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Identification of Clinical Isolates of Indole-Positive and Indole-Negative *Klebsiella* spp.▽

Maria Silvana Alves,[1,2] Rubens Clayton da Silva Dias,[2] Angela Christina Dias de Castro,[2] Lee W. Riley,[3] and Beatriz Meurer Moreira[2]*

Faculdade de Farmácia e Bioquímica, Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais,[1] and Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Rio de Janeiro,[2] Brazil, and School of Public Health, University of California, Berkeley, California[3]

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Biochemical methods employed to classify bacterial species have limitations and may have contributed to the taxonomic complexity recently reported for the genus *Klebsiella*. The objective of the present study was to apply a simple biochemical test panel to classify a collection of human *Klebsiella* isolates. We found that with only three additional tests, it is possible to place most isolates in a defined species. Analysis of a 512-bp sequence of the *rpoB* gene was used as the reference. A total of 16 conventional and 4 supplementary tests were used to evaluate 122 recent isolates identified as *Klebsiella* from 120 patients, isolated at the clinical laboratory of a university hospital in Minas Gerais, Brazil. Of these, 102 (84%) isolates were identified as *Klebsiella pneumoniae* or *Klebsiella variicola*, 19 (15%) as *Klebsiella oxytoca*, and 1 (1%) as *Raoultella planticola*. Enterobacterial repetitive intergenic consensus-PCR typing revealed a diversity of genotypes. *rpoB* gene sequencing confirmed the phenotypic identification and detected five *K. variicola* isolates among the *K. pneumoniae*/*K. variicola* group. Three additional tests that include growth at 10°C and histamine and D-melezitose assimilation should be considered essential tests for the typing of *Klebsiella* isolates.

Klebsiella spp. are opportunistic human pathogens that can be isolated from various animal and human clinical specimens (39). Between 1997 and 2002, these organisms were responsible for 7 to 10% of all hospital-associated bloodstream infections in Europe, Latin America, and North America, as reported by the SENTRY Antimicrobial Surveillance Program (3). Antimicrobial resistance represents a serious problem in this bacterial group, especially due to the increasing prevalence of extended-spectrum β-lactamase (ESBL)-producing isolates (11, 12, 26, 28, 29, 39, 43).

The phylogenetic structure of the genus *Klebsiella* has been recently reanalyzed (4, 6, 9, 13, 40), and all recent studies have shown the taxonomic complexity of this organism. Accordingly, by sequencing of the 16S rRNA genes and *phoE* genes, *Calymmatobacterium granulomatis* was renamed *Klebsiella granulomatis* (9); based on 16S rRNA genes and *rpoB* sequence analysis, the new genus *Raoultella* was proposed to accommodate *Klebsiella planticola*, *Klebsiella terrigena*, and *Klebsiella ornithinolytica* (13); two new *Klebsiella* species were described: *Klebsiella variicola*, derived from analysis of *rpoB*, *gyrA*, *mdh*, *infB*, *phoE*, and *nifH* sequences (40), and *Klebsiella singaporensis*, based on 16S rRNA genes and *rpoB* gene sequences (25); and finally, 16S rRNA gene sequences for two proposed species, *Klebsiella milletis* and *Klebsiella senegalensis*, were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (2003), but no description of these data has been published to date.

Subgroups within species were also determined. Based on nucleotide variations of the *gyrA*, *parC*, and *rpoB* genes, clinical isolates of *Klebsiella pneumoniae* fall into four phylogenetic groups, named KpI, KpII-A, KpII-B, and KpIII (6, 7, 17), with the newly described species *K. variicola* appearing to correspond to KpIII (40). In addition, *Klebsiella oxytoca* has been shown to include five distinct phylogenetic lines (KoI, KoII, KoIII, KoIV, and KoVI), which can be identified by 16S rRNA gene, *rpoB*, *gyrA*, *gapDH*, and bla_{OXY} sequencing (18).

The correct identification of *Klebsiella* species is not easily accomplished in most clinical microbiology laboratories, because several species share a similar biochemical profile. Studies have shown that a proportion of isolates classified as *K. pneumoniae* could in fact be *Raoultella planticola* (33, 45), *Raoultella terrigena* (33, 38), or *K. variicola* (5, 40), and some of the isolates classified as *K. oxytoca* actually could be *R. planticola* (33, 45). Even so, most clinical isolates classified as *Klebsiella* spp. belong to the *K. pneumoniae* (indole-negative isolates) or *K. oxytoca* (indole-positive isolates) species (22, 27, 39, 44). However, *K. variicola*, *R. planticola*, and *R. terrigena*, in addition to *K. pneumoniae*, can show a negative-indole reaction (5, 32, 34, 40, 45). On the other hand, *R. planticola* can reveal a positive-indole reaction, in addition to *K. oxytoca* (33, 45). Most test kits and automated methods do not yet include many of these organisms in their identification databases nor the substrates that would differentiate them (45). For example, *K. singaporensis* may be recognized by the ability to grow at 10°C, being positive by the Voges-Proskauer test, and having negative indole production and L-sorbose fermentation. However, to date, only one isolate of this species has been found, obtained from soil (25).

