Molecular Characterization of Extended-Spectrum β -Lactamase-Producer *Klebsiella pneumoniae* Isolates Causing Neonatal Sepsis in Peru

Coralith García,* Lizeth Astocondor, Beatriz Rojo-Bezares, Jan Jacobs, and Yolanda Sáenz

Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru; Centro de Investigación Biomédica de La Rioja, Logroño, Spain; Institute of Tropical Medicine Antwerp, Antwerp, Belgium; Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium

Abstract. *Klebsiella pneumoniae* (KP) is the most common cause of neonatal sepsis in the low- and middle-income countries. Our objective was to describe the phenotypic and molecular characteristics of extended-spectrum β -lactamase (ESBL)-producer KP in neonatal care centers from Peru. We collected 176 non-duplicate consecutive KP isolates from blood isolates of neonates from eight general public hospitals of Lima, Peru. The overall rate of ESBL production was 73.3% ($N = 129$). The resistance rates were higher among ESBL-producer isolates when compared with the nonproducers: 85.3% versus 12.8% for gentamicin ($P < 0.01$), 59.7% versus 8.5% for trimethoprim-sulfamethoxazole ($P < 0.01$), 45.0% versus 8.5% for ciprofloxacin ($P < 0.01$), and 36.4% versus 12.8% for amikacin ($P < 0.01$). A total of 359 β -lactamase-encoding genes were detected among 129 ESBL-producer isolates; 109 isolates (84.5%) carried two or more genes. Among 37 ESBL-producer isolates randomly selected, CTX-M-15 and CTX-M-2 were the most common ESBLs detected. Most of the isolates (92%) belonged to the group KpI. Pulsed-field gel electrophoresis showed that multiple KP clones were circulating among the eight neonatal units included.

INTRODUCTION

In the recent years, a remarkable increasing rate of enterobacterial microorganisms resistant to cephalosporins has been reported worldwide; their most important mechanism of resistance is the production of extended-spectrum β -lactamases (ESBLs), which confers them resistance to first-, second-, and third-generation cephalosporins and aztreonam. After the first report of ESBL-producer *Klebsiella pneumoniae* (KP) in 1983, more than 200 ESBL types have been described. Currently, ESBLs exist in every region of the world and in most genera of the Enterobacteriaceae.^{1,2}

Although ESBL-producing microorganisms are of global concern, they represent a major burden and safety problem for patients in the developing world. Different multicenter studies have showed the same trends: an increasing resistance rates to ceftriaxone among enterobacterial agents through the years correlating with an increasing detection of ESBLs worldwide and the highest rates of ESBL production among *Klebsiella* and *Escherichia coli* from Latin America when compared with other regions of the world.^{3–6} Among KP, ESBLs have been detected in an overall rate of 26% worldwide with a higher proportion in Latin American centers (35%) compared with Europe (20%) and North America (10%).⁵ Although in a regional resistance surveillance program (2011), higher ESBL rates were described among *Klebsiella* spp. isolates (52%) from different origins in Latin American nations (including Peru). That study also showed that the highest ESBL rates were detected among *Klebsiella* spp. and *E. coli* isolates of Peru (70% and 54%, respectively) and Guatemala (69% and 59%, respectively).⁷

Neonates constitute a high-risk group for developing nosocomial sepsis. Bloodstream infection rates of neonates are three to 20 times higher in developing countries than the rates of one to five per 1,000 live births reported from developed

countries.⁸ Moreover, studies in Brazil and Indonesia have reported rates of hospital-acquired infections to be more than 50% among all neonate intensive care unit admissions.^{9,10} Since KP is a leading cause of nosocomial infection and is considered the most frequent gram-negative rod causing neonatal sepsis in these settings,^{11–15} the high rates of ESBL production among KP infections in this group is of much concern.

Our objective was to assess the phenotypic and molecular profiles of ESBL-producer KP in neonatal care centers from Peru.

METHODS

From 2008 to 2011, non-duplicate consecutive KP isolates were collected from blood culture samples drawn as part of routine neonatal care at eight general public hospitals in Lima and Callao. Peru is a middle-income country with 30 million inhabitants, of which almost one-third is living in both cities. The study was approved by the Ethical Institutional Committee of Universidad Peruana Cayetano Heredia and by the ethical committees in each center.

