Characterisation of extended-spectrum β-lactamases among *Klebsiella pneumoniae* isolates causing bacteraemia and urinary tract infection in Mozambique

Maria J. Pons [a,1], Delfino Vubil [b], Elisabet Guiral [a], Dinis Jaintilal [b], Oscar Fraile [b], Sara M. Soto [a], Betuel Sigaúque [b,c], Tacilta Nhampossa [b], Pedro Aide [b], Pedro L. Alonso [a,b], Jordi Vila [a], Inacio Mandomando [b,c], Joaquim Ruiz [a,*]

[a] *ISGlobal, Barcelona Centre for International Health Research (CRESIB), Hospital Clínic–Universitat de Barcelona, Barcelona, Spain*

[b] *Centro de Investigação em Saúde da Manhiça (CISM), Manhiça, Mozambique*

[c] *Instituto Nacional de Saúde, Ministério da Saúde, Maputo, Mozambique*

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* Corresponding author. Present address: CRESIB, Ed. CEK, pl1, C/ Rosselló 153, 08036 Barcelona, Spain. Tel.: +34 932 275 400 x4547; fax: +34 932 279 853.

E-mail address: joruiz@clinic.ub.es (J. Ruiz).

[1] Present address: Universidad Peruana de Ciencias Aplicadas (UPC), Lima, Peru.

ABSTRACT

The aim of this study was to determine the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* isolated from urinary tract and bloodstream infections in a rural hospital in Manhiça, Mozambique. ESBLs were investigated among ceftriaxone-non-susceptible *K. pneumoniae* clinical isolates recovered between 2004 and 2009. Characterisation of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{OXA} and bla_{TEM} genes was performed by PCR and sequencing. Epidemiological relationships were established by phylogenetic analysis, repetitive extragenic palindromic PCR (rep-PCR), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), whilst plasmid transferability was evaluated by conjugation. In addition, the presence of class 1 and 2 integrons was studied. A total of 19 *K. pneumoniae* were analysed. The $bla_{\text{CTX-M-15}}$ gene was found in all strains. Other ESBL genes were found concomitantly, including $bla_{\text{SHV-5}}$, $bla_{\text{SHV-2}}$, $bla_{\text{SHV-2A}}$, $bla_{\text{SHV-12}}$ and $bla_{\text{SHV-38}}$. In addition, other β-lactamases such as $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-30}}$ were also detected. rep-PCR identified 15 different epidemiological profiles. MLST analysis also showed great variability of sequence types. The $bla_{\text{CTX-M-15}}$ gene showed a high transfer capacity. The presence of class 1 integrons was high. High levels of multidrug resistance were also found. In conclusion, these data show the dominance of the CTX-M-type ESBL, particularly CTX-M-15, supporting its worldwide dissemination, including in areas with limited access to third-generation cephalosporins. This finding is a matter of concern for clinical management as third-generation cephalosporins are an alternative for treating severe cases of multidrug-resistant infections in this community.

1. Introduction

Infections caused by members of the Enterobacteriaceae family are among the major causes of hospital admission and associated mortality in children, particularly in developing countries of Africa [1]. Until recent years, infections caused by these micro-organisms have been successfully treated with the available and inexpensive antibiotics present in these countries. However, the emergence and widespread development of multidrug-resistant (MDR) strains to the early effective antibiotics led to the introduction of broad-spectrum antibiotics such as fluoroquinolones or third-generation cephalosporins, which are often unaffordable or unavailable in most low-income countries [2]. Unfortunately, during the last three decades, extended-spectrum β-lactamases (ESBLs) produced by enteric pathogens have spread worldwide since their first description in 1983 [3].

The emergence and spread of ESBLs is an important public health problem and has had a tremendous impact, particularly in low-income countries. ESBL-producing micro-organisms are also often resistant to other commonly available antibiotics, such as fluoroquinolones. Knowledge of the prevalence and characterisation of ESBLs is critical for defining local empirical strategies for the treatment of infections [4]. Although in Africa the presence of ESBLs has increasingly been reported [5–7], data in Mozambique remain limited.

Klebsiella pneumoniae is among the most clinically relevant enterobacteria, being frequently reported as a cause of serious infections [3]. Moreover, an increase in the

isolation of MDR strains, especially those producing ESBLs, has also been described [3]. Infections caused by MDR *K. pneumoniae* strains have been associated with adverse clinical outcomes, including increased mortality, and prolonged hospitalisation associated with economic costs for the health system [8]. Here we characterise ESBL-producing *K. pneumoniae* isolated from a rural hospital with limited access to third-generation cephalosporins and broad-spectrum antibiotics in general in Southern Mozambique.

2. Materials and methods

2.1. Study population

Klebsiella spp. strains from blood and urine isolated during the period August 2004 to December 2009 were retrospectively analysed for the presence of ESBLs. The study was conducted by the Centro de Investigação em Saúde de Manhiça (CISM) at Manhiça District Hospital, a rural referral hospital in Manhiça District, located 80 km north of Maputo, in Southern Mozambique. Bacterial infection surveillance in the paediatric population has been conducted in the study area since 1997. A description of the study area is given elsewhere [9]. Blood cultures were collected upon hospital admission from all children under 2 years of age and from older children up to 14 years of age with an axillary temperature ≥39 °C or meeting the criteria of severe infection [1]. Urine samples were also collected in the same period among patients (adults and children) visiting the outpatient department or admitted to the wards with clinical suspicion of urinary tract infection (UTI).

