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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 *Eco*RI and *Pvu* II as restriction enzymes resulted indetection 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 ornithyne 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

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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 (bioMeriéux, 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 contaning clavulanic acid suggested the presence of ESBLs (8).

The detection of AmpC β-lactamase production was based on inducible production of β-lactamase in presence of an inducer β-lactam disk (imipenem) and a β-lactam disk test (ceftazidime and cefotaxime) as described by Sanders & Sanders (15).

The presence of the amp C gene was also probed by PCR (7). The following oligonucleotide primer sets specific for the *Enterobacter* ampC gene family were used: (forward) 5´-ATTCGTATGCTGGATCTCGCCACC-3´ (nt 413-436), (reverse) 5´- CATGACCCAGTTCGCCATATCCTG-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 polimerase (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 ressuspended in Tris-EDTA buffer to reach an optical density equivalent to 10^9 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'-CGGICTACIGCIGCIIII-5') and REP 2I (5'-ICGICTTATCIGGCCTAC-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 poliacrilamide 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 (Sequencial Agglomerative Hierarchical and Nested) of NTSYS-PC version 1.8 (Applied Biostatistics, Inc).

Ribotyping

The isolates from the outbreak were ribotyped using the RiboPrinterâ Microbial Characterization System (Qualicon, Wilmigton, DE) according to the manufacturer's instructions. This process includes cellular lysis, cleavage of DNA with restriction enzymes *Eco*RI 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 ornithyne descarboxilase, 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 ornithyne descarboxilase test. All tests were compatible with *Klebsiella pneumoniae*, subspecie *pneumoniae*, with exception of ornithyne descarboxilase.

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, ceftazidime, 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 *Eco*RI 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.

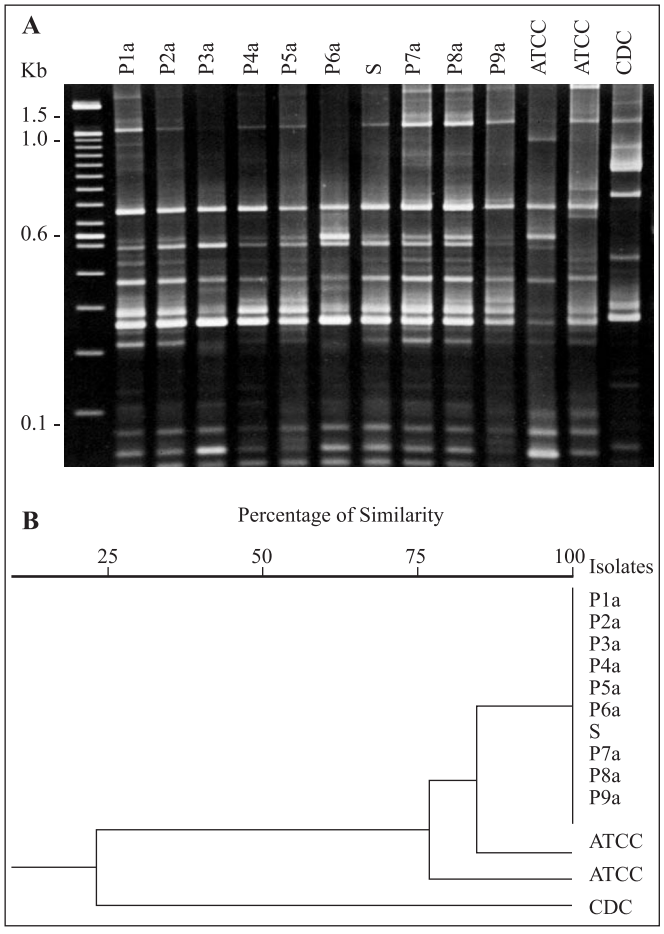

Figure 1. Genetic Variation of Extended-Spectrum β Lactamase-producing atypical *K. pneumoniae* in a Neonatal Intensive Care Unit in Brazil by means of REP-PCR. **A** - Eletrophoresis gel of REP-PCR patterns of genomic DNA from atypical *Klebsiella* isolates studied. Lanes: 1 to 10, outbreak isolates from neonates (P1a-P9b) and staff member (S), lane 11 and 12: standard *K. pneumoniae* strains (ATCC 13883 and ATCC 10031), and lane 13: *E. aerogenes* CDC 1680 strain. Molecular size markers (100-pb DNA ladder from GIBCO-Bethesda Research Loaboratories) are on the left. **B**- Dendrogram of isolates based on Simple Matching similarity coefficients, calculated from REP-PCR analysis data.

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 ornithyne descarboxilase, 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 ornithyne descarboxilase production. The differentiation between the species *K. pneumoniae* and *K. ornithinolytica* was difficult on the basis of positive ornithyne descarboxilase test and negative indole production, because *K. ornithinolytica* presente 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 cefalosporinase, 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. pneum*oniae, 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 ornithyne descarboxilase, 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 descarboxilase. 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 *Eco*RI 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.

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