The participating laboratories stored KP isolates on Trypticase Soya Agar tubes (Oxoid Ltd, Hampshire, United Kingdom). Isolates were properly stored in the hospitals and were sent to the Universidad Peruana Cayetano Heredia on weekly basis. Further identification was done by conventional biochemical testing. Antimicrobial susceptibilities were assessed by disk diffusion method for meropenem, imipenem, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole (TMP-SMX).¹⁶ Detection of ESBL phenotype was done by double disc method using the following disks: amoxicillin-clavulanate, cefotaxime, and ceftazidime. *Escherichia coli* ATCC 25922 was used for quality control. ESBL-producer KP was further analyzed by pulsed-field gel electrophoresis for genetic relatedness using the restriction enzyme XbaI with a CHEF Mapper DRIII apparatus (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom).¹⁷ Band patterns were compared visually and interpreted according to the criteria established by Tenover and

*Address correspondence to Coralith García, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Avenida Honorio Delgado 430, Lima 31, Peru. E-mail: coralith.garcia@upch.pe

others¹⁸ and analyzed with BioNumerics V.4.0 software (Applied Maths, Sint-Martins-Latem, Belgium). Detection of *bla*_{SHV}, *bla*_{TEM}, *bla*_{OXA-1-like}, *bla*_{CTX-M-2G}, *bla*_{CTX-M-1G}, *bla*_{CTX-M-9G}, and *bla*_{CTX-M-8G} genes were performed by conventional polymerase chain reaction (PCR).^{19–21} To determine the specific β -lactamase gene involved in the ESBL phenotype, 37 isolates were randomly selected for further sequencing of their PCR products on both strands (with the exception of *bla*_{OXA-1-like} amplicons). To obtain the whole sequences of the *bla*_{CTX-M} genes, their genetic environments were analyzed by specific PCRs and DNA sequencing.¹⁹ The obtained sequences were aligned and compared with those included in Lahey (<http://www.lahey.org/Studies/>) and GenBank databases (<http://www.ncbi.nlm.nih.gov/BLAST>). Phylogenetic groups were studied by PCR restriction fragment length polymorphism.²²

RESULTS AND DISCUSSION

Among 176 KP isolates collected during 2008–2011, 129 (73.3%) were ESBL producers, the overall proportion ranged from 63.6% to 90.9% among the participating hospitals (Table 1). This ESBL rate (73%) is in line with the few data previously collected from Peru (70% ESBL-positive KP).⁷ More recently, from a total of 683 KP isolates identified from different sources in one participating hospital from 2012 to mid-2015, 53.4% of them were ESBL producers (unpublished data from the hospital Annual Laboratory Report). All these data confirm that ESBL-producing *Klebsiella* is an overwhelming problem in these hospital settings.

Regarding antimicrobial resistance in the 176 KP isolates, the rates were significantly higher among ESBL producers than the nonproducers: 85.3% versus 12.8% for gentamicin ($P < 0.01$), 59.7% versus 8.5% for TMP-SMX ($P < 0.01$), 45.0% versus 8.5% for ciprofloxacin ($P < 0.01$), and 36.4% versus 12.8% for amikacin ($P < 0.01$). One isolate showed intermediate resistance to imipenem, but was susceptible to meropenem. This high level of resistance to multiple antimicrobials highly limits the treatment options for neonatal sepsis in these settings. This situation may be a consequence of multiple facts such as the indiscriminate use of cephalosporins for common infections in Peru, which is associated with the lack of (or limited) antibiotic stewardship policy in Peruvian hospital as well as the limited infection control measures to reduce the transmission of multidrug-resistant microorganisms in these hospital settings.

TABLE 1

Frequency of ESBL-positive phenotype detection among *Klebsiella pneumoniae* from eight hospitals in Lima and Callao, 2008–2011

Hospital	Total no. of isolates	% of ESBL detection				
		2008	2009	2010	2011	Overall
1	37	76.5	72.7	80.0	100	78.4
2	31	75.0	62.5	62.5	–	71.0
3	23	69.2	55.6	100	–	65.2
4	22	83.3	28.6	66.0	–	63.6
5	21	–	55.6	100	80.0	76.2
6	16	90.0	50.0	–	–	75.0
7	15	–	62.5	100	80.0	73.3
8	11	–	66.7	100	100	90.9
Total	176	77.9	57.4	91.7	82.6	73.3

ESBL = extended-spectrum β -lactamase.