2.2. Bacterial isolation

Urine samples were cultured on MacConkey agar (Laboratorios CONDA, Madrid, Spain) and blood agar media (Becton Dickinson, Heidelberg, Germany). Blood culture bottles were incubated in an automated system (BACTEC® 9050; Becton Dickinson). Positive blood cultures were subcultured on solid media after Gram staining as appropriate. In both cases, pathogens were identified according to conventional microbiology protocols [10]. All selected *Klebsiella* spp. isolates were confirmed prior to analysis using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) as described elsewhere [8].

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by a conventional disk diffusion method. The antimicrobial agents tested were: ampicillin (10 μg); amoxicillin/clavulanic acid (20/10 μg); cefoxitin (30 μg); ceftriaxone (30 μg); ceftazidime (30 μg); imipenem (10 μg); meropenem (10 μg); gentamicin (10 μg); amikacin (30 μg); tetracycline (30 μg); chloramphenicol (30 μg); trimethoprim/sulfamethoxazole (1.25/23.75 μg); nalidixic acid (30 μg); ciprofloxacin (5 μg); rifampicin (5 μg); and azithromycin (15 μg). The category of resistance was interpreted according to Clinical and Laboratory Standard Institute (CLSI) guidelines [11]. *Escherichia coli* strain ATCC 25922 was used as a control. The breakpoints for rifampicin and azithromycin have not been established for *Klebsiella*

spp., therefore resistance to azithromycin and rifampicin was considered as an inhibition halo diameter of ≤15 mm [12].

2.4. β-Lactamase characterisation

Klebsiella pneumoniae isolates showing intermediate or full resistance to ceftriaxone were phenotypically screened for the production of ESBLs by the double-disk synergy test. The presence of ESBL genes (*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{OXA}$ and *bla*$_{TEM}$ genes) was established by PCR using previously described conditions [13]. The presence of the insertion sequence IS*Ecp1* upstream of *bla*$_{CTX-M-15}$ was determined by PCR as previously reported [14]. The amplified genes were sequenced by Macrogen DNA Sequencing Service (Macrogen, Seoul, South Korea). In addition, screening for metallo-β-lactamase (MBL) production was done by the imipenem/meropenem–ethylene diamine tetra-acetic acid (EDTA) combined disk test [15].

2.5. Genetic location and transferability studies

To determine the genetic location of the genes encoding the different ESBLs, plasmids were extracted following the methodology of Kado and Liu [16]. The plasmid or chromosomal location of ESBL genes detected was determined by Southern blot using the ctx-m-1 probe. Transferability of the plasmids harbouring ESBLs was determined by conjugation using azide-resistant *E. coli* J53 as the recipient strain and all ESBL-positive isolates as donor strains [17] using Muller–Hinton agar medium plus ceftriaxone (32 μg/mL). The clonal relationship between donor strains, transconjugants and the J53

recipient strain was established by repetitive extragenic palindromic PCR (rep-PCR) [18]. Incompatibility groups of plasmids were identified in study strains and their transconjugants as previously described [19]. Co-transference of antibiotic resistance was determined in all positive transconjugants by disk diffusion methodology as previously described [11].

2.6. Integron study

Strains were screened for the presence of class 1 and class 2 integrons by PCR using two sets of primers specific for the *intI1* and *intI2* genes [20]. The gene cassettes present within the variable regions of class 1 and class 2 integrons were determined by amplification with primers annealing to the 5' and 3' ends (5'-CS and 3'-CS) [20]. Amplified products were purified and sequenced at Macrogen.

2.7. Molecular epidemiology study

Klebsiella pneumoniae phylogenetic groups were established as described by Brisse et al. [21]. Clonal relationships were determined by rep-PCR as described elsewhere [18]. In all cases in which the identity was ≥80%, strains were analysed by pulsed-field gel electrophoresis (PFGE) [14].

PFGE profiles were compared using the fingerprinting software InfoQuest^{TM} FP v.4.5 (Bio-Rad, Hercules, CA). In order to construct dendrograms from the electrophoretic patterns, the Dice coefficient was used, with clustering by the unweighted pair-group

method with arithmetic mean (UPGMA) with a 1% tolerance in band position differences. In addition, multilocus sequence typing (MLST) was performed using the sequences of the *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *ton* genes [22]. MLST sequences were compared on the Institut Pasteur website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) to obtain the sequence type (ST) profile. The MLST tree was built from the allelic profile t using the neighbour-joining method (http://pubmlst.org/analysis/).

2.8. Statistical analysis

Statistical analysis was performed using STATA software v.12.0 (Stata Corp., College Station, TX). Proportions were compared using the χ^2 or Fisher's exact test, as appropriate. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Study strains

During the study period, *Klebsiella* spp. were identified as only 8.2% (30/368) and 25% (20/81) of the total Enterobacteriaceae isolated from blood and urine samples, respectively. A total of 19 ceftriaxone-resistant ESBL-producing *K. pneumoniae* isolates were included in this study, including 5 from urine samples (yielding a proportion of 26% of *K. pneumoniae* clinical isolates causing UTI), 11 from blood samples (proportion 58%) and 3 strains (16%) from unspecified sources (Fig. 1).

3.2. Antimicrobial susceptibility

The isolates were characterised as being dramatically resistant to the most commonly available antibiotics in this community. All of the isolates (100%) were resistant to ampicillin, cefoxitin, ceftriaxone, ceftazidime, chloramphenicol, gentamicin and rifampicin, 89% were resistant to amoxicillin/clavulanic acid and trimethoprim/sulfamethoxazole and 63% were resistant to tetracycline. Meanwhile, resistance levels to the remaining antimicrobial agents were lower; 32% of the isolates showed resistance to nalidixic acid and 16% showed resistance to amikacin, azithromycin and ciprofloxacin. No carbapenem-resistant isolates were found (Table 1).

