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Atypical *Klebsiella* Species in a Third Level Hospital as Cause of Neonatal Infection

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Abstract

Background: The opportunistic pathogen *Klebsiella pneumoniae* is one of the main causes of pediatric bacterial blood stream infections (BSI), which is complicated with sepsis and high mortality.
Objectives: To identify atypical *Klebsiella* species affecting a sample of infected neonates with low antimicrobial response.
Methods: Multidrug resistant blood cultures for *Klebsiella* from a Neonatal Service, were submitted to molecular identification by sequencing analysis of 16S ribosomal RNA.
Results: The mean age of the newborns was 14.7 ± 5.6 days. A total of 6 out of 8 cases were sepsis, 1 case of pneumonia, and 1 a catheter-related infection. The molecular identification showed 3 cases of *K. pneumoniae* subsp. *ozaenae*, 2 of *K. pneumoniae* and *K. variicola*, and 1 case of *K. oxytoca*. The highest antimicrobial resistance was against cephalosporins and Trimethoprim/sulfamethoxazole.
Conclusions: *Klebsiella pneumoniae* subsp. *ozaenae* was responsible for multidrug resistant strains of *Klebsiella* even in 37.5% of cases. In our clinical setting, the use of Amikacin and carbapenems are still useful to treat neonatal infections by *Klebsiella* even against *K. variicola*, which is the most resistant.

Keywords: Amikacin, Neonatal Sepsis, Multidrug Resistance, *Klebsiella pneumoniae* subsp. *ozaenae*, *K. variicola*

1. Background

Pediatric bacterial Blood Stream Infections (BSI) are a major cause of morbidity and mortality worldwide (1, 2). In this regard, *Enterobacteriaceae* are the group with most common agents causing BSI, among which, the genus *Klebsiella* is a major infectious agent (3). The opportunistic pathogen *Klebsiella pneumoniae* has been classified into 3 phylogenetic groups: KpI; KpII-A/KpII-B, and KpIII (4), and chromosomal class A β-lactamase *blaSHV*, *blaOKP-A*/*blaOKP-B*, and *blaLEN* genes have been directly associated with each of these groups, respectively (5). These groups correspond to the species *K. pneumonia* (KpI), *K. quasipneumoniae* subspp. *quasipneumoniae* (KpII-A), *K. quasipneumoniae* subspp. *similipneumoniae* (KpII-B), and *K. variicola* (KpIII) (6). At present, *K. oxytoca* can cause infection in healthcare settings, with outbreaks of multidrug-resistant infection being increasingly reported in hospitalized patients (7-12). Moreover, for pediatric population, infections due to extended spectrum beta lactamase (ESBL)-resistant Enterobacteriaceae are an emerging problem (13).

2. Objectives

The aim of this work was to identify atypical *Klebsiella* species affecting a sample of infected neonates with sepsis, catheter-related infection, or pneumonia.

3. Methods

3.1. Ethics Statement

The study was approved by the ethics and research committee of the HMPMPS (code: 217B500402016058) and we followed the mexican regulations of the general health law in the field of research.

3.2. General Data

Information of blood cultures positive for *Klebsiella* with a diagnosis of sepsis was retrieved from the epidemiology service "Monica Pretelini Saenz" maternal-perinatal hospital (HMPMPS), Health institute of the State of Mexico (ISEM), Toluca, Mexico, from August to October 2016. Standardized collection of epidemiological data included age,

clinical features, antimicrobial treatment, and final outcome. All the information was managed on an Excel data sheet.

At the neonatal intensive care unit (NICU) of our hospital, the diagnosis of sepsis was made if a patient exhibited clinical manifestations and had any of the following criteria: (1) white blood cell count (WBC) reduction to $< 5 \times 10^9$/L, platelet count (PLT) $\leq 100 \times 10^9$/L, and erythrocyte sedimentation rate $\geq$ 15 mm/h. The diagnosis of pneumonia was based on unexplained worsening of the patient's respiratory status and a change in the quality of the respiratory secretions supported by the chest x-ray, pulse oximetry, blood cultures, and Gram stain and culture of tracheal aspirate. The suspicion of catheter-related bloodstream infection (CRBSI) was based on clinical finding, fever, inflammation, or purulence around the insertion site. In the 2 conditions, blood cultures had to be positive.

