Title: FIRST REPORT OF A *Klebsiella pneumoniae* ST466 STRAIN CAUSING NEONATAL SEPSIS HARBOURING THE $bla_{CTX\text{-}M\text{-}15}$ GENE IN RABAT, MOROCCO.

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Abstract

Klebsiella pneumoniae is one of the Gram-negative bacilli most commonly found in urine of pregnant women and causing neonatal sepsis. The aim of this study was analyze in terms of epidemiology and antimicrobial resistance of 23 *K. pneumoniae* isolates collected from vaginal swabs or urine of pregnant women, from pharyngeal and ear swabs of apparently healthy newborns, and from peripheral cultures and hemocultures of newborns with suspected invasive neonatal infection in Rabat, Morocco. The prevalence of *K. pneumoniae* was 0.6% and 0.9% among pregnant women and neonates, respectively. These strains showed lower antimicrobial resistance levels regarding to developed countries. Thus, only one strain from a neonate presented an ESBL. This is the first report of a *K. pneumoniae* strain causing neonatal sepsis harbouring the *bla*CTX-M-15 gene in an IncFII plasmid and belonging to ST466 in this area.

Introduction

Contrarily to what occurs in developed countries, the impact of infectious diseases in middle and low-income countries remains huge as a cause of morbidity of the mothers, the foetus and the newborns. Recent estimates suggest that infectious diseases may account for at least 30% of the deaths occurring in newborns (1 million deaths annually) and 50% or more of all stillbirths in low and middle income countries (Goldenberg *et a*l., 2010). The microorganisms most frequently involved in these infections include, among others, group B *Streptococcus* (GBS), *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus, Klebsiella pneumoniae* and *Haemophilus influenzae.* Neonatal infections caused by microorganisms harbouring ESBLs are usually acquired during hospitalization and associated with invasive procedures (catheters, etc.). *E. coli* and *K. pneumoniae* are the two Gram-negative bacterial pathogens involved in neonatal sepsis in developing countries (Roy *et al*., 2013).

Pregnant women are at increased risk of developing urinary tract infections (UTIs) including asymptomatic bacteriuria, cystitis or pyelonephritis. Several factors may contribute to the development of UTIs during pregnancy, such as the increase in urinary volume within the bladder which helps spread the infection from the bladder to the kidneys or increase in urine pH and in urinary progestin and estrogens favouring bacterial growth (Patterson & Andriole, 1987). In consequence, these all can lead to adverse pregnancy outcomes such as preterm birth, and even neonatal sepsis. Among the Gram-negative microorganisms involved in these infections are *E. coli, K. pneumoniae, Proteus mirabilis* and *Enterobacter* spp. Additionally, untreated asymptomatic bacteriuria has been associated with intrauterine growth retardation and low-birth-weight infants (Harris *et al*., 1991).

K. pneumoniae may be cause of sepsis in the newborn, mainly in patients with some predisposing factors, including prematurity or those carrying an intravenous catheter. Oropharyngeal colonization could act as the main reservoir for nosocomial outbreaks caused by *K. pneumoniae* that have been reported in the literature (Rastogi *et al*., 2010; Ruiz *et al*., 2010).

Klebsiella spp. were the most common bacterial pathogens in newborns in Tel Aviv, Ethiopia, India, and Mexico showing a mortality rate of approximately 66.6% (Ghotaslou *et al*., 2007).

Since the initial description of extended-spectrum β-lactamase (ESBL) production by *K. pneumoniae* strains in 1983 (Knothe *et al*., 1985), *K. pneumoniae* strains resistant to broad spectrum cephalosporins are being increasingly recognized (Jacoby & Medeiros, 1991) and spread worldwide.

In this study, we describe the prevalence and antimicrobial resistance of *K. pneumoniae* isolates collected from pregnant women and newborns in Rabat, Morocco, emphasizing the first report of a CTX-M-15 *K. pneumoniae* strain causing neonatal sepsis belonging to sequence type ST466 in this Northern African region.