Several groups of biochemical tests have been proposed to allow differentiation among some of the most common *Klebsiella* species. A scheme was proposed by Monnet and Freney

* Corresponding author. Mailing address: Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco I, Cidade Universitária, Rio de Janeiro, RJ 21941-590, Brazil. Phone: 55-21-25626745. Fax: 55-21-22608344. E-mail: bmeurer@micro.ufrj.br.

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in 1994 (32) and included four carbon substrate assimilation tests: ethanolamine, histamine, D-melezitose, and DL-3-hydroxybutyrate, in addition to indole production and ornithine decarboxylation. All *Raoultella* isolates tested in this study were histamine and DL-3-hydroxybutyrate positive and ethanolamine and D-melezitose negative, with the exception for *R. terrigena*, which revealed a positive D-melezitose test (32). The limitation of this scheme is that the authors used five different combinations of the Analytical Profile Index system for preliminary identification, a procedure unfeasible in the routine work of most clinical microbiology laboratories. Another important test was proposed by Drancourt and collaborators: the ability to grow at 10°C would be a hallmark of the genus *Raoultella*, while all species of *Klebsiella*, with the exception of *K. oxytoca*, would not grow at this temperature (13). Rosenblueth and collaborators found that the inability to ferment adonitol could be characteristic of *K. variicola*, while most *K. pneumoniae* strains were able to ferment this carbohydrate (40). However, Brisse and collaborators (2004) reported later that KpI, KpII, and KpIII are adonitol variable (7), putting into question the usefulness of adonitol fermentation as a differential test. Finally, in 2004, Hansen and collaborators (23) proposed a test panel consisting of 18 biochemical tests to differentiate *Klebsiella* species. However, no reference taxonomic methods were employed by those authors to validate their proposal.

The development of methods for rapid routine differentiation of *Klebsiella* isolates in clinical microbiology laboratories is necessary to clarify whether or not significant epidemiological or pathogenic differences exist between the *Klebsiella* species (27). According to Brisse and Verhoef (2001) (6), the association between genetic variability and virulence and transmissibility of *Klebsiella* strains is not well understood, but there is clear evidence for varied behavior of this bacterial group. In addition, unlike the case with community outbreaks, typing of organisms in hospital outbreaks may be essential for identifying chains of transmission because of the ubiquitous nature of microorganisms in the hospital environment.

The objective of the present study was to apply a simple biochemical test panel to identify a recent collection of human *Klebsiella* isolates and to propose that with only three additional tests, it is possible to place most isolates into a defined species. We used the analysis of the *rpoB* gene sequence as the reference method for the final identification of isolates to the species level.

MATERIALS AND METHODS

Bacterial isolates. From March 2002 to March 2004, 122 clinical *Klebsiella* isolates were sequentially recovered from 120 patients (58 inpatients and 62 outpatients) from Hospital Universitário of Universidade Federal de Juiz de Fora, in the state of Minas Gerais, southwest region of Brazil. Only one isolate per patient was included in this study, except for two patients, who had one *K. pneumoniae* isolate and one *K. oxytoca* isolate each. In addition, two isolates classified as *R. planticola* obtained from the same patient were included, but only for the analysis of the *rpoB* gene sequence. Isolates were obtained from urine (86 isolates; 71%), blood (15 isolates; 12%), various secretions (12 isolates; 10%), catheter tip (4 isolates; 3%), peritoneal fluid (3 isolates; 2%), and other sites (2 isolates; 2%). Reference strains included *Escherichia coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *K. pneumoniae* ATCC 700603, *R. planticola* ATCC 33531, *R. terrigena* ATCC 33257, and one *K. oxytoca* isolate previously characterized in our laboratory (*K. oxytoca* AP). All bacterial isolates were grown on MacConkey agar and stored as suspensions in a 10% (wt/vol) skim milk solution containing 10% (vol/vol) glycerol at −20°C for later analysis.