3.3. Culture and Identification

Blood-culture tubes were incubated at 35°C and inspected daily for signs of bacterial growth for 7 days. The seeding was repeated 3 times during this period. All positive cultures were characterized by colony characteristics, Gram stain, and standard biochemical tests. Routine subculture was undertaken at 24 hours, 48 hours, and 7 days. Organisms were identified using Gram-staining/microscopy, in-house biochemical testing, and commercial biochemical-analytical profile-index kits. Subcultures were performed with supplemented chocolate agar and 5% sheep blood agar.

The MicoScan 4 (Beckman Coulter, Inc., USA) was used to identify species of *Klebsiella* (all negative for hemolysis in agar blood, positive for lactose and catalase, and negative for oxidase) and antimicrobial susceptibility. Drug susceptibility testing was performed against Amikacin, Ampicillin, Cefepime, Ceftriaxone, Cefotaxime, Cefotetam, Cefuroxime, Ciprofloxacin, Ertapetem, Gentamicin, Imipenem, Levofloxacin, Meropenem, Moxifloxacin, Tetracycline, Tigecycline, and Trimethoprim/Sulfamethoxazole, reporting the minimum inhibitory concentration (MIC) for each of the isolates. In this study, multidrug resistance was defined as simultaneous resistance to 2 or more drugs of different classes of antimicrobial agents.

3.4. Molecular Identification

The samples were transported to the Laboratory of Microbiology of the faculty of medicine, autonomous university of the state of Mexico (UAEMEx), in enriched media such as blood agar or brain heart infusion (BHI) broth. To obtain biomass, the bacteria were inoculated in the BHI medium and incubated at 37°C for 3 days. Isolated strains were extracted with DNA, according to the Wizard® Genomic DNA purification kit (Promega A1120) protocol. The 8 strains previously classified as *K. pneumoniae* were characterized by 16S rRNA (rrs) gene sequencing. For this procedure, 2 sets of universal primers were used: 27f 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492r: 5'- TAC GGY TAC CTT GTT ACG ACT T-3'.

Polymerase chain reaction (PCR) was performed using Taq DNA polymerase in storage buffer B Bioline® Brand (BIO 21105). The conditions of the thermal cycle were as follows: a pre-denaturation cycle of 5 min (94°C); denaturation for 30 seconds (94°C); coupling for 20 seconds (52°C), and elongation for 1.5 minutes (72°C); 34 cycles were repeated and then a post-elongation cycle of 7 minutes (72°C) was performed. The amplified products were purified using the Amicon ultra filter kit (Millipore UFC901008) and checked for presence and quality on 1% agarose gel. The 16S ribosomal RNA amplification products were sent to the sequencing service of macrogen sequencing service (Maryland, USA). The sequences obtained were assembled and corrected using the Chromas Pro version 1.5 program. The sequences obtained were compared to the sequences validated and deposited in the GenBank databases of the national center for biotechnology information (NCBI) through the BLAST program. Nucleotide sequence accession numbers were the following: 1, KY711154.1; 2, KY711153.1; 3, KY711152.1; 4, KY711151.1; 5, KY711150.1; 6, KY711149.1; 7, KY711148.1 and 8, KY711147.1.

4. Results

The mean age of the newborns (2 females and 6 males) was of 14.7 ± 5.6 days. At the moment of identification by using the 16S rRNA sequence, 5 patients were discharged from the hospital and 3 remained hospitalized. In total, 6 of the 8 cases were sepsis and there was 1 case of pneumonia and 1 attributed to an infection at the catheter-insertion site. MicroScan 4 reported the identification of *K. pneumoniae* from 7 isolates and only 1 of *K.* spp. In contrast, molecular identification revealed 1 case of *K. oxytoca*, 2 of *K. variicola*, 2 of *K. pneumoniae*, and 3 cases of *K. pneumoniae* subspp. *ozaenae*. A list of isolates and molecular identification is provided in Table 1.

From the antibiogram (Table 2), we can see that, fortunately, there were no ESBL; however, there was resistance to cephalosporins, Trimethoprim/Sulfamethoxazole and, in 3rd place, to quinolones. Trying to define more objective criteria to determine the bacterial aggressiveness, for each bacterium the antibiotic sensibilities (S) were divided between the antibiotic resistances (R) = S/R. Subsequently a mean was obtained for the same type cultures, obtaining

Table 1. General Data of the Strains with the MicroScan4 and Molecular Identification

