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Research Article

CHARACTERIZATION AND HORIZONTAL TRANSFER OF ENHANCED SPECTRUM BETA-LACTAMASES PRODUCTION IN KLEBSIELLA PNEUMONIAE CLINICAL STRAINS FROM 2011 TO 2016 IN ABIDJAN (CÔTE D'IVOIRE)

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A B S T R A C T

Klebsiella pneumoniae is a commensal human bacterium that has become one of the major causative agents of hospital infections in recent decades. The aim of this study was to characterize broad-spectrum beta-latamase genes of the *TEM*, *CTX-M* and *SHV* type in *Klebsiella pneumoniae* on the one hand and to test their transferability. 91 strains of *Klebsiella pneumoniae* were collected at the observatory of the resistance of microorganisms to anti-infectives in Côte d'Ivoire. Antibacterial sensitivity was determined by the solid-media diffusion method of antibiotic discs. Extended spectrum beta-lactamase production was confirmed by the double synergy method. The presence of *blaTEM*, *blaCTX-M* and *blaSHV* genes was detected by the polymerase chain reaction. The diffusion of genetic support for antibiotic resistance was tested by bacterial conjugation. Results from this study showed a high resistance of clinical strains of *Klebsiella pneumoniae* to the majority of marker antibiotics used, particularly beta-lactams. Of the isolates, 28 (33.73%), 14 (16.86%), and 41 (49.39%) strains carried respectively the *blaTEM*, *blaCTX-M* and *blaSHV* genes. The conjugation test was positive because the *blaTEM* and *blaCTX-M* genes were transferred to the recipient strain *Escherichia coli* CIP 104886. Although these observed prevalences are of small proportions, this can be considered as a warning signal for the future, hence the need for increased surveillance of anti-infectives in Abidjan.

INTRODUCTION

Klebsiella pneumoniae is one of the major opportunistic pathogens commonly isolated in community and hospital settings that cause high rates of mortality and morbidity in hospitals (Bao *et al*., 2013). It is implicated in urinary tract infections, pneumonia, bloodstream infections, surgical site infections and meningitis (Ahn *et al*., 2016). Selective pressure due to intensive use of antibiotics facilitated the emergence of multiresistant *Klebsiella pneumoniae*. The production of broad-spectrum beta-lactamases (ESBL) is the main mechanism for beta-lactam resistance. At present in this microorganism, this production contributes to the emergence and dissemination of resistance genes (Ben Redjeb *et al*., 2000). Therefore, it promotes an increase in resistance to beta-lactams, fluoroquinolones and aminoglycosides (Fair and Tor, 2014).

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In addition, transmission by conjugation of antibiotic resistance genes across bacterial species has exacerbated the problem of antibiotic resistance. Moreover, the determinism of the resistance mechanisms concerns either the bacterial chromosome or the exogenous genetic material (plasmids, transposons and integrons) (Pogdlajen, 2006). But plasmid transfer has been considered one of the most important mechanisms for horizontal multidrug resistance transfer (Zhao *et al*., 2010). Previous studies indicate that Klebsiella pneumoniae has several large plasmids that carry a large number of ESBL genes and carbapenemases, as well as aminoglycoside resistance genes coding for co-resistance (Tokajian *et al*., 2015). The *blaTEM*, *blaSHV*, *blaCTX-M* and *blaOXA* genes have been described in several epidemiological studies in Africa. ESBL prevalence rates of 1.3% in Morocco (Bourjilat *et al*., 2011), 3.8% in Senegal (Sire *et al*., 2007), and 16% in Cameroon (Lonchel *et al*., 2012) and 22% in Benin (Ahoyo *et al*., 2007) have been reported. In Burkina Faso, the prevalence of strains resistant to at least one third-generation cephalosporin (C3G) and ESBL-producing strains is 67.22%

(Metuor, 2014). In Côte d'Ivoire, in a study carried out in the intensive care unit of the cocody CHU, Klebsiella pneumoniae ESBL was isolated in nasal carriage (Kouamé-Elogne *et al.,* 2013). However, no literature is related to the horizontal transfer of bla genes in this locality. The objective of this study was to detect the genetic support for beta-lactam resistance and to demonstrate the horizontal transfer of resistance genes in this bacterial species.