Biochemical methods. All bacterial isolates were identified as *Klebsiella* spp. in the clinical laboratory by nonautomated biochemical tests. All isolates were reevaluated in 16 well-established biochemical tests for *Enterobacteriaceae* (16) and four supplementary tests selected from those recommended in references 13, 23, and 32. Initial tests followed the protocols suggested by MacFaddin (30) and included the oxidase test; glucose and lactose or sucrose fermentation, gas and H_2S production in triple sugar iron agar; motility and indole production in sulfide indole motility medium; citrate and malonate utilization; arginine, lysine, and ornithine decarboxylation; phenylalanine deamination; urease production; adonitol fermentation; and methyl red and Voges-Proskauer tests. Supplementary tests included growth at 10°C and L-sorbose fermentation (15) and histamine and D-melezitose assimilation (32). Carbohydrates were obtained from Sigma Chemical Co., St. Louis, MO. The bacterial inocula were prepared as 0.5 McFarland standard turbidity suspensions obtained from cells grown in solid medium; 10-μl aliquots were used for growth at 10°C and adonitol and L-sorbose tests, and 100-μl aliquots were used for histamine and D-melezitose tests. Incubation times were 2 days for the histamine and D-melezitose assimilation tests, 3 days for growth at 10°C, and up to 7 days for L-sorbose and adonitol tests. The incubation temperature was 35°C, except for histamine and D-melezitose (30°C) and growth at 10°C. A bromothymol blue pH indicator was used for the adonitol test (19) and Andrade's indicator for the L-sorbose test (15). For one isolate (K124) with a profile suggestive of *K. oxytoca* that was repetitively indole negative in sulfide indole motility medium, indole production was evaluated by dropping Kovac's reagent over colonies grown overnight on Fluorocult ECD Agar (Diagnostica Merck, Darmstadt, Germany).

Sequence analysis of the *rpoB* gene. A total of 47 isolates (30 putative *K. pneumoniae*/*K. variicola*, 15 putative *K. oxytoca*, and 2 putative *R. planticola*) were included in the analysis of *rpoB*. DNA was extracted by thermal lysis (36), and ~1,000-bp fragments of the *rpoB* gene were PCR amplified with oligonucleotide primers CM_7 (5′-AACCAGTTCCGCGTTGGCCTGG-3′) and CM_{31b} (5′-CCTGAACAACACGCTCGGA-3′) (13). Amplicons were purified with the QIAquick gel extraction kit (QIAGEN, Inc., Valencia, CA), and DNA sequencing of both strands was carried out at the DNA Sequencing Facility of the University of California, Berkeley. Fragments of 512-bp overlapping sequences were analyzed by BLASTn (http://www.ncbi.nlm.nih.gov) to confirm the identity of the isolates and then aligned by the ClustalW Multiple Alignment of BioEdit Sequence Alignment Editor, version 7.0.4.1. The alignment data were assessed by bootstrap analyses based on 1,000 resamplings. Aligned sequences were examined for molecular evolutionary relationships by the neighbor-joining distance method with the Jukes-Cantor parameter model. A phylogenetic tree was obtained by TREECON for Windows version 1.3b (University of Konstanz). The significance of the branching order was evaluated by bootstrap analysis with 100 replicates. The *rpoB* sequences of the following strains were obtained from the GenBank database and included in the phylogenetic analysis: *K. pneumoniae* subsp. *pneumoniae* ATCC 13883 (accession no. U77444), *K. pneumoniae* subsp. *ozaenae* ATCC 11296 (accession no. AF129445), *K. pneumoniae* subsp. *rhinoscleromatis* ATCC 13884 (accession no. AF129446), *K. oxytoca* ATCC 13182 (accession no. U77442), *K. variicola* ATCC BAA-830 (accession no. AY367356), *R. planticola* ATCC 33531 (accession no. AF129449), *R. terrigena* ATCC 33257 (accession no. AF129448), *Raoultella ornithinolytica* ATCC 31898 (accession no. AF129447), and *Staphylococcus sciuri* subsp. *carnaticus* ATCC 700058 (accession no. DQ120748) as an outgroup. *K. granulomatis* and *K. singaporensis* were not included in the phylogenetic analysis because isolates of these species cannot be cultured on conventional media (8) or are not obtained from human clinical specimens (25).

Antimicrobial susceptibility testing and ESBL detection. Bacterial susceptibility was determined by disk diffusion in accordance with CLSI guidelines (10, 35) for the following agents: amikacin, amoxicillin-clavulanate, ampicillin, aztreonam, cefepime, cefotaxime, cefoxitin, ceftazidime, cephalothin, ciprofloxacin, gentamicin, imipenem, and trimethoprim-sulfamethoxazole. ESBL production was determined by the combination of oxiimino-cephalosporins and clavulanate (10, 35).

