ATYPICAL PHENOTYPIC CHARACTERISTICS OF KLEBSIELLA PNEUMONIAE ISOLATES FROM AN OUTBREAK IN A NEONATAL INTENSIVE CARE UNIT IN BRAZIL

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ABSTRACT

Extended-spectrum β-Lactamase-producing (ESBL) Klebsiella sp. isolates from an outbreak in a Neonatal Intensive Care Unit (NICU) at a teaching hospital in Londrina, Paraná State, Brazil, presented atypical phenotypic characteristics that hampered their identification and the distinction between Klebsiella and Enterobacter species. Ten isolates were identified as K. pneumoniae due to negative reactions for motility and inducible β-lactamase test (ESBL and AmpC) despite being positive for ornithyne descarboxilase. These isolates were genotyped by ribotyping and polymerase chain reaction (PCR) with repetitive extragenic palindromic sequences (REP). Ribotyping by means of an automated instrument and EcoRI and Pvu II as restriction enzymes resulted in detection of K. pneumoniae subspecie pneumoniae RIBO1 222-36-S-5 ribotype. Typing by REP-PCR showed that the 17 isolates from the outbreak were highly similar, belonging to one cluster with 100% of similarity, and that they presented more than 70% of similarity with K. pneumoniae ATCC 13883 and ATCC 10031, and 25% of similarity with E. aerogenes CDC 1680. In conclusion, the isolates of the outbreak were identified as Klebsiella pneumoniae, despite presenting ornithynye descarboxilase enzyme, which is an atypical characteristic of this Klebsiella species.

Key words: Klebsiella pneumoniae, REP-PCR, genetic variation, ornithinolytic

INTRODUCTION

Klebsiella spp., particularly K. pneumoniae, is an important cause of nosocomial infections, and the main population at risk is neonates and immunocompromised hosts. Outbreaks of multiple resistant Klebsiella, which caused systemic infections and death, were widely reported (14). Klebsiella became resistant to broad-spectrum β-lactam antibiotics due to the emergence and spread of plasmid-mediated β-lactamases such as extended-spectrum β-lactamases (ESBLs) (17).

The genus Klebsiella comprises five species, K. pneumoniae, K. oxytoca, K. planticola, K. terrigena and K. ornithinolytica (1) that are usually identified and differentiated according to their biochemical reactions. If the complete identification of the species of Klebsiella is not possible by traditional biochemical tests, comparison of data of the intrinsic resistance of species known, β-lactamases production, and genotypic methods are alternatives to help in the identification (6).

K. pneumoniae that produce ESBL has been associated with infection acquired in neonatal intensive care units (NICU). The frequent use of antimicrobial agents in NICUs increases the likelihood of infection with resistant strains (9,12).

We previously described an outbreak of atypical Klebsiella sp. causing sepsis and death of newborns in a NICU at a tertiary teaching hospital in Londrina, Brazil (11); although the bacteria responsible for that outbreak was identified as Klebsiella sp. by manual procedures, it was identified as E. aerogenes by analysis on Microscan (Microscan Dade, West Sacramento, CA). Due to this uncertainty concerning the genus of the isolated microorganism, in this study we analysed...
the isolates from that outbreak by means of biochemical tests, drug resistance, ribotyping and by the percentage of similarity of them with standard strains, using the fingerprinting generated by REP-PCR.

**MATERIALS AND METHODS**

**Clinical Isolates and Reference Strains**

*Klebsiella* sp. isolates were obtained from an outbreak in a NICU, in Londrina, Brazil, out of which 15 isolates were from tracheal secretions of 9 neonates, one isolate from nasopharynx of a NICU staff member, and one isolate from environment surfaces of the hospital (11). The reference strains *Klebsiella pneumoniae* ATCC 13883 (American Type Culture Collection), *Klebsiella pneumoniae* ATCC 10031 and *Enterobacter aerogenes* CDC 1680 (Center of Diseases Control) were used as controls.

**Bacterial identification and biotyping**

Identification and biotyping of strains were made by standard techniques (6), and subsequently by Microscan Gram-negative panel (Neg Combo 20) according to the manufacturer’s instructions (Microscan Dade, West Sacramento, CA) and by API20E system (bioMerieux, Marcy L’Etoile, France).