MATERIAL AND METHODS

Collection of strains

Strains of *Klebsiella pneumoniae* isolated from various biological products for diagnostic purposes were collected from January 2011 to June 2016 at the Observatory of resistance of microorganisms to anti-infectious agents in Côte d'Ivoire (ORMICI). The confirmation of the identity of the strains was carried out in MALDI TOF at the Pasteur Institute of Côte d'Ivoire (IPCI).

Bacterial sensitivity

Antibiogramme

The susceptibility to antibiotics was determined by the method of diffusion of disks in agar medium (Müeller-Hinton) according to the recommendations of the Committee of the Antibiogram of the French Society of Microbiology (EUCAST-CASFM, 2016). The antibiotic discs tested are amikacin (30 µg), gentamicin (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), norfloxacin (10 µg), µg), cefotaxime (5 µg), ceftazidime (10 µg), ceftriaxone (30 µg), aztreonam (30 µg), cefalotine (30 µg) cefoxitine (30 µg), tobramicine (10 µg) and amoxicillin-clavulanic acid (20/10 µg). Reference strain E. coli ATCC 25922 was used during antibiograms for the purpose of carrying out the positive control.

Broad Spectrum Beta-Lactamase Production Detection (ESBL)

The double synergism method was used for the detection of Klesbiella pneumoniae ESBL according to Jarlier *et al.* (1988). This consisted of placing the 3rd generation cephalosporin (cefotaxime, ceftriaxone and ceftazidime) and aztreonam discs at 30 mm around a central amoxicillin + clavulanic acid disc as recommended by EUCAST-CASFM (2016). The presence of ESBL is materialized by a distortion of the zone of inhibition and those with regard to the disk containing clavulanic acid, thus describing a "champagne cork" image. Only isolates of *Klebsiella pneumoniae* ESBL with resistance to aminoglycosides and fluoroquinolones will be included in the study.

Genotyping strains of Klebsiella pneumoniae ESBL

Extraction of plasmid DNA from *Klebsiella pneumoniae* strains and reference strains (Table I) was performed by the alkaline lysis method with phenolysis. A conventional Chain Polymerization Reaction (PCR) detected the beta-lactam resistance genes (*blaTEM, blaCTX-M and blaSHV*). Specific primer pairs were used to amplify genes (Table II). The PCR amplification was carried out in a volume of 50 µl with the thermal cycler (Perkin Elmer Gen Amp Lapplied Biosystems 9700). The amplification conditions are summarized in Table III. The reaction medium is composed of 5 µl of plasmid DNA, 0.3 U of Taq polymerase (Promega), 10 µM of dNTP mixture, 10 µM of MgCl 2, 10 µM of each specific primer for each target, and a 5X PCR buffer (final concentration). Another reaction mixture without DNA was used for the negative control. The amplified products were analyzed by electrophoresis in a 1.5% agarose solution Gel (Invitrogen) stained with ethidium bromide. The reading was done on the ultra-violet plate (Gel doc).