Strain typing by ERIC2-PCR. DNA was extracted by thermal lysis (36). PCRs were prepared in a total volume of 25 μl, containing 3 mM $MgCl_2$, 0.3 μM ERIC2 primer, and 3 μl of bacterial DNA. Amplification conditions were 94°C for 2 min; 40 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 4 min; and a final step at 72°C for 1 min (36). Banding profiles were analyzed by GelComparII, version 3.5 (Applied Maths, Kortrijk, Belgium) by the Dice index and the unweighted-pair group method with arithmetic averages.

GenBank accession numbers. The *rpoB* gene sequences analyzed are deposited under numbers DQ415473 to DQ415498 (*K. pneumoniae*), DQ415458 to

TABLE 1. Biochemical reactions for differentiation of 122 *Klebsiella* isolates

Biochemical test	No. (%) of isolates with positive test[a]				
	K. pneumoniae/*K. variicola*	*K. pneumoniae*[b]	*K. variicola*[b]	*K. oxytoca*	*R. planticola*
Lactose/sucrose	101 (99)	25 (100)	5 (100)	19 (100)	1
H_2S	0	0	0	0	0
Indole	0	0	0	19 (100)	1
Motility	0	0	0	0	0
Citrate	102 (100)	25 (100)	5 (100)	19 (100)	1
Lysine	99 (97)	23 (92)	4 (80)	19 (100)	1
Ornithine	0	0	0	0	0
Arginine	0	0	0	0	0
Phenylalanine	0	0	0	0	0
Methyl red	5 (5)	3 (12)	2 (40)	3 (16)	1
Voges-Proskauer	98 (96)	23 (92)	3 (60)	19 (100)	0
Urease	100 (98)	23 (92)	5 (100)	19 (100)	1
Adonitol	89 (87)	22 (88)	1 (20)	NT[c]	NT
Malonate	96 (94)	21 (84)	5 (100)	18 (95)	1
L-sorbose	47 (46)	13 (52)	5 (100)	19 (100)	1
Growth at 10°C	0	0	0	19 (100)	1
Histamine	0	0	0	0	1
D-melezitose	0	0	0	16 (84)	0

[a] For *K. pneumoniae*/*K. variicola*, $n = 102$; for *K. pneumoniae*, $n = 25$; for *K. variicola*, $n = 5$; for *K. oxytoca*, $n = 19$; for *R. planticola*, $n = 1$.
[b] Includes only isolate with identification to species level by *rpoB* sequencing.
[c] NT, not tested.

DQ415472 (*K. oxytoca*), DQ415499 to DQ415503 (*K. variicola*), and DQ415504 to DQ415505 (*R. planticola*).

RESULTS

Biochemical identification. All 122 clinical isolates but 1 formed typical red colonies, indicating fermentation of lactose and acid production on MacConkey agar. All of these isolates were oxidase, H_2S, arginine, ornithine, and phenylalanine negative, glucose fermenters, and citrate positive and were nonmotile typical gram-negative bacilli. Therefore, all were confirmed as belonging to the genus *Klebsiella* or *Raoultella*. A total of 102 (84%) of the 122 isolates were negative for indole production, unable to assimilate histamine and D-melezitose or to grow at 10°C. All of these 102 isolates were identified as possible *K. pneumoniae*/*K. variicola*, as shown in Table 1. Of the total, 25 isolates (25%) showed from 1 to 3 variations in other biochemical tests. Another 19 isolates (15%) were positive for indole and urease production, L-sorbose fermentation, lysine decarboxylation, Voges-Proskauer test, and growth at 10°C and negative for histamine assimilation. They were identified as possible *K. oxytoca* (Table 1). Finally, one isolate (1%) was positive for indole production, histamine assimilation, and growth at 10°C and negative for ornithine and D-melezitose. This isolate was identified as possible *R. planticola*. No isolates corresponding to *K. singaporensis*, *R. ornithinolytica*, or *R. terrigena* species were identified in the present study.

***rpoB* gene sequences and phylogenetic analysis.** Among 30 possible *K. pneumoniae*/*K. variicola* isolates sequenced, the BLASTn search revealed, for 25 isolates, 98% to 100% *rpoB* sequence similarity to known *K. pneumoniae* strains, and for the other 5 isolates, 99 to 100% similarity to known *K. variicola* strains, including the type strain *K. variicola* ATCC BAA-830. The *K. variicola* strains were obtained from urine (three), catheter tip (one), and wound (one). The results for adonitol fermentation (40), the only test proposed to date to differentiate *K. pneumoniae* (positive) from *K. variicola* (negative), revealed that the 25 *K. pneumoniae* isolates included 22 positive and 3 negative ones, and the 5 *K. variicola* isolates included 4 negative isolates and 1 positive isolate. Likewise, the *rpoB* sequences of 15 putative *K. oxytoca* isolates exhibited 98 to 100% similarity to known *K. oxytoca* strains, including the type strain *K. oxytoca* ATCC 13182. Finally, the two putative *R. planticola* isolates (K111 and K112) exhibited 98% *rpoB* sequence similarity to known *R. planticola* strains, including the type strain *R. planticola* ATCC 33531.