**Detection of ESBL and AmpC β-lactamase production**

The presence of ESBL was determined by double-disk synergy test. A zone of inhibition between any one of the β-lactam disks and the disk containing clavulanic acid suggested on inducible production of β-lactamase. A zone of inhibition between any one of the β-lactam disks and the disk containing clavulanic acid suggested the presence of ESBLs (8).

The presence of the amp C gene was also probed by PCR (7). The following oligonucleotide primer sets specific for the *Enterobacter* ampC family were used: (forward) 5’-ATTCGTATGGCTGATCGGCCACC-3’ (nt 413-436), (reverse) 5’-CATGACCCAGTTCGCCATCTCCTG-3’ (nt 808-785). Amplification reactions were performed in a final volume of 25 µl containing DNA from outbreak isolates and from *Enterobacter* sp. strain, 0.2mM deoxynucleoside triphosphate, buffer and 1.25 of Taq DNA polymerase (GIBCO-BRL, Life Technology). The ampC reactions were incubated for 2 min at 96ºC, followed by 30 cycles of 94ºC for 1 min, 64ºC for 1 min, and 70ºC for 1 min and 30 sec. High purity water was used as negative control. The ampC specific PCR products were resolved in 1.5% agarose gel.

**REP-PCR**

Total genomic DNA was prepared from strains grown in Luria-Bertani (LB) medium at 37ºC. Cultures were washed with Tris EDTA buffer (50 mM Tris, 20 mM EDTA [pH 8.0]) and resuspended in Tris-EDTA buffer to reach an optical density equivalent to 10⁶ cells/ml by MacFarland standard. To 400 µl of suspension of cells 50 µl of SDS 10%, 10 µl lysozyme 5 mg/ml, 1 µl of RNase A (Sigma) 10 mg/ml, and 5 µl of Proteinase K 20mg/ml were added. The cells were then incubated for 1 h, at 37ºC. NaCl 250 mM was added and the preparation was incubated for 1h at 4ºC. DNA was recovered by ethanol (Merck) precipitation, and quantified by comparison with lambda DNA (GIBCO-BRL). REP-PCR were obtained with the oligonucleotide primers REP 1RI (3’-CGGICTAC1GCGCIIIII-5’) and REP 21 (5’-ICGICTTATCIGGCTAC-3’) at a concentration of 50 rmol, as previously described (18), using a thermal cycler (Gene Amp PCR System 9700/Perkin Elmer). Amplification reactions were performed in a final volume of 25 µl with 25 ng isolates and standards strains DNA. The amplification cycles were as follows: 1 cycle at 95ºC for 7 min; 30 cycles at 90ºC for 30 s, 45ºC for 1 min, 65ºC for 8 min; and 1 cycle at 68ºC for 16 min. High purity water was used as negative control. The amplified products (10 µl) were electrophoretically separated in pociarlamide 3.5 to 8.0% gel in Tris-Borate-EDTA buffer pH 8.0, stained with ethidium bromide and photographed on a UV-transluminator.

**Analysis of REP-PCR**

The fingerprints obtained were compared for similarity by visual inspection of band patterns. Sizes of DNA fragments amplified were determined by direct comparison with the DNA marker. The REP-PCR products were converted to binary matrix (presence or absence of band), which were subjected to analysis of correlation by unweighted pair group method with arithmetic averages (UPGMA), generating the dendrograms by SAHN (Sequential Agglomerative Hierarchical and Nesting) of NTSYS-PC version 1.8 (Applied Biostatistics, Inc).

**Ribotyping**

The isolates from the outbreak were ribotyped using the RiboPrinter Microbial Characterization System (Qualicon, Wilmington, DE) according to the manufacturer’s instructions. This process includes cellular lysis, cleavage of DNA with restriction enzymes EcoRI and Pvu II, electrophoresis to separate the bands and southern blotting. The DNA fragments were hybridised with probe derived of ribosomal RNA from *Escherichia coli* and the bands were detected with chemiluminescence’s substrate. The patterns were electronically imaged, stored and transferred to a computer system. Each line representing the data of strain was compared to standard marker based to intensity of bands (2,13). Similarity coefficient was calculated according to position and relative weight of bands. All isolates that presented similarity coefficient equal to or more than 0.93 (93%) were considered as having the same ribotype; those that presented similarity coefficient ≤0.92 were considered as different isolates with a distinctive ribotyping pattern (2).
RESULTS

Biochemical Identification and drug resistance

The isolates from the outbreak lacked motility, presented negative indole test, and positive Voges-Proskauer test, produced urease and lysine descarboxylase, and therefore produced ornithine descarboxylase, as analysed by standard laboratory procedures, and were considered as atypical K. pneumoniae. The results from Microscan Gram-negative panel ascribed 71.09% of probability to E. aerogenes, 26.45% to K. pneumoniae and 2.45% to K. ornithinolytica. The API20E system could not identify this isolate due to the positive ornithine descarboxylase test. All tests were compatible with Klebsiella pneumoniae, subspecie pneumoniae, with exception of ornithine descarboxylase.