3.3. Identification of β-lactamases by PCR and sequencing

The *bla*$_{\text{CTX-M-15}}$ gene was the most frequently detected, and IS*Ecp1* was found upstream of the *bla*$_{\text{CTX-M-15}}$ gene in all cases except two. Other ESBL genes concomitantly detected were exclusively from the *bla*$_{\text{SHV}}$ family (*bla*$_{\text{SHV-2}}$, *bla*$_{\text{SHV-2A}}$, *bla*$_{\text{SHV-5}}$, *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{SHV-38}}$), sometimes found in isolates carrying the *bla*$_{\text{CTX-M-15}}$ gene. In addition, other β-lactamases such as *bla*$_{\text{TEM-1}}$ (84%; 16/19), *bla*$_{\text{OXA-30}}$ (37%; 7/19) and different non-ESBL *bla*$_{\text{SHV-like}}$ genes (SHV-1, -11, -32, -33 and -121) were also detected. Analysis of the proportion of ESLBs according to the source of infection showed that ESBL-producing *K. pneumoniae* isolates were more prevalent in bloodstream strains compared with uropathogenic strains [11/30 (37%) vs. 5/20 (25%), respectively; $P < 0.05$]. No MBLs were detected (Table 1).

3.4. Transferability study

A total of 13 transconjugants were obtained, with the *bla*$_{\text{CTX-M-15}}$ gene being present in all cases. The plasmid incompatibility groups amplified in the parental isolates were IncFIC, IncFrep, IncA/C, IncY, IncFIIA, IncFIB, IncHI1 and IncHI2, showing a high variability of plasmid groups in these isolates. Meanwhile, in only 2 of the 13 transconjugants were the transferred incompatibility groups identified: IncA/C in the K4 transconjugant and IncFrep and IncFIB in the K13 transconjugant (Table 2). In addition, in 5 cases in which the ESBL phenotype was transferred, no incompatibility group was detected either in the parental or transconjugant strains.

3.5. Analysis of integrons

Twelve strains (63%) harboured class 1 integrons. Eight isolates had an integron of ca. 800 bp containing the gene cassettes *dfrA7* and *dfrA15* in three and five isolates, respectively, conferring resistance to trimethoprim. Five strains had an integron of 2000 bp. The first contained *dfrA5–ereA2* conferring resistance to trimethoprim and macrolides; three isolates had *dfrA16–aadA2*; and the last had *dfrA17–aadA5*, both conferring resistance to trimethoprim and streptomycin–spectinomycin, respectively. It is important to highlight that two types of gene cassettes (*dfrA15* and *dfrA16–aadA2*) were present in one strain (Table 1). No class 2 integrons were found.

3.6. Molecular epidemiological analysis

Restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) of the *gyrA* gene showed that all *K. pneumoniae* isolates belonged to the same phylogenetic group (KpI). rep-PCR showed 15 different epidemiological profiles (considering the same profile as having ≥80% similarity), with a maximum of three isolates per profile, suggesting that most strains were not epidemiologically related, as shown by different strain clusters. This result was confirmed by PFGE, which showed that all isolates belonged to different clusters and thus no related profiles were found. MLST analysis also showed a large variability of ST groups as well as the emergence of a new ST group (*gapA*-2, *infB*-1, *mdh*-1, *pgi*-1, *phoE*-16, *rpoB*-4, *tonB*-12) (Fig. 2). The tree developed with allele variants also showed a high diversity of STs (Fig. 3).

4. Discussion

This study reports a high prevalence of ESBLs in *Klebsiella* isolates both in bacteraemia and UTIs. ESBL data in *Klebsiella* from Mozambique are scarce. In fact, only one study showed a high prevalence of ESBLs in the Central region (Beira City) for both *Klebsiella* spp. (75–100%) and *E. coli* (63.6–66.7%) causing UTI in children admitted to the malnutrition and paediatric wards [6].

The high prevalence of antibiotic resistance is a matter of special concern as antibiotic therapies are often instituted empirically in the country as well as due to the lack of available second-line alternatives [2,23]. The rates of ESBLs and antibiotic resistance observed in this study may be associated with a complex scenario of treatments derived

from the high prevalence of infectious diseases [tuberculosis (TB), malaria, human immunodeficiency virus (HIV), etc.] [24,25]. Therefore, bacteria present in the area are under direct pressure from trimethoprim/sulfamethoxazole not only in people treated with trimethoprim/sulfamethoxazole or sulfadoxine/pyrimethamine, but also in HIV-infected and HIV-exposed subjects. In addition, there is high rifampicin pressure due to its use in the treatment of TB. In this line of thought, the analysis of integrons has shown the high number and variability of trimethoprim resistance genes. A serious parallel event is that trimethoprim/sulfamethoxazole pressure could co-select other resistance genes such as the *ereA* gene that confers resistance to macrolides. In the future, this fact may compromise the use of macrolides in the area. In addition, co-selection of resistance to antibiotics not in use in the area cannot be ruled out.