Material and Methods

Study population

The study formed part of a bacteriological screening programme for *K. pneumoniae* among pregnant women and sick newborns carried out from March to July 2013. Vaginal swabs and urine samples of 349 pregnant women attending antenatal visits during weeks 35 to 37 of their pregnancies, or from pregnant women delivering at the maternity ward in the Maternité des Orangers (Rabat, Morocco) with no prior sampling conducted were included in the study. Pharyngeal and ear swabs were obtained from 135 newborns apparently healthy born from recruited mothers. In addition, peripheral cultures and hemocultures were obtained from 86 newborns admitted in the first 6 hours of life to the neonatal ward of the Hôspital d'Enfants of Rabat with suspected invasive neonatal infection. Vaginal, pharyngeal and ear swabs were spread into MacConkey agar and suspected *K. pneumoniae* colonies were confirmed using API 10S system.

Determination of phenotypic and genotypic resistance

Resistance phenotypes were carried out by disk-plate diffusion agar method using the Clinical and Laboratory Standards Institute guidelines (2011). The antimicrobial agents analyzed were cefotaxime (CTX 30µg), ampicillin (AM 10µg), gentamicin (GM 10µg), tetracycline (Te 30µg), chloramphenicol (C 30µg), ciprofloxacin (CIP 5µg) and trimethoprim-sulphamethoxazole (SXT 30µg). ESBL production was verified by a double-disc confirmation test (EUCAST, 2013) and ESBL producers were screened for *bla*CTX-M-type by PCR and sequencing (Calbo *et al.*, 2005).

Conjugation experiments

Conjugation experiments using an *E. coli* K12 strain resistant to kanamycin (Km) were performed to determine transferability. The possible transconjugants were selected onto MacConkey agar plates supplemented with 32mg/ml of cefotaxime and 256mg/ml of kanamycin.

REP-PCR (Vila *et al*., 1996) of the obtained colonies, as well as PCR specific for the $bla_{\text{CTX-M-15}}$ (Calbo *et al*., 2005) were carried out in order to determine if they share the same band profile than the receptor strain but containing the ESBL gene under study.

Plasmids analysis of transconjugants

The location of $bla_{\text{CTX-M-15}}$ gene was studied by plasmid extraction using the S1 digestion method (Durmaz *et al*., 2009), which allows to separate chromosomal DNA from plasmidic DNA. In addition, southern blot and hybridization using the $bla_{\text{CTX-M-15}}$ probe was performed.

Five different multiplex-PCRs recognizing three different replicon types, and three simplex-PCRs for F, K and B/O were used to assign plasmids from donor and transconjugant strains to the incompatibility groups (Carattoli *et al*., 2005).

Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was performed according to Diancourt *et al*. (2005). The *rpoB, gapA, mdh, pgi, phoE, infB,* and *tonB* genes were amplified and sequenced. Allele sequences were analysed with a database available online (www.pasteur.fr/mlst).

Results

The prevalence of *K. pneumoniae* on pregnant women in this study was 0.6% (20/349). Among these isolates, 12 were collected from vaginal swabs and 8 from urine samples. In addition, *K. pneumoniae* was isolated from the ear swab of one asymptomatic newborn birth from one of the recruited mothers. Two isolates from blood and pharyngeal swab, confirmed to be the same strain, were collected from one newborn presenting early-onset neonatal sepsis (EONS). No samples from the mother of the last newborn were available, as she had not been recruited to the study (Table 1).

All the *K. pneumoniae* isolates studied were resistant to ampicillin, seven to tetracycline (30%), four to trimethoprim-sulphamethoxazole (17%), four to ciprofloxacin (17%), two to gentamicin (8.7%) and only one was resistant to chloramphenicol (4.3%). Only the strain collected from the newborn showing EONS was resistant to cefotaxime and presented a resistance phenotype by double-disk synergy test indicating ESBL production. Apart from β-lactam resistance (ampicillin and cefotaxime), this strain also showed resistance to gentamicin (Table 1). Amplification with specific primers for $bla_{\text{CTX-M-1}}$ group and sequencing provided positive genotypic confirmatory test results for ESBL production, showing the presence of the $bla_{\text{CTX-M-15}}$ gene.

S1 digestion showed that the strain causing EONS presented two plasmids of about 145.5 kb and 60 kb (Figure 1A). Southern blot and hybridization with CTX-M-15 probe of the S1 digestion showed that the $bla_{\text{CTX-M-15}}$ gene was located in the plasmid of about 60 kb. This plasmid belonged to the IncFII incompatibility group (Figure 1B).

MLST determined that this strain presented the alleles: *gapA*-2, *infB*-1, *mdh*-2, *pgi*-1, *phoE*-10, *rpoB*-50, *tonB*-120, corresponding to the ST466 that has not been described in this area yet.