Most isolates from the outbreak showed ESBL production, and none of strains showed AmpC β-lactamase production by means of PCR and by inducible β-lactamase test. All isolates were uniformly susceptible to imipenem (IPM) and ciprofloxacin (CIP), and showed resistance to cephalothin, sulbactam-ampicillin, gentamicin, chloranphenicol, cefazidime, aztreonam, cefotaxime, piperacillin-tazobactam and cefepime.

Genotypic analysis

REP-PCR patterns of each isolate on three different days were consistent. By REP-PCR, Klebsiella from the outbreak generated patterns of 11 distinct amplification DNA bands ranging in size from 0.05 to 1.5 Kb and the standards strains K. pneumoniae ATCC and Enterobacter aerogenes CDC generated 11 bands ranging in size from 0.05 to 2.5 Kb (Fig. 1A). Dendrograms were constructed from REP amplification to show the degree of relatedness between the isolates from the outbreak studied and standard strains, K. pneumoniae ATCCs and E. aerogenes CDC, by UPGMA analysis and coefficient simple matching (Fig. 1B). All isolates grouped into one cluster with 100% of similarity and showed more than 70% of similarity to the two K. pneumoniae ATCC strains and less than 25% to E. aerogenes CDC 1680 strain. K. pneumoniae ATCC 13883 and ATCC 10031 were very similar to each other (Fig. 1B).

The ribotyping analysis, using EcoRI and Pvu II as restriction enzyme, revealed that the isolates from the outbreak had the ribotype of K. pneumoniae subspecie pneumoniae RIBO1 222-36-S-5.

DISCUSSION

The identification of a microorganism is based on the comparison between biochemical tests which reflect the metabolic activities of the isolate, and data published for known genera and species (6). Klebsiella genus is defined as containing Gram-negative bacilli of the family Enterobacteriaceae, not motile, usually encapsulated and rod-shaped, which produce lysine descarboxylase but do not produce ornithine descarboxylase (14). The phenotypic differentiation between the genus Klebsiella and Enterobacter, specially the species K. pneumoniae and E. aerogenes, is based on tests of ornithine descarboxylase, urease production and motility. K. pneumoniae presents ornithine negative test (100% of strains), positive urease production (95% of strains) and lack of motility (100% of strains); and E. aerogenes presents positive ornithine test (98%
of strains), negative urease production (98% of strains) and positive motility (97% of strains) (6).

In this work, these standard techniques of phenotypic differentiation showed that the isolates presented all characteristics of *K. pneumoniae*, with exception of ornithine decarboxylase production. The differentiation between the species *K. pneumoniae* and *K. ornithinolytica* was difficult on the basis of positive ornithine decarboxylase test and negative indole production, because *K. ornithinolytica* presents 100% of positive indole test.

Automated analysis on Microscan consists of performing biochemical tests and comparing the results with a printed or a computerized database; the isolates studied presented 71.09% of probability of being *E. aerogenes* by Microscan. However, this percentage is considered unsatisfactory for a definitive identification because *E. aerogenes* strains present low percentage (2%) of urease production, and this system does not consider the motility test, which is very important for differential identification of those two genera, demonstrating that this method is inadequate for complete and accurate identification. Moreover, the API20 system, frequently utilized in the bacterial identification, failed to identify the isolates from the outbreak, indicating the presence of an atypical *Klebsiella* strain.

The system API20 also misidentified as *Klebsiella pneumoniae* or *K. terrigena* ten clinical isolates of *E. aerogenes*, which were identified by tDNA-PCR. These isolates were identified as *K. pneumoniae* due to negative reactions for motility and ornithine decarboxylase, therefore all isolates possessed an inducible cephalosporinase, as detected on the antibiogram using a disk approximation test, a finding which strongly contradicts an identification as *K. pneumoniae* (4).