All of the isolates in this study were related to the KpI phylogenetic group, in accordance with the idea that this group is the most common in clinical specimens and is related to highest levels of resistance. Thus, the high prevalence of KpI isolates could be a consequence of the high resistance rates, since antibiotic resistance is a critical parameter for the transmission of this pathogen [21]. The lack of clonality of the studied strains in conjunction with high heterogeneity in the ST groups shows the presence of non-related ESBL-producing MDR *K. pneumoniae* strains circulating in the community. The finding of $bla_{\text{CTX-M-15}}$ as the most prevalent ESBL in this community confirms its important dissemination worldwide. In addition, five ESBLs belonging to the bla_{SHV} group were detected. These bla_{SHV} genes might be plasmid-encoded and then, due to the universal nature of the used primers, be co-amplified with the chromosomal bla_{SHV}

but detected in a preferential manner because of the high copy number. However, all *bla*$_{SHV}$ detected in the present study were unable to conjugate, thus the possible presence in the area of *K. pneumoniae* carrying different chromosomal *bla*$_{SHV}$ variants cannot be excluded. In fact, some of them, such as *bla*$_{SHV-38}$, have been previously described as encoded on the chromosome of *K. pneumoniae* [26].

Although ESBLs have been previously reported in sub-Saharan Africa [5–7,27–30], the report of ESBLs in a rural hospital with limited access to third-generation cephalosporins, such as in Manhiça, Mozambique, is relevant, suggesting again the co-selection of antibiotic resistance genes. Despite access to third-generation cephalosporins being limited in this community, a high prevalence of β-lactamase genes, such as *bla*$_{TEM-like}$ and *bla*$_{OXA-like}$, has been reported in *Shigella* and *Salmonella* isolates in previous studies, reflecting of the high use of ampicillin in the area [23].

No MBLs were detected and no isolates were resistant to carbapenems. However, *bla*$_{SHV-38}$ is able to hydrolyse imipenem [26], and the combination of *bla*$_{CTX-M-15}$ or *bla*$_{SHV-12}$ plus loss of expression of OmpK35 and/or OmpK36 results in full resistant to carbapenems [31]; thus, the use of carbapenems in the area needs to be done with caution.

These findings are of special concern since ceftriaxone is the main third-generation cephalosporin used in the area for severe infections by MDR pathogens. In addition, it

was the only antibiotic found to be effective in vitro for MDR *E. coli* strains with reduced susceptibility to fluoroquinolones [2].

The reported prevalence of ESBLs is high compared with that reported in neighbouring countries such as Tanzania [27] and South Africa [5]. In another sub-Saharan country (Ethiopia), a high level of ESBL-producers (36%; 46% among inpatients and 33% among outpatients) was reported among *E. coli* strains from different samples (urine, sputum, stool, wound swabs) [28]. Similarly, ESBLs were found in 247 (27%) of 912 *E. coli* isolates, mainly $bla_{\text{CTX-M-14}}$ (29%) and $bla_{\text{CTX-M-15}}$ (24%), in hospital- and community-acquired infections in Kenya, whilst non-ESBLs were found in 30% (54% $bla_{\text{TEM-1}}$, 35% $bla_{\text{SHV-1}}$ and 11% both $bla_{\text{TEM-1}}$ and bla_{SHV}) [29]. ESBLs have also been detected in Gabon, where 49.4% of all *K. pneumoniae* were ESBL-producers [7].

The ESBLs isolated from the bloodstream are likely to be community-acquired since blood cultures were collected upon admission and no extra blood cultures were taken during hospitalisation. Moreover, data from the demographic and morbidity databases showed that none of the patients in whom ESBL-producing micro-organisms were isolated had been hospitalised in the preceding 6 months. In contrast, we cannot rule out hospital-acquired ESBLs for those detected in urine since some samples were collected ≥48 h after admission.

The community is predominately rural; non-prescribed antibiotic use is uncommon and third-generation cephalosporins are rarely used even in the hospital owing to their

limited availability. One plausible explanation for the high prevalence of ESBLs reported here is their possible association with international dissemination through the introduction of strains harbouring ESBLs and posterior dissemination within the community. Moreover, ESBL maintenance in the bacterial population may be due to the high levels of use of ampicillin or by co-selection related to the presence in the same genetic structure of mechanisms of resistance for other non-related antimicrobial agents used in the area. In this line of thought, carriage of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* isolates was reported in children living in a remote village with no data known of antibiotic exposure [30]. These data agree with the current massive spread of ESBLs worldwide.

Treatment of infections caused by ESBL-producing organisms is difficult because of their resistance to other antibacterial agents, including fluoroquinolones. On the other hand, antibiotics used as second-line treatment such as carbapenems, which are still effective in vitro in the Manhiça area, are not available in most developing countries. Use of fluoroquinolones could be compromised in areas with a high prevalence of malaria that use chloroquine as treatment owing to the selection of quinolone resistance during antimalarial therapy in the absence of quinolone exposure [32], although, actually, in Manhiça chloroquine is not in use.

The availability of effective antibiotics is a challenge, with this fact being of special concern in developing countries where the antibiotic armamentarium is limited. Steadily increasing resistance among Gram-negative bacteria associated with the emergence and spread of MDR strains including ESBL-producers is an important public health

problem with serious implications in low-income countries, which bear 95% of the global infectious disease burden and in which the use of empirical antimicrobial treatment plays a strategic role to counteract these diseases. This has resulted in many once easily curable infectious diseases becoming untreatable [7].

The present data are important to guide empirical treatment in the area and compromise the use of third-generation cephalosporins. This study highlights the spread of different ESBLs in rural Mozambique, despite the low levels of cephalosporin use, thereby demonstrating the need to establish antimicrobial resistance surveillance within the country to monitor the trends and new types of resistance mechanisms in order to promote adequate therapeutic guidelines.

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References

[1] Sigaúque B, Roca A, Mandomando I, Morais L, Quintó L, Sacarlal J, et al. Community-acquired bacteremia among children admitted to a rural hospital in Mozambique. Pediatr Infect Dis J 2009;28:108–13.