Table I Characteristics of reference strains taken as controls

bacteria	numbers	Characteristics	Positive witnesses
Salmonella sp	U2A 1446	TEM-1 + SHV-12	Genes bla_{TEM} et bla_{SHV}
E. coli	U2A 1790	CTX-M1	Genes bla_{CTXM}
E. coli	ATCC 29522	BLSE−	CQI
K. pneumoniae	ATCC 70603	BLSE+	(susceptibility)

Table II List of primers to be used for detection

GENES		SEQUENCES 5'-3'
bla TEM	Amorce F	ATGAGTATTCAACATTTCCGTG
	Amorce R	TTACCAATGCTTAATCAGTGAG
bla CTX	Amorce F	TTTGCGATGTGCAGTACCAGTAA
	Amorce R	CGATATCGTTGGTGGTGCCATA
bla SHV	Amorce F	TTTATGGCGTTACCTTTGACC
	Amorce R	AAC ACCTCGACTTAAGTCTGA

Table III Conditions of amplification of the genes of the study

Amplification steps	Condition / term
Initial denaturation	95 °C / 15 min
Cyclical denaturation	94 °C / 1 min
Hybridization	55 °C / 50 s
Cyclic elongation	72 °C / 90 s
Final elongation	72 °C / 7 min
Number of cycles	35

Bacterial Conjugation

Conjugation experiments were performed according to the modified Touati protocol (2006). Strains of Klebsiella pneumoniae ESBL carrying the *blaCTX-M* gene and the *blaTEM* gene were used as the donor strains, whereas azide-resistant *Escherichia coli* CIP 104886, as the recipient strain. The transconjugants were selected on Müller Hinton agar medium supplemented with ceftazidime (16 µg / ml) plus azide (200 µg / ml).

RESULTS

Bacterial strains

A total of 91 strains of *Klebsiella pneumoniae* producing ESBL were collected from January 2011 to June 2016. The distribution of ESBL-producing strains according to organic products is summarized in Figure 1. Of these biological products, *Klebsiella pneumoniae* is elevated in the urine (36.2%), followed by pus (24.2%) and blood (20.8%). The most highly charged hospital services are respectively the outpatient department and the pediatric ward, with respectively 35.2% and 14.3%.

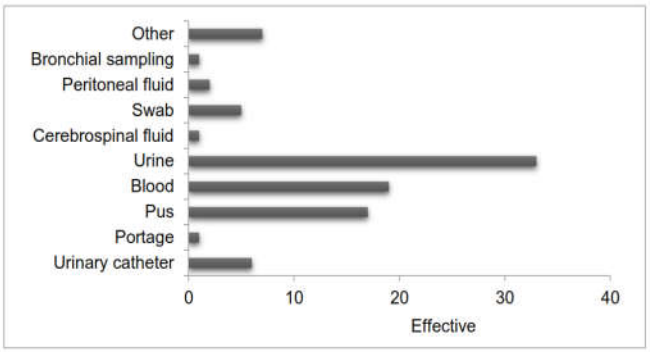

Figure 1: Distribution of strains of *Klebsiella pneumoniae* ESBL in the different pathological products.

Susceptibility of strains of Klebsiella pneumoniae ESBL to antibiotics

For beta-lactams

The strains tested were all susceptible to imipenem (99%) with the exception of one strain. Resistance was 31.8% for cefoxitin, 92.3% for ceftazidine and cefotaxime, 93.4% for ceftriaxone and 100% for cefalotin. As for aztreonam, the level of resistance was 90.1% and finally 54.9% for amoxicillin + clavulanic acid.

For Floroquinolones

The prevalence of quinolones was 67% for ciprofloxacin, 50.5% for nalidixic acid and 73.6% for norfloxacin.

For aminoglycosides

The resistance of strains to aminoglycosides revealed that only amikacin was the most active molecule with only 4.4% resistance. As for tobramycin and gentamicin, all the strains were resistant with respectively 84.6% and 74.7%.

Research of beta-lactam resistance genes

The PCR technique detected from the 91 analyzed, 83 strains that possess the bla gene, ie a prevalence of 91.20% with predominance of the *SHV* type. Of the 83 strains, 14 (16.86%) have the *blaCTX-M* gene (750 bp), 41 (49.39%) have the *blaSHV* gene (680 bp) and 28 (33.73) have the *blaTEM* (500 bp) (Figure 2).