All 176 KP isolates were assigned to one of the three phylogenetic groups: 92% belonged to the group KpI and 4.6% and 3.4% to the groups KpII and KpIII, respectively. Among the 129 ESBL producers, 95.3% belonged to the group KpI, 2.4% to KpII, and the remaining 2.4% to KpIII. The isolates were distributed among the three groups, but the phylogenetic group KpI was the predominant one, which is in line with what has been previously described among the clinical KP isolates recovered from Brazil.²²

The clonal relationship among 129 ESBL-producer KP isolates was further analyzed; 87 different pulsotypes were found (see Supplemental Figure 1). The most predominant clone involved nine isolates that were distributed in four hospitals. In addition, three clones involved four or five isolates each, and the remaining pulsotypes involved two or three isolates. These findings indicate an extensive genetic heterogeneity among the ESBL-positive strains circulating in the different neonatal units studied.

Among ESBL producers ($N = 129$), the frequency of β -lactamase-encoding genes by group was as follows: SHV group: 125 (96.8%); OXA-1-like: 77 (59.8%); CTX-M: 74 (57.4%); and TEM: 69 (53.5%). A total of 109 (84.5%) isolates carried at least two genes, and 29 (22.5%) carried genes from all the four groups of β -lactamase genes assessed (Table 1).

One hundred and six PCR products from 37 randomly selected isolates were sequenced (Table 2). The *bla*_{SHV} gene was detected in 36 of them (97.3%) encoding at least nine SHV types (SHV 1, 2, 11, 12, 27, 32, 33, 60, and 129). The isolate that lacked the *bla*_{SHV} gene was run twice, but the results were negative in both tests. *bla*_{TEM} gene was detected in 21 isolates (56.8%), all of them harbored the *bla*_{TEM-1} variant. Among the CTX-M producers, the most commonly detected genes belonged to group 2 ($N = 24$, 64.9%), all the six *bla*_{CTX-M-2} genes completely determined were surrounded by classical *ISCRI* and *qacEAI* + *sulI* structures. It was not possible to differentiate the CTX-M type 2 from the type 97 among the remaining 18 isolates. The other most common CTX-M producers belonged to the group 1 ($N = 20$, 54.1%, all of them harbored the *bla*_{CTX-M-15} gene linked to *ISEcp1* and *orf477* genetic elements). The *bla*_{CTX-M-14a} genetic variant surrounded by *ISEcp1* and *IS903* sequences was found in four KP isolates. The ESBL phenotype in most of these selected isolates (34/37, 91.9%) were justified by the presence of at least one CTX-M type ESBL (Table 2). In two (5.4%), the presence of ESBL type SHV-2 justified this phenotype. Only in one isolate we could not determine the ESBL variant.

The most frequent CTX-M-types among KP isolates in this study were CTX-M-15 and CTX-M-2. CTX-M-15 has been described worldwide, but some CTX-M types are typically distributed in specific regions such as CTX-M-9 and CTX-M-14 in Spain and CTX-M-2 in several South American countries.²³ Besides, a study in Argentina showed changes in the molecular epidemiology of ESBL types among KP isolates: CTX-M-2 was maintained endemically but the emergence of CTX-M-15 was shown.²⁴ In Brazil, several studies have shown that CTX-M-15 is one of the most frequent ESBLs and is widely distributed in the country.²⁵ In Peru, limited investigations have included the molecular characteristics of *Klebsiella*, however, CTX-M-2 and CTX-M-15 were observed among commensal *E. coli* from healthy children in 2002.²⁶

TABLE 2

Types of β -lactamases based on sequencing of *bla* genes and phylogenetic groups among 37 phenotypically ESBL-producing *Klebsiella pneumoniae* isolates