The comparison of intrinsic resistance data of known species and of the data of β-lactamases production can be an alternative to aid in the identification of the microorganism. Essentially all strains of *Enterobacter* have intrinsic resistance to cephalothin and all strains of *K. pneumoniae* have intrinsic resistance to ampicillin and carbenicillin (6). In this work, the isolates were resistant to ampicillin and did not present intrinsic resistance to cephalothin, suggesting that the isolates studied belong to genus *Klebsiella*. Also, bacteria of group CESP (Citrobacter, *Enterobacter*, *Serratia*, *Providencia*, and *Pseudomonas*) usually produce an inducible β-lactamase known as AmpC, which is codified by a chromosomal gene and confer resistance to a variety of β-lactam drugs, including cefoxitin, and is poorly inhibited by β-lactamases inhibitors such as clavulanic acid (3). As all isolates studied were susceptible to cefoxitin, did not show AmpC induction, and the ampC gene was not found by PCR, these isolates probably do not present constitutive AmpC β-lactamase production, suggesting that they belong to genus *Klebsiella* and not to *Enterobacter*, which has a chromosomal gene that codifies AmpC. Genes for AmpC have also recently been found on a plasmid that transfers no inducible cephalosporin resistance to *K. pneumoniae*, but were not described in chromosomes (5).

REP sequences are the most commonly used targets for DNA typing. These sequences seem to be highly conserved, their chromosomal situation varies among species and/or strains of the same species, and this variation can be detected by PCR, allowing an understanding about the relationship between the bacteria (18). REP-PCR was utilized to differentiate between a relapse of *K. pneumoniae* urinary tract infection caused by the same bacterial strain and reinfection with a different bacterial strain by DNA fingerprinting of clinical isolates (10).

In this work, the REP-PCR fingerprint presented by all outbreak isolates showed more than 70% of similarity to the REP-PCR fingerprints from standard *K. pneumoniae* strains, ATCC 13883 and ATCC 10031 (Fig. 1B). On the other hand, *E. aerogenes* CDC 1680 showed only 25% of similarity to the cluster of outbreak isolates (Fig. 1B). These results revealed a clonal relationship among the isolates of *Klebsiella* studied, and that they are closer to *K. pneumoniae* than to *E. aerogenes*, in agreement with the ribotyping, which is an important typing method that has revealed high power of differentiation of strains and has helped in the surveillance of infections (16).

In conclusion, the isolates of ESBL-*Klebsiella* sp from an outbreak in a NICU in Brazil produced ornithine decarboxylase, which is atypical of the specie pneumoniae, presented the ribotype of *K. pneumoniae* subspecie *pneumoniae*, and were closer to *K. pneumoniae* than to *E. aerogenes* by REP-PCR.

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**RESUMO**

Características fenotípicas atípicas de isolados de *Klebsiella pneumoniae* de um surto na unidade de terapia intensiva neonatal, no Brasil

Isolados de *Klebsiella* sp. produtora de β-lactamase de espectro estendido (ESBL), responsável por um surto na Unidade Neonatal de Terapia Intensiva (UNTI) do Hospital Universitário de Londrina, Paraná, Brasil apresentaram características fenotípicas atípicas que dificultaram sua identificação e a diferenciação entre as espécies *Klebsiella pneumoniae* e *Enterobacter aerogenes*. Dez isolados foram identificados como *K. pneumoniae* devido às reações negativas para motilidade e produção de enzimas β-lactamases (ESBL e AmpC). Embora apresentassem teste positivo para ornitina decarboxilase. Estes isolados foram genotipados por ribotipagem e por reação em cadeia da polimerase (PCR) com
oligonucleotídeos para “repetitive extragenic palindromic sequences” (REP). A ribotipagem com as enzimas de restrição EcoRI e Pvu II detectou o ribotipo de *K. pneumoniae* subespécie *pneumoniae* RIBO1 222-36-S-5. A técnica de REP-PCR mostrou que os isolados do surto foram similares, pertencentes a um grupo com 100% de similaridade, e apresentaram mais de 70% de similaridade com amostras padrão de *K. pneumoniae* (ATCC 13883 e 10031), e 25% de similaridade com *E. aerogenes* CDC 1680. Concluindo, os isolados do surto da NICU mostraram-se geneticamente relacionados e foram identificados como *Klebsiella pneumoniae*, embora apresentassem ornitina descarboxilase, característica atípica para esta espécie de *Klebsiella*.

**Palavras chave:** *Klebsiella pneumoniae*, REP-PCR, variabilidade genética, ornitolítica.

### REFERENCES