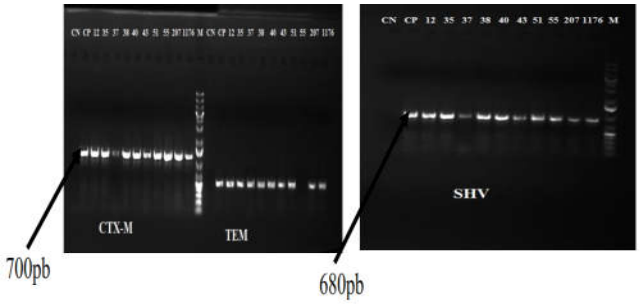

Figure 2: 1.5% agarose gel electrophoresis showing monoplex PCR for detection of *blaCTX-M*, *blaTEM* and *blaSHV* genes
Lane M: molecular weight marker (Invitrogen, 1 Kb DNA Ladder); CN Channel: Negative Control; CP Lane: *CTX-M* Positive Control (750 bp), Lane 12, 35, 37, 38, 40, 43, 51, 55, 207 and 1176: positive samples of *blaCTX-M*; CP channel: positive control TEM (500 bp); Lanes 12, 35, 38, 40, 43, 51, 207 and 1176: positive samples of *blaTEM*. Positive control *SHV* (680 bp), lanes 12, 35, 37, 38, 40, 43, 51, 55, 207 and 1176: positive samples

Horizontal Transfer: The Conjugation

Six strains were selected based on their genotypic aspects. Indeed, those are strains that possess after molecular characterization the genes: *blaCTX-M*, *blaTEM* and *blaTEM*. The identification of transconjugants by the API 20E gallery has shown that they are Escherichia coli strains. Transconjugants had the same pattern of antibiotic susceptibility of donor strains. After screening of the selective medium, the antibiogram (Figure 3) and the PCR reactions, there was the simultaneous presence of the *blaCTX-M* gene and the *blaTEM* gene which codes for beta-lactam resistance in all transconjugants (Figure 4).

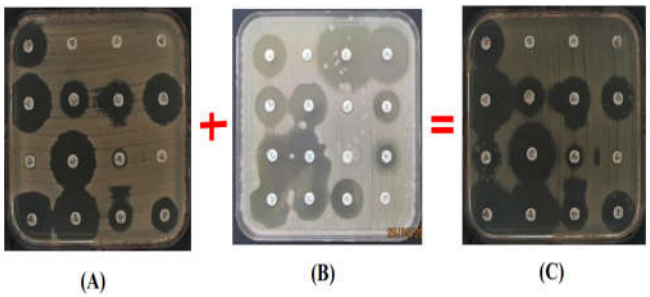

Figure 3: Antigenogram images of the three strains showing the transfer of determinants of resistance by a transconjugant plasmid.
(**A**) *Klebsiella pneumoniae* (donor strain); (**B**): *Escherichia coli* CIP 104886 (recipient strain); (**C**): *Escherichia coli* (transconjugant)

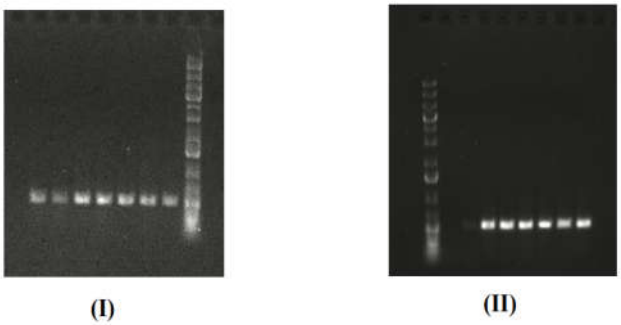

Figure 4: Polymerization reaction of 6 strains of Escherichia coli transconjugants.
(**I**) amplification of the blaTEM gene by standard PCR, (**II**) amplification of the *blaCTX-M* gene by standard PCR.