Sample ID	Year of isolation	Hospital of origin	Phylogenetic group	β -lactamase gene variant(s)*
Rgai981	2010	1	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2} , <i>bla</i> _{CTX-M-15}
Rgai144	2008	1	I	<i>bla</i> _{SHV-60} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rhu131	2009	7	I	<i>bla</i> _{SHV-33} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rhu208	2011	7	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-15} , <i>bla</i> _{CTX-M-14}
Rhu090	2009	7	I	<i>bla</i> _{SHV} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rch145	2008	6	I	<i>bla</i> _{SHV-32} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rhu211	2011	7	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rch056	2008	6	I	<i>bla</i> _{SHV-12} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rch098	2008	6	I	<i>bla</i> _{SHV-12} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rseb136	2011	2	I	<i>bla</i> _{SHV-27} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rseb109	2010	2	I	<i>bla</i> _{SHV-1} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rch102	2008	6	I	<i>bla</i> _{SHV-129} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Rgai213	2008	1	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-15}
Rgai120	2011	1	I	<i>bla</i> _{SHV-33} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Ral019	2008	8	I	<i>bla</i> _{SHV-11} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-15}
Rma078	2009	5	I	<i>bla</i> _{SHV-12} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-15}
Rerm144	2008	3	III	<i>bla</i> _{SHV-32} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-15}
Rerm609	2009	3	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-15}
Rass033	2008	4	I	<i>bla</i> _{SHV-12} , <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-2/97} , <i>bla</i> _{CTX-M-15}
Ral290	2011	8	I	<i>bla</i> _{SHV-1} , <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-15}
Rma174	2011	5	II	<i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2/97}
Rseb030	2008	2	I	<i>bla</i> _{SHV-11} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2}
Rseb020	2008	2	I	<i>bla</i> _{SHV-11} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2}
Ral085	2009	8	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-14}
Rseb037	2008	2	I	<i>bla</i> _{SHV-11} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2}
Rass111	2008	4	I	<i>bla</i> _{SHV-12} , <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-2/97}
Rch424	2010	6	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-2}
Rass157	2008	4	I	<i>bla</i> _{SHV-12} , <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-2/97}
Rma135	2010	5	I	<i>bla</i> _{SHV-12} , <i>bla</i> _{CTX-M-2/97}
Rma130	2010	5	I	<i>bla</i> _{SHV-11} , <i>bla</i> _{CTX-M-14}
Rma141	2010	5	I	<i>bla</i> _{SHV-11} , <i>bla</i> _{CTX-M-14}
Rseb047	2009	2	I	<i>bla</i> _{SHV-1} , <i>bla</i> _{CTX-M-2}
Rerm247	2008	3	I	<i>bla</i> _{SHV-11} , <i>bla</i> _{CTX-M-2/97}
Rass072	2008	4	I	<i>bla</i> _{SHV} ND , <i>bla</i> _{CTX-M-2/97}
Rseb087	2009	2	II	<i>bla</i> _{SHV-2}
Rma095	2009	5	I	<i>bla</i> _{SHV-2}
Rerm024	2008	3	III	<i>bla</i> _{SHV} ND

ESBL = extended-spectrum β -lactamase; ND = not determined; PCR = polymerase chain reaction.*The *bla*_{OXA-1} gene was amplified by PCR, but none of the 22 *bla*_{OXA-1}-positive amplicons were sequenced. *bla*_{OXA-1} instead of *bla*_{OXA-1-like} is used in this table.

This study has several limitations. First, clinical data including antibiotic treatment and outcome were not recorded. Second, sequencing all β -lactamase genes was not possible, considering the high number of positive isolates and the multiple genes detected per isolate. This was particularly important for the group of CTX-M negative isolates ($N = 55$, 42.6%), since we were not able to determine which ESBL variant justified the ESBL phenotype. We selected a subset of 37 isolates for full molecular analysis. Although they were randomly selected, most of them carried the ESBL type CTX-M in contrast to only 56.4% of the entire group of ESBL isolates.

In conclusion, we found more than 70% of production of ESBL among KP isolates from neonates; these isolates also showed higher resistance rates to other antimicrobial agents. CTX-M-15 and CTX-M-2 were the most frequently detected ESBL types. Besides, multiple KP clones were circulating among the units involved. Antibiotic stewardship and cost-effective infection control measures are needed in these settings to decrease the risk of transmission of these multidrug-resistant microorganisms among neonates.

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Authors' addresses: Coralith García and Lizeth Astocondor, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru, E-mails: coralith.garcia@upch.pe and lizeth1226@hotmail.com. Beatriz Rojo-Bezales and Yolanda Sáenz, Centro de Investigación Biomédica de La Rioja (CIBIR), Logroño, Spain, E-mails: brojo@riojasalud.es and ysaenz@riojasalud.es. Jan Jacobs, Institute of Tropical Medicine Antwerp, Antwerp, Belgium, E-mail: jjacobs@itg.be.

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