[2] Mandomando I, Sigauque B, Morais L, Espasa M, Vallès X, Sacarlal J, et al. Antimicrobial drug resistance trends of bacteremia isolates in a rural hospital in Southern Mozambique. Am J Trop Med Hyg 2010;83:152–7.

[3] Downie L, Armiento R, Subhi R, Kelly J, Clifford V, Duke T. Community-acquired neonatal and infant sepsis in developing countries: efficacy of WHO's currently recommended antibiotics—systematic review and meta-analysis. Arch Dis Child 2013;98:146–54.

[4] Tumbarello M, Sali M, Trecarichi EM, Leone F, Rossi M, Fiori B, et al. Bloodstream infections caused by extended-spectrum-β-lactamase-producing *Escherichia coli*: risk factors for inadequate initial antimicrobial therapy. Antimicrob Agents Chemother 2008;52:3244–52.

[5] Usha G, Chunderika M, Prashini M, Willem SA, Yusuf ES. Characterization of extended-spectrum β-lactamases in *Salmonella* spp. at a tertiary hospital in Durban, South Africa. Diagn Microbiol Infect Dis 2008;62:86–91.

[6] van der Meeren BT, Chhaganlal KD, Pfeiffer A, Gomez E, Ferro JJ, Hilbink M, et al. Extremely high prevalence of multi-resistance among uropathogens from hospitalised children in Beira, Mozambique. S Afr Med J 2013;103:382–6.

[7] Alabi AS, Frielinghaus L, Kaba H, Kösters K, Huson MA, Kahl BC, et al. Retrospective analysis of antimicrobial resistance and bacterial spectrum of infection in Gabon, Central Africa. BMC Infect Dis 2013;13:455.

[8] Berrazeg M, Diene SM, Drissi M, Kempf M, Richet H, Landraud L, et al. Biotyping of multidrug-resistant *Klebsiella pneumoniae* clinical isolates from France and Algeria using MALDI-TOF MS. PLoS One 2013;8:e61428.

[9] Nhacolo AQ, Nhalungo DA, Sacoor CN, Aponte JJ, Thompson R, Alonso P. Levels and trends of demographic indices in southern rural Mozambique: evidence from demographic surveillance in Manhiça District. BMC Public Health 2006;6:291.

[10] Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA. *Manual of clinical microbiology.* 9th ed. Washington, DC: ASM Press; 2007.

[11] Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial disk susceptibility tests; approved standard—tenth edition*. Document M02-A10. Wayne, PA: CLSI; 2009.

[12] Ochoa TJ, Ruiz J, Molina M, Del Valle LJ, Vargas M, Gil AI, et al. High frequency of antimicrobial drug resistance of diarrheagenic *Escherichia coli* in infants in Peru. Am J Trop Med Hyg 2009;81:296–301.

[13] Jouini A, Vinue L, Slama KB, Sáenz Y, Klibi N, Hammami S, et al. Characterization of CTX-M and SHV extended-spectrum β-lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia. J Antimicrob Chemother 2007;60:1137–41.

[14] Durmaz R, Otlu B, Koksal F, Hosoglu S, Ozturk R, Ersoy Y, et al. The optimization of a rapid pulsed-field gel electrophoresis protocol for the typing of *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiell*a spp. Jpn J Infect Dis 2009;62:372.

[15] Tsakris A, Poulou A, Pournaras S, Voulgari E, Vrioni G, Themeli-Digalaki K, et al. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. J Antimicrob Chemother 2010;65:1664–71.

[16] Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 1981;145:1365–73.

[17] Pérez-Moreno MO, Picó-Plana E, de Toro M, Grande-Armas J, Quiles-Fortuny V, Pons MJ, et al. β-Lactamases, transferable quinolone resistance determinants, and class 1 integron-mediated antimicrobial resistance in human clinical *Salmonella enterica* isolates of non-Typhimurium serotypes. Int J Med Microbiol 2013;303:25–31.

[18] Navia MM, Capitano L, Ruiz J, Vargas M, Urassa H, Schellemberg D, et al. Typing and characterization of mechanisms of resistance of *Shigella* spp. isolated from feces of children under 5 years of age from Ifakara, Tanzania. J Clin Microbiol 1999;37:3113–7.

[19] Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 2005;63:219–28.

[20] Sáenz Y, Briñas L, Domínguez E, Ruiz J, Zarazaga M, Vila J, et al. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. Antimicrob Agents Chemother 2004;48:3996–4001.

[21] Brisse S, van Himbergen T, Kusters K, Verhoef J. Development of a rapid identification method for *Klebsiella pneumoniae* phylogenetic groups and analysis of 420 clinical isolates. Clin Microbiol Infect 2004;10:942–5.

[22] Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol 2005;43:4178–82.

[23] Mandomando I, Jaintilal D, Pons MJ, Vallès X, Espasa M, Mensa L, et al. Antimicrobial susceptibility and mechanisms of resistance in *Shigella* and *Salmonella* isolates from children under five years of age with diarrhea in rural Mozambique. Antimicrob Agents Chemother 2009;53:2450–4.

[24] González R, Munguambe K, Aponte J, Bavo C, Nhalungo D, Macete E, et al. High HIV prevalence in a southern semi-rural area of Mozambique: a community-based survey. HIV Med 2012;13:581–8.

[25] World Health Organization. *Global tuberculosis report 2013*. Geneva, Switzerland: WHO; 2013.

[26] Poirel L, Héritier C, Podglajen I, Sougakoff W, Gutmann L, Nordmann P. Emergence in *Klebsiella pneumoniae* of a chromosome-encoded SHV β-lactamase that compromises the efficacy of imipenem. Antimicrob Agents Chemother 2003;47:755–8.

