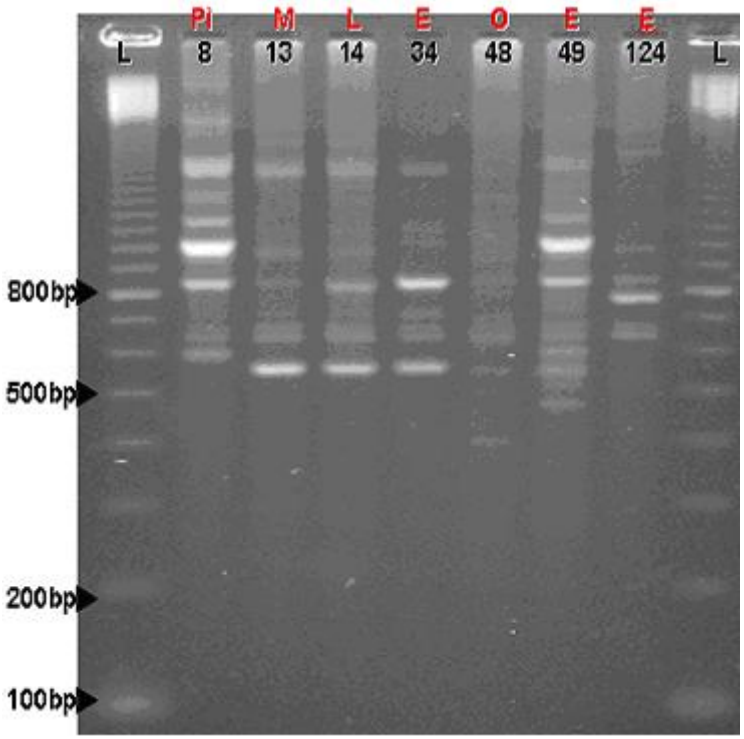
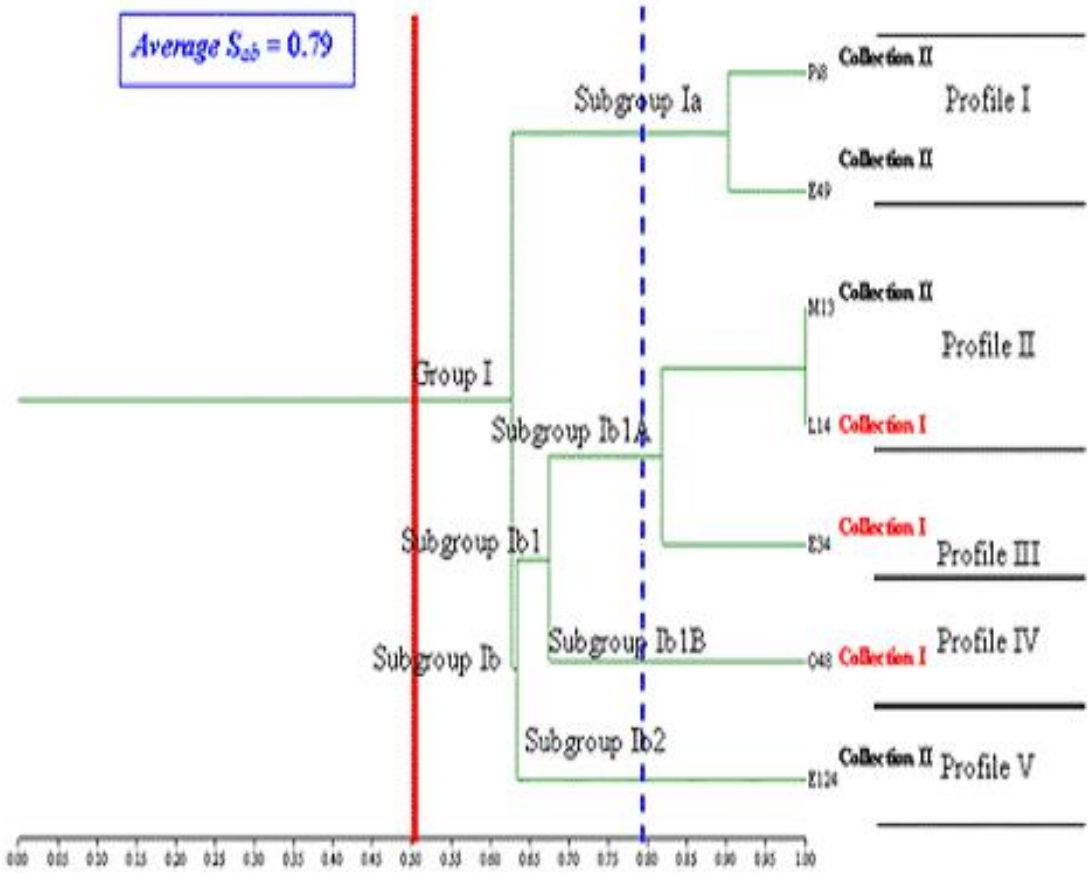


Figure 4. Gel electrophoresis and dendrogram of the RAPD profiles obtained from *Klebsiella pneumoniae* isolates with primer Operon 18.