Patient	Gender	Age (Days)	Evolution	Clinical Infection	Identification MicroScan4	Blood Culture Key	Molecular Identification Length Fragment (pb)	Similarity % Strain
1	M	10	HD	Sepsis	*Klebsiella pneumoniae*	H1157	*Klebsiella pneumoniae* subsp. *ozaenae*, 1273pb	99, ATCC 11296
2	F	19	HD	Catheter-related infection	*Klebsiella pneumoniae*	H821	*Klebsiella variicola*, 1320pb	98, F2R9
3	M	9	HD	Sepsis	*Klebsiella pneumoniae*	H814	*Klebsiella pneumonia*, 1362pb	99, DSM 30104
4	M	15	HD	Sepsis	*Klebsiella pneumoniae*	H1064	*Klebsiella pneumoniae* subsp. *ozaenae*, 1318pb	98, ATCC 11296
5	M	21	HD	Pneumonia	*Klebsiella sp.*	H793	*Klebsiella oxytoca*, 1207pb	99, ATCC 13182
6	M	9	SH	Sepsis	*Klebsiella pneumoniae*	H776	*Klebsiella variicola*, 1206pb	100, LX3
7	F	23	SH	Sepsis	*Klebsiella pneumoniae*	H1078	*Klebsiella pneumonia*, 1213pb	100, DSM 30104
8	M	12	SH	Sepsis	*Klebsiella pneumoniae*	H759	*Klebsiella pneumoniae* subsp. *Ozaenae*, 1357pb	99, ATCC 11296

Abbreviations: HD, hospital discharge (improvement); SH, still hospitalized.

the next S/R values: *K. oxytoca*: 3, *K. pneumoniae* subsp. *ozaenae*: 0.643, *K. pneumoniae*: 0.607, and *K. variicola*: 0.452.

5. Discussion

Tariq and Rasool published that, in cases of culture-proven sepsis, among Enterobacteriaceae, 31.82% were *K. species* (*K. pneumonia* and *K. oxytoca*) (14). This becomes more relevant because *K. pneumoniae*, *K. variicola*, and *K. quasipneumoniae* are difficult to differentiate phenotypically, leading to misinterpretation of their infection prevalence (6, 15, 16). In fact, to our knowledge, there is no biochemical test capable of differentiating these 3 *Klebsiella* species concomitantly during routines in clinical microbiology laboratories. A very important issue to take into account is that *Klebsiella* singaporensis is a junior heterotypic synonym of *K. variicola* and some studies, even with the technique we used, still report the first as a different species (17). Even more, *K. oxytoca* is known to have a similar antimicrobial resistance profile to that of *K. pneumonia*.

Surprisingly in this survey the first was even less resistant to the antibiotics usually indicated in the Neonatal Service. Contrastingly, *K. variicola* seems to be the most dangerous strain as one of the registered cases showed resistance against 9 antibiotics, and probably more, however, in the moment of the analysis, the equipment ran out of reagents. With the available information the SR ratio reflected the severity of *K. variicola* in this exercise of neonatal infections. Our results illustrates the possibility that as *K. variicola* was very recently described as a new bacterial species and is very closely related to *K. pneumoniae*, it might be the case that some isolates, which were initially classified as *K. pneumoniae*, were actually *K. variicola* (18), being the cases of greatest suspicion to those with higher mortality (19).

Research concerning antibiotic resistance profiles of *Klebsiella* isolates are diverse, for example, Vasaikar et al. reported, in South Africa, high antibiotic resistance in decreasing order to penicillins, cephalosporins, folate pathway inhibitors, monobactams, and aminoglycosides. A percentage of low resistance was observed in carbapenems, aminoglycosides (only Amikacin), glycylcyclines (Tigecycline), cephamycins (Cefoxitin), quinolone (Levofloxacin), phosphonic acids (Fosfomycin), antipseudomonal penicillins + β-lactamase inhibitor (pip/tazo), and fluoroquinolones (Ciprofloxacin), which can be considered for treatment of *Klebsiella* species (3). In contrast, in Mexico, the option of quinolones would not be so useful.