[27] Blomberg B, Jureen R, Manji KP, Tamim BS, Mwakagile DS, Urassa WK, et al. High rate of fatal cases of pediatric septicemia caused by Gram-negative bacteria with extended-spectrum β-lactamases in Dar es Salaam, Tanzania. J Clin Microbiol 2005;43:745–9.

[28] Mulualem Y, Kasa T, Mekonnen Z, Suleman S. Occurrence of extended spectrum β-lactamases in multi-drug resistant *Escherichia coli* isolated from a clinical setting in Jimma University Specialized Hospital, Jimma, southwest Ethiopia. East Afr J Public Health 2012;9:58–61.

[29] Kiiru J, Kariuki S, Goddeeris BM, Butaye P. Analysis of β-lactamase phenotypes and carriage of selected β-lactamase genes among *Escherichia coli* strains obtained from Kenyan patients during an 18-year period. BMC Microbiol 2012;12:155.

[30] Ruppe E, Woerther PL, Diop A, Sene AM, Da Costa A, Arlet G, et al. Carriage of CTX-M-15-producing *Escherichia coli* isolates among children living in a remote village in Senegal. Antimicrob Agents Chemother 2009;53:3135–7.

[31] Tsai YK, Liou CH, Fung CP, Lin JC, Siu LK. Single or in combination antimicrobial resistance mechanisms of *Klebsiella pneumoniae* contribute to varied susceptibility to different carbapenems. PLoS One 2013;8:e79640.

[32] Davidson RJ, Davis I, Willey BM, Rizg K, Bolotin S, Porter V, et al. Antimalarial therapy selection for quinolone resistance among *Escherichia coli* in the absence of quinolone exposure, in tropical South America. PLoS One 2008;3:e2727.

Fig. 1. Flowchart of *Klebsiella* isolates and the prevalence of extended-spectrum β-lactamases (ESBLs) in blood and urine samples. CRO, ceftriaxone.

Fig 2. Epidemiological relationships between *Klebsiella pneumoniae* isolates. rep-PCR, repetitive extragenic palindromic PCR; ST, sequence type.

Fig. 3. Tree of allelic profile using multilocus sequence typing (MLST) in *Klebsiella pneumoniae* isolates.

Highlights

- We showed the dominance of the CTX-M-type extended-spectrum β-lactamase (ESBL) in Mozambique, supporting its worldwide dissemination.
- We report a high prevalence of ESBLs in *Klebsiella* isolates both in bloodstream and urinary tract infections.
- We report the presence of ESBLs in an area with limited access to third-generation cephalosporins.
- Third-generation cephalosporins are an alternative for treating multidrug-resistant infections in Mozambique.
- Rates of antibiotic resistance are very high.

Table 1

Study strains, β-lactamase analysis, resistance profile and class 1 integron characterisation

Isolate	Source	ESBL genes	Other β-lactamases genes	IS*Ecp1* upstream of CTX-M	Antibiotic resistance pattern [a]	Integron
K1	Indeterminate	CTX-M-15, SHV-5	TEM-1	Absent	AMP-AMC-CHL-GEN-TET-RIF-SXT	*dfrA7*
K2	Indeterminate	CTX-M-15	TEM-1, SHV-1	Present	AMP-AMC-CHL-GEN-TET-RIF-SXT-AZM	*dfrA15*
K4	Blood	CTX-M-15	TEM-1, SHV-1	Present	AMP-AMC-CHL-GEN-TET-RIF-SXT	*dfrA5–ereA2*
K5	Blood	CTX-M-15	TEM-1, SHV-33, OXA-30	Present	AMP-AMC-AMK-CHL-GEN-TET-RIF-SXT	–
K6	Blood	CTX-M-15, SHV-2	TEM-1	Present	AMP-AMC-CHL-GEN-TET-RIF-SXT	*dfrA7*
K8	UTI	CTX-M-15	TEM-1, SHV-121	Present	AMP-AMC-CHL-GEN-TET-RIF-SXT	*dfrA7*
K9	Blood	CTX-M-15	TEM-1, SHV-11	Present	AMP-AMC-CHL-GEN-TET-RIF-SXT	*dfrA15*/*dfrA16–aadA2*
K10	Indeterminate	CTX-M-15	TEM-1, SHV-11, OXA-30	Present	AMP-AMC-CHL-GEN-TET-RIF	*dfrA16–aadA2*

K12	Blood	CTX-M-15	TEM-1, SHV-1, OXA-30	Present	AMP-CHL-AMK-GEN-RIF-SXT	*dfrA15*
K13	Blood	CTX-M-15	TEM-1, SHV-32	Present	AMP-AMC-CHL-GEN-NAL-TET-RIF-SXT-AZM	*dfrA17–aadA5*
K15	Blood	CTX-M-15, SHV-38	TEM-1, OXA-30	Present	AMP-CHL-AMK-GEN-RIF-SXT	–
K16	Blood	CTX-M-15, SHV-2A	TEM-1	Present	AMP-AMC-CHL-GEN-RIF-SXT	*dfrA15*
K18	UTI	CTX-M-15, SHV-2	–	Present	AMP-AMC-CHL-GEN-RIF-SXT	*dfrA15*
K19	Blood	CTX-M-15	TEM-1, SHV-11	Present	AMP-AMC-CHL-GEN-NAL-RIF-SXT	–
K24	UTI	CTX-M-15	–	Present	AMP-AMC-CHL-GEN-NAL-CIP-RIF-SXT	–
K25	Blood	CTX-M-15	TEM-1, SHV-1, OXA-30	Absent	AMP-AMC-CHL-GEN-NAL-CIP-TET-RIF-SXT	*dfrA16–aadA2*
K26	Blood	CTX-M-15	–	Present	AMP-AMC-CHL-GEN-NAL-RIF-SXT	–
K27	UTI	CTX-M-15	TEM-1, SHV-11, OXA-30	Present	AMP-AMC-CHL-GEN-TET-RIF-SXT-AZM	–