Discussion

K. pneumoniae strains harbouring the *bla*$_{\text{CTX-M-15}}$ gene and causing neonatal sepsis have been reported worldwide but belonged to different sequence types (ST48, ST11, ST17, ST341, ST15) and presented different plasmids with different sizes and incompatibility groups from those presented in this study (Mshana *et al*., 2013; Oteo *et al.*, 2009; Rettedal *et al.*, 2012). Two references about a *K. pneumoniae* isolates belonging to ST466 were compiled in the Institute Pasteur webpage in 2010 and 2013, respectively (http://www.pasteur.fr/cgi-bin/genopole/PF8/mlstdbnet.pl?page=profile-query&file=klebs_profiles.xml).

Characterization of the plasmids harbouring resistance determinants is necessary for further surveillance and global epidemiology in order to understand that not only a dissemination of bacterial clones can happen but also a dissemination of plasmids harbouring resistant determinants is possible intra- and interspecies. For instance, the *bla*$_{\text{CTX-M-15}}$ genes have been found into transferable plasmids between 40-350 kb and belonged to IncF, IncI, IncN, IncP, IncA/C, and IncL/M incompatibility groups (Carattoli, 2009). Thus, in the presented study, this gene was found in an IncFII plasmid. The IncF plasmids are largely distributed among Enterobacteriaceae clinical isolates. The high versatility of these plasmids with regard to cellular adaptation and evolution of their mechanism of replication of the IncF plasmid, are related to their high capacity to spread the *bla*$_{\text{CTX-M-15}}$ gene in humans (Carattoli, 2009) as well as to disseminate of other resistance determinants (Villa *et al*., 2010).

Treatment failures of neonatal sepsis have been observed in the last years. Empirical treatment of neonatal sepsis consists on ampicillin plus an aminoglycoside such as gentamicin, and sometimes cephalosporins. The emergence of neonatal pathogens, including *E. coli* and *K. pneumoniae*, harbouring resistance mechanisms against

ampicillin and gentamicin have been reported (Guiral *et al*., 2012; Saleem *et al.*, 2013). In addition, the emergence of strains additionally showing resistance against cephalosporins is a serious problem in both developed and developing countries.

Although surveillance to assess further dissemination of this strain in the neonatal unit was not possible to perform, our study shed a light in bacterial infections caused by *K. pneumoniae* among pregnant women and newborns in Rabat, being relevant due to the scarcity of data concerning this issue in this geographic area in spite of Morocco is classed as a middle-income country (rather than low-income country).

In conclusion, this is the first study on the prevalence of *K. pneumoniae* in pregnant women and their presence in newborns in Rabat, Morocco, as well as the first report of a *K. pneumoniae* strain causing neonatal sepsis harbouring the $bla_{\text{CTX-M-15}}$ gene in an IncFII plasmid and belonging to ST466. Although the prevalence of *K. pneumoniae* is low among pregnant women and neonates, the spread of a strain or the plasmid containing the $bla_{\text{CTX-M-15}}$ gene among newborns and specially among those presenting prematurity, could be a serious problem in a neonatal intensive care unit. For this reason, epidemiological and antimicrobial resistance surveillance of *K. pneumoniae* will enable monitoring of its emergence/spread and allow implementation of infection prevention and control procedures that will impact on whether the strain/plasmid will spread.