Although our sample is low, in a previous work done by Dong et al., (20), from a sample of 96 cases of neonatal sepsis, 19 were Gram-negative bacteria and only 10

Table 2. Antibiograms

	Patient								
Antibiotic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**% Resistance[a]**
Amikacin	NE	S	S	S	R	S	S	S	14.28
Ampicilin	NE	R	R	R	R	R	R	R	100
Cefepime	NE	R	WR	R	WR	R	R	R	100
Ceftriaxone	NE	R	R	R	R	R	R	R	100
Cefotaxime	NE	R	R	R	WR	R	R	R	100
Cefotetam	NE	WR	WR	WR	S	WR	WR	WR	0
Cefuroxime	NE	R	R	R	WR	R	R	R	100
Ciprofloxacin	NE	R	S	R	S	R	R	R	28.57
Ertapetem	NE	WR	WR	WR	S	WR	WR	WR	0
Gentamicin	NE	R	R	R	WR	R	R	R	100
Imipenem	NE	S	S	S	S	S	S	S	0
Levofloxacin	NE	R	S	S	S	S	R	S	28.57
Meropenem	NE	S	S	S	S	S	S	S	0
Moxifloxacin	NE	WR	R	WR	WR	WR	S	S	33.33
Tetracycline	NE	WR	WR	WR	S	WR	WR	WR	0
Tigecycline	NE	WR	WR	WR	S	WR	WR	WR	0
Trimethoprim/sulfamethoxazole	NE	R	R	WR	S	WR	WR	WR	66.66
Total tests		12	12	11	12	11	12	12	
Sensitivity		3	5	4	9	4	4	5	
Resistance		9	7	7	3	7	8	7	
S/R		0.33	0.71	0.57	3.00	0.57	0.50	0.71	

Abbreviations: NE, Non evaluated; R, resistant; S, Sensitive; WR, without reactive.
[a] Taking into account only those samples with report of antibiogram.

cases were *K. pneumoniae* subsp., which exhibited full resistance (100%) to amoxicillin, amoxicillin/clavulanate, cefepime, cefoxitin, ceftazidime, cefotaxime, cefuroxime, and piperacillin, a 70.0% cephalothin resistance rate, and no resistance to amikacin, meropenem, or netilmicin. Resistance to carbapenems with varying prevalence has been reported at sites worldwide (21). Thus, screening Enterobacteriaceae for ESBL production is essential for better antibiotic selection and preventing its further emergence and spread. In our study, fortunately, there was no case of carbapenemase-producing isolation. However, our findings did not consider other types of Enterobacteriaceae.

Finally, talking about the strain identification techniques, nowadays, PCR diagnosis can confirm the presence or absence of bacterial genomic DNA, giving a diagnosis to the clinician in a few hours (22). Furthermore, molecular tests based on multiplex real-time quantitative PCR (23) could be more useful in cases of neonatal sepsis. However, this technique is very expensive for most of the general hospitals and not affordable for the health systems of many low income countries. In the case of Mexico, a medium income country, most of the samples of multidrug resistance are not processed by failures in reagent supply or lack of specialized laboratories in all the Mexican states. This study is limited by its retrospective observational design, and nonsystematic sampling of hospital staff may underestimate the true burden of BSI. Despite the limitations, we have determined through molecular techniques that *Klebsiella* species are scarcely known and hardly reported in Latin America.

A future action should be the design of antibiotic-decision flowcharts based on the resistance and clinical parameters as well as studies available in general hospitals. It remains pending the use of new alternatives to treat neonatal infections. For example, our group has explored the use of triazoles obtained by "Via click" against *Candida albicans* (24), however, this type of compounds are far from being clinically approved yet. Another point of view has been the re-evaluation of known antibiotics such as colistin (25).

6. Conclusion

First, within our clinical setting of Neonatal care, multidrug resistant strains of *Klebsiella* can be attributed to *K. pneumoniae* subsp. *ozaenae* even in 37.5% of cases. Second, as a result of this study, we conclude that in our clinical setting, the use of Amikacin and carbapenems, are still useful to treat neonatal infections by *Klebsiella* even against *K. variicola*, which is the most resistant. Two main

future actions should be implemented a, ensure the correct equipment of state laboratories and their adequate provision of reagents and b, the design of antibiotics decision flowcharts based on the resistance, clinical parameters, and studies available in general hospitals.

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Footnotes

Authors' Contribution: Study concept and design: Hugo Mendieta Zeron and Ninfa Ramirez Duran; acquisition, analysis, and interpretation of data: Hugo Mendieta Zeron, Iliana Espinoza Rivera, Luz Marcela Caro Gonzalez, Martin Pablo Antonio Moreno Perez, and Damian David Cifuentes Castaneda; drafting of the manuscript: Hugo Mendieta Zeron and Ninfa Ramirez Duran; critical revision of the manuscript for important intellectual content: Hugo Mendieta Zeron and Ninfa Ramirez Duran.

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