K28	UTI	CTX-M-15, SHV-12	TEM-1, OXA-30	Present	AMP-AMC-CHL-GEN-NAL-CIP-TET-RIF-SXT	–

ESBL, extended-spectrum β-lactamase; UTI, urinary tract infection; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CHL, chloramphenicol; GEN, gentamicin; TET, tetracycline; RIF, rifampicin; SXT, trimethoprim/sulfamethoxazole; AZM, azithromycin; AMK, amikacin; NAL, nalidixic acid; CIP, ciprofloxacin.

[a] All isolates were additionally resistant to cefoxitin, ceftazidime and ceftriaxone.

Table 2

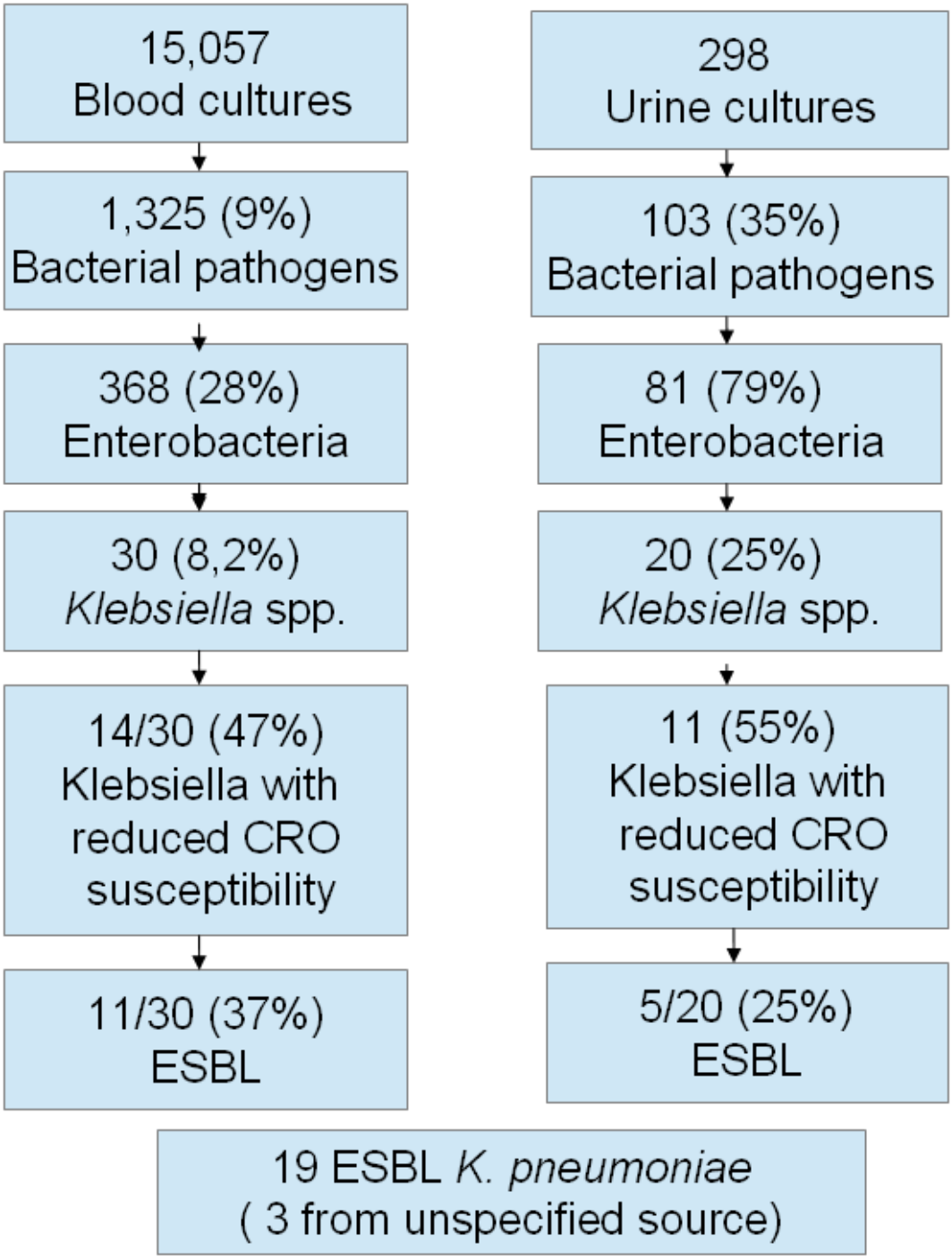
Conjugation assay and plasmid characterisation