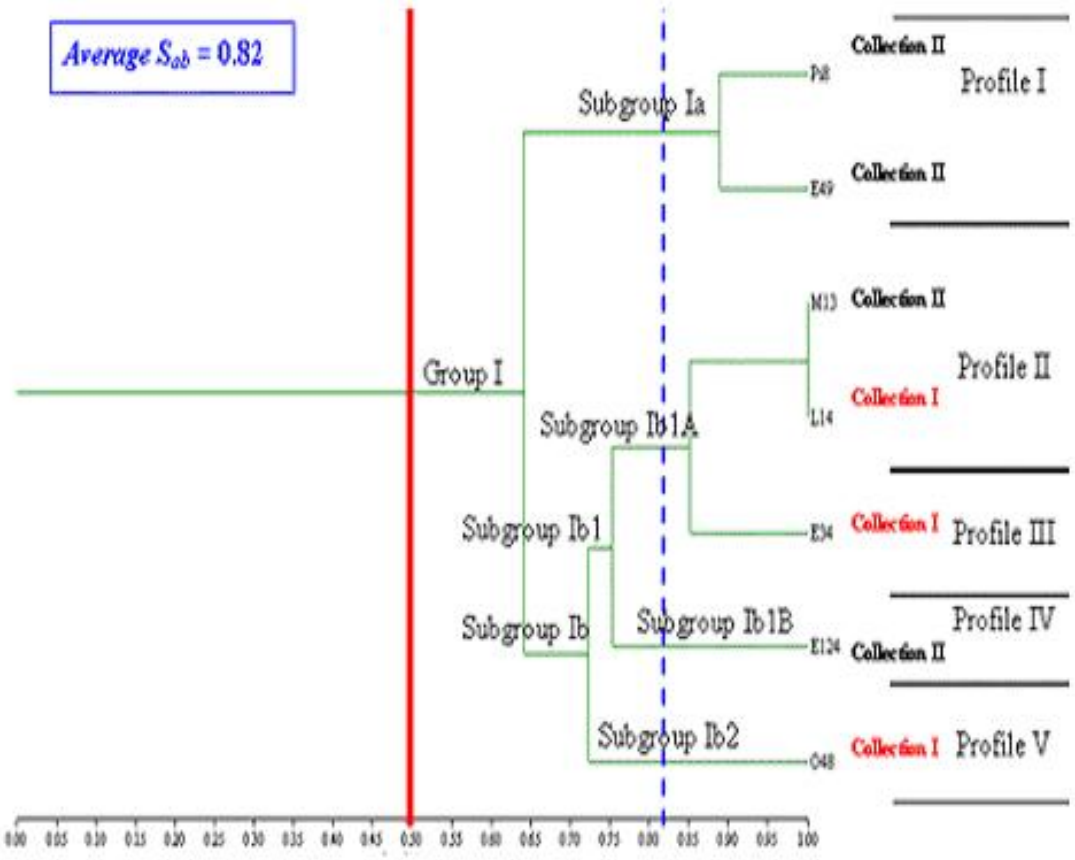
L*: ladder 100 bp; Pi: isolates from lavatory basin; L: isolates from gloves; O: isolates from eyeglasses; E: isolates from hand skins of surgeon-dentists; M: isolates from masks.



	Pi8	M13	L14	E34	O48	E49	E124
Pi8		0.71	0.71	0.5	0.56	0.9	0.66
M13	0.71		1	0.81	0.69	0.68	0.72
L14	0.71	1		0.81	0.69	0.68	0.72
E34	0.5	0.81	0.81		0.63	0.56	0.55
O48	0.56	0.69	0.69	0.63		0.61	0.52
E49	0.9	0.68	0.68	0.56	0.61		0.56
E124	0.66	0.72	0.72	0.55	0.52	0.56	

Figure 5. Dendrogram based on the S_{ab} coefficients showing the genetic similarity developed from RAPD-PCR standards of *Klebsiella pneumoniae* with primer Operon 18 by Sorensen-Dice coefficient. S_{ab} average indicated by the dotted line.