Phylogenetic analysis of *rpoB* gene sequences demonstrated the separation of possible species into four distinct clusters, which we named Kp, Kv, Rp/Ro, and Ko (Fig. 1). Cluster Kp included the sequences of 25 *K. pneumoniae* isolates and strains *K. pneumoniae* subsp. *pneumoniae* ATCC 13883, *K. pneumoniae* subsp. *ozaenae* ATCC 11296, and *K. pneumoniae* subsp. *rhinoscleromatis* ATCC 13884; cluster Kv included the sequences of the 5 *K. variicola* isolates and strain *K. variicola* ATCC BAA-830; cluster Rp/Ro included the *R. planticola* isolates and strains *R. planticola* ATCC 33531 and *R. ornithinolytica* ATCC 31898; and finally, cluster Ko included all but 1 (K82) of the *K. oxytoca* isolates and strain *K. oxytoca* ATCC 13182. Isolate K82 showed a biochemical profile compatible to that of *K. oxytoca*, with one test variation (malonate negative). The BLASTn search revealed an *rpoB* sequence similarity of 99% between this isolate and *K. oxytoca* strain SG266 (accession no. AJ871806). In conclusion, *rpoB* sequencing validated the phenotypic results, but *K. variicola* was not separated from *K. pneumoniae* by the biochemical tests. Table 2 summarizes test results that could separate *Klebsiella* species.

To discriminate phylogenetic groups within the *K. pneumoniae*/*K. variicola* and *K. oxytoca* strains, we obtained new trees (data not shown) that included the *rpoB* sequences analyzed by Fevre and collaborators (17, 18) (GenBank accession numbers AM051162 to AM051186 and AJ871801 to AJ871818).