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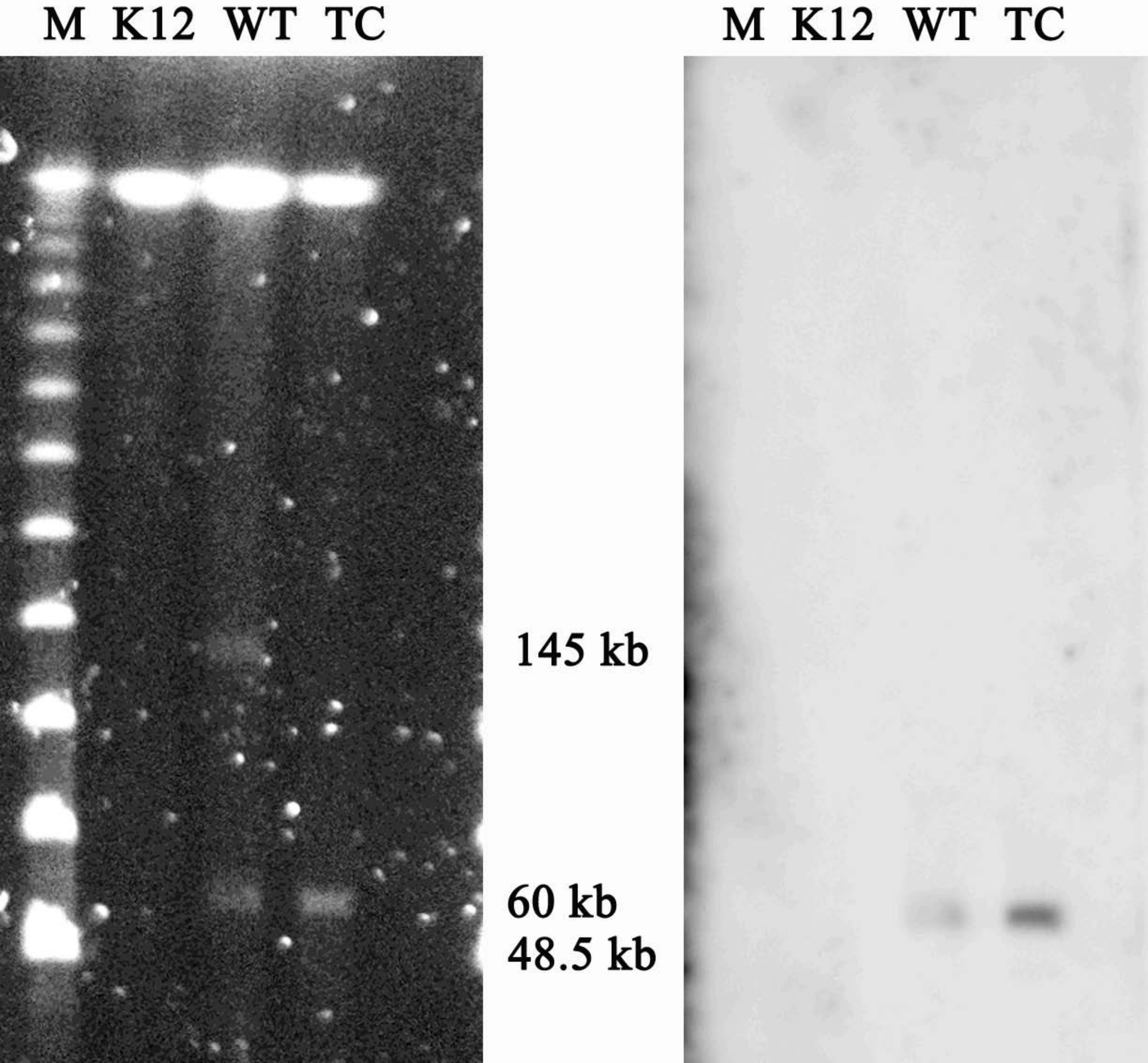
Table 1. Characteristics of *Klebsiella pneumoniae* isolates.

Origin	Number	Resistance profile
Urine	6	AM
	2	AM-CIP-Te
Vaginal swabs	6	AM
	1	AM-SXT
	2	AM-CIP-Te
	2	AM-SXT-Te
	1	AM-C-SXT-Te
Neonatal peripheral Swabs	1	AM
	1*	AM-CTX-GM
Neonatal blood	1*	AM-CTX-GM

* They are the same strain and derived from the same patient.

Figure 1. Plasmid location of the *bla*CTX-M-15 gene.

Panel A, S1-PFGE of strains. M, PFGE size marker (Innolabs, Spain); K12, *E. coli* K12-Km receptor strain; WT, *K. pneumoniae* donor strain; TC, transconjugant strain. **Panel B,** Southern-blot and hybridization of S1-PFGE using the *bla*CTX-M-15 gene probe. M, PFGE size marker (Innolabs, Spain); K12, *E. coli* K12-Km receptor strain; WT, *K. pneumoniae* donor strain; TC, transconjugant strain.


M K12 WT TC
M K12 WT TC
145 kb
60 kb
48.5 kb

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	2	AM-SXT-Te
	1	AM-C-SXT-Te
Neonatal peripheral Swabs	1	AM
	1*	AM-CTX-GM
Neonatal blood	1*	AM-CTX-GM

* They are the same strain.