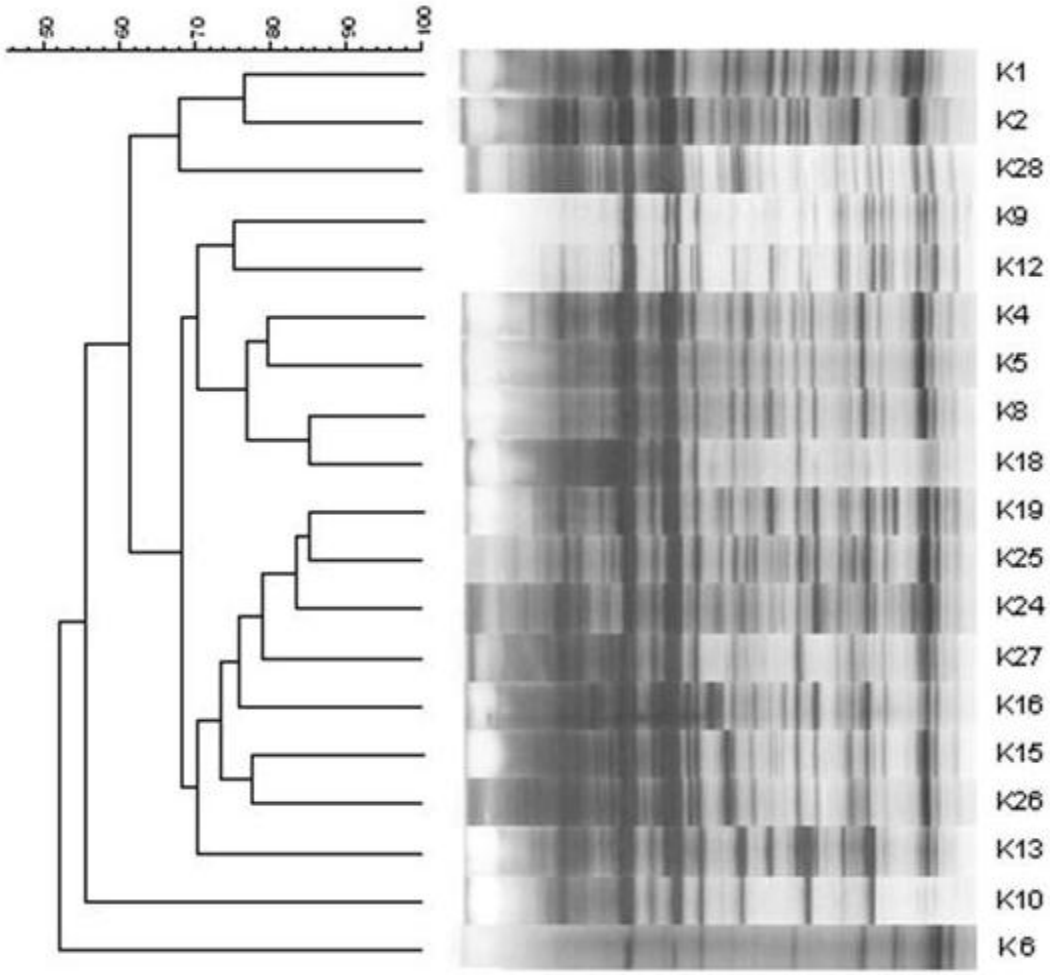
Donor isolate		Transconjugant		
Isolate	Plasmid replicon	Plasmid replicon	Co-selection of antibiotic resistance [a]	ESBL gene
K1	FIC	–	**AMP-AMC-CHL-GEN**-TET-RIF-SXT	*bla*$_{CTX-M-15}$
K2	Frep			
K4	A/C	A/C	**AMP-AMC-CHL-GEN-TET**-RIF-**SXT**	*bla*$_{CTX-M-15}$
K5	–	–		
K6	–	–	**AMP-AMC-CHL-GEN-TET**-RIF-**SXT**	*bla*$_{CTX-M-15}$
K8	A/C/Frep	–	**AMP**-AMC-CHL-GEN-TET-RIF-SXT	*bla*$_{CTX-M-15}$
K9	Y			
K10	Y	–	**AMP-AMC-CHL-GEN**-TET-RIF	*bla*$_{CTX-M-15}$
K12	FIIAs	–	**AMP-CHL**-AMK-GEN-**RIF**-SXT	*bla*$_{CTX-M-15}$
K13	FIB/Frep	FIB/Frep	**AMP-AMC-CHL-GEN**-NAL-TET-**RIF-SXT**-AZM	*bla*$_{CTX-M-15}$
K15	–	–	**AMP-CHL-**AMK-**GEN**-RIF-**SXT**	*bla*$_{CTX-M-15}$
K16	–	–	**AMP-AMC-CHL-GEN**-RIF-**SXT**	*bla*$_{CTX-M-15}$
K18	–			
K19	–			
K24	–			
K25	HI1, HI2	–	**AMP**-AMC-CHL-GEN-NAL-CIP-TET-RIF-SXT	*bla*$_{CTX-M-15}$
K26	–	–	**AMP**-AMC-**CHL-GEN**-NAL-RIF-**SXT**	*bla*$_{CTX-M-15}$

K27	Frep	–	**AMP-AMC-CHL-GEN**-TET-RIF-**SXT**-AZM	*bla*$_{CTX-M-15}$
K28	–	–	**AMP**-AMC-**CHL**-GEN-NAL-CIP-TET-RIF-SXT	*bla*$_{CTX-M-15}$

ESBL, extended-spectrum β-lactamase; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CHL, chloramphenicol; GEN, gentamicin; TET, tetracycline; RIF, rifampicin; SXT, trimethoprim/sulfamethoxazole; AMK, amikacin; AZM, azithromycin; NAL, nalidixic acid; CIP, ciprofloxacin.

[a] Bold letters indicate the antibiotic resistance phenotype that was found in the transconjugant strains.





rep-PCR profile group	ST	Phylogenetic group
1	298	KpI
2	45	KpI
3	334	KpI
4	111	KpI
5	76	KpI
6	626	KpI
6	218	KpI
6	218	KpI
7	54	KpI
8	ND	KpI
8	17	KpI
8	101	KpI
9	17	KpI
10	29	KpI
11	29	KpI
12	29	KpI
13	234	KpI
14	268	KpI
15	54	KpI