Pi: isolates from lavatory basin; L: isolates from gloves; O: isolates from eyeglasses; E: isolates from hand skins of surgeon-dentists; M: isolates from masks.



	Pi8	M13	L14	E34	O48	E49	E124
Pi8		0.7	0.7	0.55	0.59	0.88	0.7
M13	0.7		1	0.85	0.74	0.66	0.77
L14	0.7	1		0.85	0.74	0.66	0.77
E34	0.55	0.85	0.85		0.74	0.59	0.7
O48	0.59	0.74	0.74	0.74		0.62	0.66
E49	0.88	0.66	0.66	0.59	0.62		0.59
E124	0.7	0.77	0.77	0.7	0.66	0.59	

Figure 6. Dendrogram based on the S_{ab} coefficients showing the genetic similarity developed from RAPD-PCR standards of *Klebsiella pneumoniae* with primer Operon 18 for simple matching coefficient. S_{ab} average indicated by the hatched line.

Pi: isolates from lavatory basin; L: isolates from gloves; O: isolates from eyeglasses; E: isolates from hand skins of surgeon-dentists; M: isolates from masks.

The opportunistic pathogen *Klebsiella* ssp usually originate in immunocompromised individuals that are hospitalized and carry underlying illnesses such as *diabetes mellitus* or chronic pulmonary obstruction. The principal reservoirs of *Klebsiella* ssp are the gastrointestinal tract and the hands of hospital personnel (7). Based on a simple technique, RAPD is viable for large-scale use in epidemiological studies, in

association with bacterial samples that help to identify possible origins of contamination and infection. This method is sufficiently sensitive when parameters of reaction optimization and choice of primers are applied adequately (5, 12, 15, 19).

In the present study the isolate similarity profile was evaluated by two methods. In general terms, it was observed that, although the simple matching coefficient considers band joint absences, this method did not present considerable differences in relation to the Sorensen-Dice coefficient, which does not consider joint absence of bands. This fact can be explained on the basis that joint absences do not necessarily mean that DNA regions are identical, which suggests the use of any coefficient that does not consider the joint absence, as does Sorensen-Dice.

The phylogenetic analysis generated by the dendrograms enabled the characterization of *K. oxytoca* individuals. Through evaluation of S_{ab} values, it can be concluded that all isolates belonging to Ia, except for the sample Pi199, were the same strain; therefore, they present S_{ab} higher than 0.80 in relation to the isolates with S_{ab} 1.0. These isolates were collected from skin, eyeglasses, gloves, reflectors and places near lavatory basins, which suggests dissemination by hands. Additionally, as these isolates were present in different collections, we believe that this bacterial strain persisted in the clinical environment.

The examination of *K. pneumoniae* by RAPD permitted – through observation of S_{ab} values in Subgroup Ia – detection of the presence of two isolates, with S_{ab} 0.90, collected from lavatory basins and hand skins. This similarity coefficient value showed that these isolates were part of the same strain. Meanwhile, in Subgroup Ib1A that contained three isolates, two presented S_{ab} 1.0 and were derived from masks and gloves; and the other, with S_{ab} around 0.81, was from hand skins. These data imply dissemination by hands, since they present high similarity among isolates found on hand skins of dentists. These strains are supposed to be prevalent in clinical environments, since isolates from gloves were obtained in Collection I and those derived from masks, accquired in Collection II, present S_{ab} 1.0. Eisen *et al*. (3), through the phylogenetic analysis of *K. pneumoniae* isolates, found that handling (by medical staff) in newborn units was the usual non-environmental source of dissemination for these microorganisms.

Due to the close connection among these isolates, another hypothesis suggested that instead of being contaminated by infected hands, the environment itself could

disseminate microorganisms to hands, gloves and masks. This supposition agrees with Podschun *et al.* (10) who demonstrated the incidence of *Klebsiella* species on surface waters and proved its ability to express virulence factors, thus evidencing the importance of the presence of these species in clinical environments. In this case, both dissemination routes of closely related pathogenic strains offer risks of cross-contamination.

Knowledge of the genetic interactions among species of the genus *Klebsiella* provides a framework for studies on the distribution of phenotypic properties implicated in virulence or in epidemiological differences between clones (2). RAPD studies would be useful to determine whether the *K*. *pneumoniae* and *K*. *oxytoca* clusters correspond to distinct genomic species and given the proof that it is a useful technique to distinguish between related and unrelated isolates of *K. pneumoniae* and *K. oxytoca*. The capacity to detect genetic heterogeneity in different strains is important for the surveillance of odontological-acquired infections and may be used in epidemiologic studies, in association with strains that help identify the origin of contamination and infections.

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