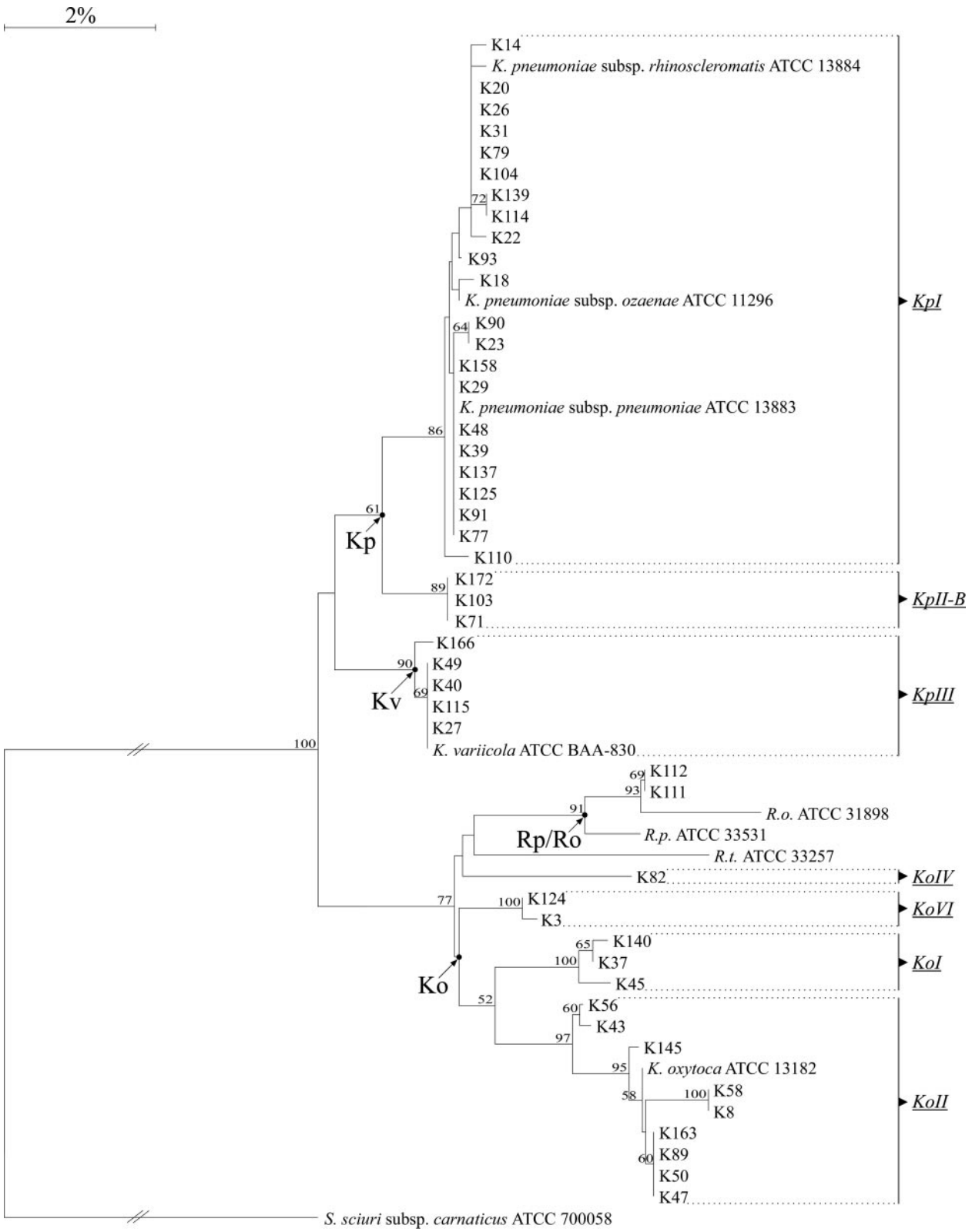

FIG. 1. Phylogenetic tree derived from partial *rpoB* sequences of 47 *Klebsiella* isolates and type strains determined by the neighbor-joining distance method, using the Jukes-Cantor parameter model. Numbers within the tree indicate the occurrence (%) of the branching order in 100 bootstrapped trees. Only values above 50 are shown. The scale bar indicates 2% divergence. Clusters Kp, Kv, Rp/Ro, and Ko are indicated by arrows. Intraspecies groups determined in two other trees (not shown) that included also the *rpoB* sequences studied by Fevre and collaborators (17, 18) are indicated by square brackets.

TABLE 2. Differentiation of *Klebsiella* and *Raoultella* species obtained from human clinical specimens according to results of the present study and references 13, 23, and 32

Bacterial species[a]	Test result[b]				
	Indole	Growth at 10°C	Histamine	D-melezitose	Ornithine
K. pneumoniae/*K. variicola*	−	−	−	−	−
K. oxytoca	+	+	−	+/−	−
R. planticola	+/−	+	+	−	−
R. terrigena	−	+	+	+	−
R. ornithinolytica	−	+	+	−	+

[a] *K. granulomatis* and *K. singaporensis* are not included because isolates of these species cannot be cultured on conventional media (8) or are not obtained from human clinical specimens (25).

[b] −, negative reaction; +, positive reaction; +/−, either positive or negative reaction.

TABLE 3. Antimicrobial resistance of 121 *Klebsiella* isolates evaluated in the present study

Antimicrobial agent	No. (%) of resistant isolates[a]	
	K. pneumoniae/*K. variicola*	*K. oxytoca*
Amikacin	33 (32)	5 (26)
Amoxicillin-clavulanate	7 (8)	6 (32)
Ampicillin	102 (100)	19 (100)
Aztreonam	19 (19)	9 (47)
Cefepime	9 (9)	5 (26)
Cefoxitin	9 (9)	3 (16)
Ceftazidime	14 (14)	1 (5)
Cefotaxime	25 (25)	8 (42)
Cephalothin	43 (42)	12 (63)
Ciprofloxacin	12 (12)	6 (32)
Gentamicin	23 (23)	9 (47)
Imipenem	0	0
Trimethoprim-sulfamethoxazole	36 (35)	8 (42)

[a] For *K. pneumoniae*/*K. variicola*, $n = 102$; for *K. oxytoca*, $n = 19$.

Clusters obtained in these trees are indicated in Fig. 1. Of all subgroups described, only KpII-A and KoIII were not found within our study collection. Of note, the KpI subgroup comprised 22 (73%) of the 30 *K. pneumoniae*/*K. variicola* isolates sequenced. The KpIII subgroup included all five *K. variicola* isolates. Considering the five Ko genetic groups described by Fevre and collaborators (18), in our collection we observed that 9 isolates (60% of the 15 isolates sequenced), together with the *K. oxytoca* ATCC 13182 strain, belong to KoII group, and a few other isolates belong to groups KoI, KoVI, and KoIV.

Antimicrobial susceptibility testing and extended spectrum β-lactamase production. Antimicrobial resistance rates among *K. pneumoniae*/*K. variicola* and *K. oxytoca* isolates are described in Table 3. All isolates were resistant to ampicillin and susceptible to imipenem. Trimethoprim-sulfamethoxazole and cephalotin resistance rates were very high (more than 30%) for both bacterial groups. ESBL production was observed in 28% (29 of 102) of the *K. pneumoniae*/*K. variicola* isolates and 47% (9 of 19) of the *K. oxytoca* isolates. The *R. planticola* isolate was resistant only to ampicillin and did not reveal ESBL production.