DISCUSSION

The incidence of multidrug-resistant *Klebsiella pneumoniae* infections has increased over the last decade, reflecting the extensive use of antimicrobial agents (Wu *et al.,* 2012). It has been reported according to the work of Bao *et al.* (2013) and Rangaiahagari *et al.* (2013) that *Escherichia coli* and *Klebsiella sp* are the most commonly encountered species in hospitals. In this study, 36.2% of *Klebsiella pneumoniae* was isolated from the urine. This observed predominance has been reported respectively by Hashemi et al. (2013) in Iran with 69.3% and Raji *et al.* (2013) with 64.7% in Nigeria. However, high levels of blood cultures positive for *Klebsiella pneumoniae* ESBL have been described by some authors (Elhani *et al.*, 2006 ; Rebuck *et al.*, 2000).

The susceptibility of *Klebsialla pneumoniae* strains is characterized by a high level of resistance to marker antibiotic molecules (beta-lactams, aminoglycosides and quinolones). In

an earlier study by Xiang-hua *et al.* (2015), 89.5% was recorded as strains of *Klebsiella pneumoniae* ESBL.

In the present study, 100% of the ESBL positive strains were cephalothin resistant, 84% were cefotaxime and ceftazidime resistant, 85% were ceftriaxone resistant and 82% were aztreonam resistant. These observed results are indicative of the overall high rate of third-generation cephalosporin resistance in Côte d'Ivoire. This discovery can be seen as an epidemic warning for the future, highlighting the need for increased surveillance. To avoid this risk, antibiotic prescriptions must be carefully considered by the actors of human, animal and agropastoral health.

With regard to genetic support for beta-lactam resistance, the present study further showed that the blaSHV gene was very high (45.05%) in ESBL isolates. This result corroborates that of Al-Agamy *et al*. (2009) who showed that 214 (97.3%) of the 220 strains isolated in Saudi Arabia carried the *blaSHV* gene. This dominance was also reported by Ghafourian in 2011 in Iran. His study found that 94% of multidrug-resistant *Klebsiella pneumoniae* carried the blaSHV gene (Ghafourian *et al.,* 2011).

In addition to the *blaSHV* gene, there is also the *blaCTX-M* gene, which has a lower level than the blaTEM gene. Significantly, blaSHV was more responsible for the production of ESBL as evidenced by several publications, notably those of Feizabadi *et al*. (2010) and Ghafourian *et al.* (2011).

The dissemination of ESBL genetic support is a first of its kind in Côte d'Ivoire. The results from the horizontal conjugation experiment showed that 6 transconjugants expressed the same level of resistance to commonly used beta-lactams. The *blaCTX-M* gene was transferred with the *blaTEM* gene, suggesting that the strains harbored a conjugative plasmid. Certainly the integron cassette on this plasmid houses more than one resistance gene. These results are consistent with previous studies that have shown that the genes that code for beta-lactamases and other genes are localized transposable genetic structures responsible for many epidemics (Naas *et al*., 2011).

We can deduce from these observations that the multidrug-resistant bacteria constitute in our establishment a growing infectious risk. Indeed, these genes carried by plasmids whose character is transferable could be responsible for clonal diffusion or not within the species. The development of multi-family antibiotic resistance can seriously compromise the clinical use of antibiotics to treat infections caused by Gram-negative bacteria (Hidalgo *et al*., 2013).

CONCLUSION

This study showed that antibiotic resistance levels were high for most of the molecules tested. Experimental horizontal transfer was positive for *blaCTX-M* and *blaTEM* gene transfer. It is important to have good hygiene practices in our health centers and also be sharp in the treatment and selection of the antibiotic molecules of choice. This attitude would be necessary to reduce the progression of ESBL micro-organisms. The emergence and spread of resistance genes in Côte d'Ivoire could pose a long-term public health problem. Hence the need to put in place a relevant resistance monitoring policy for better control of Klebsiella pneumoniae ESBL circulation.

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