Enterobacterial repetitive intergenic consensus-PCR typing. We typed 94 *Klebsiella* isolates: 73 of *K. pneumoniae*/*K. variicola*, including all 29 ESBL-producing isolates and the 5 *K. variicola* isolates; 19 *K. oxytoca* isolates, and the 2 *R. planticola* isolates. Three type strains (*K. pneumoniae* ATCC 700603, *R. planticola* ATCC 33531, and *R. terrigena* ATCC 33257) and one strain previously characterized in our laboratory (*K. oxytoca* AP) were also analyzed. Strain *R. planticola* ATCC 33531 was included as a control in each amplification. Each isolate presented 4 to 12 bands, ranging from 200 to 5,000 bp. Among the *K. pneumoniae*/*K. variicola* isolates, one cluster was observed with two (K62 and K65) isolates. Each one of the isolates with a final identification of *K. variicola* presented a different profile. Among the *K. oxytoca* isolates, only one cluster of two isolates (K43 and K56) was observed. The two *R. planticola* isolates presented identical profiles (not shown).

DISCUSSION

Klebsiella spp. are difficult to identify and are often misclassified in clinical microbiology laboratories (23, 32, 33, 38, 45). However, the correct identification of *Klebsiella* isolates is important for improved taxonomic and molecular epidemiologic characterization of this bacterial group.

In the present study, we characterized a collection of 122 recent *Klebsiella* isolates with a panel of 20 biochemical tests, including 4 supplementary tests not routinely used in the identification of enterobacterial isolates: growth at 10°C, L-sorbose fermentation, and histamine and D-melezitose assimilation. Phenotypic results were compared with those of *rpoB* sequencing. *K. pneumoniae*/*K. variicola*, *K. oxytoca*, and *R. planticola* were easily and accurately identified. However, the *K. pneumoniae*/*K. variicola* isolates could be differentiated in *K. pneumoniae* or *K. variicola* species only by genotyping. The isolate classified as *R. planticola*, originally identified as *K. oxytoca*, had positive histamine and negative ornithine and D-melezitose test results (32). Based on the results of the present study and those obtained by others (13, 23, 32), we propose that three supplemental tests, growth at 10°C and histamine and D-melezitose assimilation, should be routinely used in the identification of *Klebsiella* isolates, as summarized in Table 2. Our results demonstrated that the L-sorbose fermentation test was not essential for the correct identification of *Klebsiella* isolates at the species level. The same observation was earlier made by Hansen and collaborators (23). We agree with those authors that if the number of tests is to be reduced, L-sorbose could be omitted.

According to Rosenblueth and collaborators (40), *K. variicola* isolates do not ferment adonitol, a general characteristic of *K. pneumoniae* strains. However, some *K. pneumoniae* isolates can be adonitol negative (16, 46), but *K. variicola* (or KpIII isolates) can be adonitol positive (7). Therefore, it does not seem possible to identify *K. variicola* based only on a negative adonitol test result. In the present study, of the five isolates that clustered with *K. variicola* ATCC BAA-830, four were adonitol negative but one was positive. On the other hand, among 25 *K. pneumoniae* isolates confirmed by *rpoB* gene sequencing, 3 (12%) tested adonitol negative. Currently, *K. variicola* is distinguished from *K. pneumoniae* and other *Klebsiella* species only by *rpoB*, *gyrA*, *mdh*, *phoE*, *infB*, and *nifH*

gene sequencing or by low levels of DNA hybridization with other species (40).

K. pneumoniae/*K. variicola* were the most common isolates (102 isolates; 84%), followed by *K. oxytoca* (19 isolates; 15%) and *R. planticola* (1 isolate; 1%). The relative frequencies of isolation of *K. pneumoniae*/*K. variicola* and *K. oxytoca* in our population are in agreement with those described by other authors (21, 44). However, the relative frequency of *R. planticola* isolation is much lower than that reported by Monnet and collaborators in France (8% of 204 isolates obtained from cardiology and neurology hospitals) (33), by Mori and collaborators in Japan (19% of 439 isolates from the Central Laboratories, Nagoya University Hospital, and several regional general hospitals) (34), and by Podschun and collaborators in Germany (9% of 131 isolates from a pediatric clinic, University of Heidelberg) (37). A small percentage of *R. planticola* isolates, as we observed, was also detected by Westbrook and collaborators in the United States: 0.4% of a collection of 352 stock isolates from the Centers for Disease Control and Prevention, including 43 stock isolates of *K. oxytoca* obtained from newborns and 84 fresh clinical isolates randomly collected from four hospital laboratories in Atlanta (45).

The *rpoB* gene, which encodes the bacterial RNA polymerase β-subunit, has been used as a signature for bacterial identification and as a locus for phylogenetic analysis (31). In 2005, Drancourt and Raoult proposed that bacterial isolates with less than 97% similarity in the 16S rRNA genes or *rpoB* gene sequences belong to different species (14). In the present study, the BLASTn search revealed *rpoB* sequence similarities greater than 98% for the classification suggested by the biochemical profiles. Therefore, *rpoB* sequencing confirmed the biochemical identification for all 47 isolates analyzed: 25 *K. pneumoniae*, 5 *K. variicola*, 15 *K. oxytoca*, and 2 *R. planticola* isolates. It is noteworthy that only a genotypic method was able to separate *K. variicola* from *K. pneumoniae*. In previous studies, *K. variicola* (KpIII) had not been observed in urinary tract infections (7). In our collection, three of the five *K. variicola* isolates were obtained from urine.

The proposed classification was supported by BLASTn high similarities. In addition, the phylogenetic tree with all sequences obtained allowed for the separation of *K. pneumoniae*, *K. variicola*, *K. oxytoca*, and *R. planticola* in clearly defined clusters sustained by moderate bootstrap values. These values are in the range of those previously reported by analysis of 16S rRNA genes for *Enterobacteriaceae* (13, 24, 42) and the *rpoB* gene for *Klebsiella* (13, 17, 40).

In the present collection, 22 (73%) *K. pneumoniae* isolates clustered with KpI, 3 (10%) with KpII-A, and 5 (17%) with KpIII. A predominance of KpI isolates was also found by Brisse and collaborators (7). Isolates we determined to be *K. variicola* in the present study were clustered together with the *K. variicola* ATCC BAA-830 strain and also with KpIII strain SB1. Therefore, it supports that KpIII corresponds to *K. variicola* as reported by Rosenblueth et al. (40). *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* appear to be closely related to *K. pneumoniae* subsp. *pneumoniae* on the tree (Kp group), in agreement with earlier reports (4, 6, 13).

Fevre and collaborators (18) have recently revised the phylogenetic tree organization of *K. oxytoca*. In the tree obtained with the sequences analyzed by these authors, isolate K82, which did not group with other *K. oxytoca* isolates in our first tree, was shown to belong to KoIV group.

The phylogenetic tree included type strain *R. ornithinolytica* ATCC 31898 in cluster Rp/Ro with the two *R. planticola* isolates of the present study (K111 and K112) and type strain *R. planticola* ATCC 33531. Therefore, the analysis based on the *rpoB* sequencing with 512 bp could not separate these species. A similar observation was made by Brisse and Verhoef (2001) based on the analysis of *gyrA* and *parC* gene sequences and ribotyping data (6). However, a BLASTn search performed with about 1,000 bp of the *rpoB* gene is able to distinguish *R. ornithinolytica* from *R. planticola*. Furthermore, the ornithine decarboxylation test allows for the separation of isolates belonging to these two species (16, 23, 32).

All 122 isolates evaluated in the present study were resistant to ampicillin, as expected for *Klebsiella* (21, 28, 43). All variants of the *K. pneumoniae* chromosomal β-lactamase confer resistance to ampicillin, amoxicillin, carbenicillin, and ticarcillin but not to extended-spectrum β-lactams (1, 2, 20). *K. oxytoca* is naturally resistant to amino- and carboxy-penicillins, and many isolates are resistant to aztreonam and third-generation cephalosporins, with the characteristic exception of ceftazidime (28). As described with other isolates of *K. pneumoniae* and *K. oxytoca*, *K. variicola* isolates were described as naturally resistant to ampicillin and carbenicillin (40). *Klebsiella* clinical isolates are often multidrug resistant, with an increasing proportion of ESBL production (28, 39, 41). In the present study, 28% (29 of 102) of the *K. pneumoniae*/*K. variicola* isolates and 47% (9 of 19) of the *K. oxytoca* isolates were ESBL producers. We also observed that three (K40, K115, and K166) of the five *K. variicola* isolates revealed ESBL production: this is the first report of ESBL production for the newly described *K. variicola* species. The *R. planticola* isolate was resistant only to ampicillin and did not reveal ESBL production, the same profile previously described for 13 *R. planticola* isolates from neonates (37).

Enterobacterial repetitive intergenic consensus-PCR typing revealed a diversity of genotypes in the present collection, indicating that cross-transmission of isolates among patients was not important. Genotypic diversity as observed in our strain collection suggests that our results are not affected by any unique epidemic strains and might be applicable to other collections of isolates.

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