SUSCEPTIBILITY OF DISSEMINATION OF THE GENES CTX-M-15 AND SHV-187, ISOLATED IN MULTI-ANTIBIOTIC RESISTANT *KLEBSIELLA PNEUMONIAE* AND UROPATHOGENIC *ESCHERICHIA COLI* IN CÔTE D'IVOIRE.

ABSTRACT

Enterobacteriaceae are ubiquitous commensal bacteria of humans that have become major causative agents of hospital infections. The objective of the study was to characterise the extended-spectrum beta-lactam resistance genes isolated from multi-resistant uropathogenic clinical strains in Côte d'Ivoire. 266 enterobacterial strains were collected during this study. Antibacterial susceptibility and the presence of *bla* genes were determined by the solid-state diffusion method and by PCR, respectively. The production of ESBL was confirmed by the double synergy method and Sequencing was performed. Of the strains collected, the most isolated were *Escherichia coli* 53 (39.25%) and *Klebsiella pneumoniae* 36 (26.66%). Antibiotic resistance of more than 50% was observed for gentamicin, norfloxacin, third generation cephalosporins and tobramicin in *E coli*. Only imipenem had a low resistance rate of 5.6%. However, apart from norfloxacin, the *Klebsiella pneumoniae* strains tested expressed resistance to aztreonam and second generation cephalosporins in excess of 50%. The genes *blaCTX*-M, *blaSHV, blaTEM, qnr* B and a class I integron were detected. After sequencing, the SHV-1, SHV-28, SHV-187 and CTX-M15 variants were detected. Although these results are of low proportions, this may be considered critical for the future, hence the need for a better antibiotic surveillance strategy in Abidjan.

Key words : Uropathogens, antibiotic, anterobacteriaceae, multidrug-resistant, Côte d'Ivoire

1- INTRODUCTION

Antibiotics represent the most widely prescribed therapeutic class in Africa. Among them, β-lactams are the most frequently used family because of their broad antibacterial spectrum, bactericidal activity, low toxicity and the wide choice of molecules available [1].

Over the past decade, a significant increase in resistance to these antibacterials has been observed in Enterobacteriaceae. According to the WHO Global Antimicrobial Resistance Surveillance Report 2014, antibiotic resistance is a reality worldwide and now poses a serious threat to public health [2]. Indeed, the emergence and spread of multidrug-resistant bacteria through the hyperproduction of Cephalosporinase (AmpC) or production of Broad Spectrum Beta-Lactamases (ESBL) or Carbapenemases, through the misuse of Beta-lactams in human health, animal health and agriculture are increasingly observed [3].

Since the discovery of ESBL-producing bacteria and until 1990, most of the ESBLs detected were the classical Temoniera (TEM) and Sulfydryl variable (SHV) types that were predominantly disseminated within hospital clones of certain germs. However, in recent years, a new type of enzyme, CTX-M, has emerged and has been disseminated among community strains of *Escherichia coli*, the main cause of urinary tract infections [4].

Moreover, bacterial agents regularly exchange genetic information between themselves through the horizontal transfer of antibiotic resistance genes, thanks to plasmids or transposons that harbour integrons [5]. The latter, play a major role in the emergence and spread of antibiotic resistance by capturing resistance genes and transferring them from one DNA molecule to another [6]. The functional platform of integrons includes an insertion site capable of inserting gene-bearing cassettes. At this site, specific recombinations take place leading to a reorganisation of gene expression [7]. Once mobilised, genes can be hosted by numerous mobile elements other than integrons: insertion sequences (IS) such as Ecp1, IS26

and IS903 [8]. Thus, these mobile elements are a major problem in the management of anti-infective resistance.

In order to better understand the mechanisms of resistance emergence and to fight more effectively against the dissemination of multidrug-resistant bacteria (MRB), it is necessary to consider the interactions between bacterial clones harbouring antibiotic resistance genes and the genetic elements that carry them.

2- MATERIALS AND METHODS

2.1- Type and study site

This is a retrospective study on the characterisation and dissemination of antibiotic resistance genes in uropathogenic strains. Most of the methods of this study were performed at the Institut Pasteur de Côte d'Ivoire (IPCI). Only the sequencing of ESBL genes was performed at the EA7361 unit of the Faculty of Medicine of the University of Paris-Sud (France).

2.2- Collection and verification of the identity of strains

Bacterial strains isolated from various biological products were collected from January 2011 to June 2016 at the Observatoire de la résistance des micro-organismes aux anti-infectieux en Côte d'ivoire (ORMICI). Confirmation of the identity of the strains was performed by MALDI TOF at the Institut Pasteur de Côte d'ivoire (IPCI).

2.3- Bacterial sensitivity

2.3.1- Antibiogramme

Antibiotic susceptibility was determined by the agar disc diffusion method (Müeller-Hinton) according to the recommendations of the Antibiogram Committee of the French Society of Microbiology [9]. The antibiotic discs tested were amikacin (30 µg), gentamicin (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), norfloxacin (10 µg) cefotaxime (5 µg), ceftazidime (10 µg), ceftriaxone (30 µg), aztreonam (30 µg), cefalotin (30 µg), cefoxitin (30 µg), tobramicin (10 µg), and amoxicillin/clavulanic acid (20/10 µg) The

reference strain *E. coli* ATCC 25922 was used in the course of the susceptibility testing for the purpose of positive control.

2.3.2- Detection of extended spectrum beta-lactamase (ESBL) production

The double synergy method was used for ESBL detection 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 the central amoxicillin + clavulanic acid disc according to [9]. The presence of ESBLs is indicated by a distortion of the inhibition zone and those in front of the clavulanic acid disc, thus describing a "champagne cork" image.

2.4- Genotyping

Plasmid DNA extraction from the strains and reference strains (Table I) was performed by the alkaline lysis method with phenolysis. A Polymerase Chain Reaction (PCR) was used to detect beta-lactam resistance genes (blaTEM, blaCTX-M and blaSHV), quinolones (qnr A, B and S) and class 1, 2 and 3 integrons. Specific primer pairs were used for the amplification of these genes (Table II). The 50 µl reaction medium consisted of 5 µl of plasmid DNA, 0.3 U of Taq polymerase (Promega), 10 µM of dNTP mixture, 10 µM of MgCl2, 10 µM of each target-specific primer, and 5X PCR buffer. Another reaction mixture without DNA was used as a negative control. Amplification was performed with the thermal cycler (Perkin® Elmer Gen Amp Lapplied Biosystems 9700). The amplification conditions are summarised in Table III. The amplified products were analysed by electrophoresis in a 1.5% agarose gel solution (Invitrogen) stained with ethidium bromide. The reading was carried out in an automaton (Gel doc) incorporated with an ultraviolet plate.

Table I : Characteristics of reference strains taken as controls

Bacteria	Number	Characteristic	Positive control
Salmonella sp	U2A 1446	TEM-1 + SHV-12	**Genes bla_{TEM} and bla_{SHV}**
E. coli	U2A 1790	CTX-M1	**Genes bla_{CTXM}**
E. coli	ATCC 29522	BLSE−	**IQC**
K. pneumoniae	ATCC 70603	BLSE+	**(antibiogram)**

IQC = Internal Quality Control ; **CTX-M** = CefoTaXimase-Munich ; **SHV** = SulfHydryl Variable ; **TEM** = TEMoneira ; **ATCC** = American Type Culture Collection ; **ESBL** = Extended Spectrum Beta-Lactamase

Table II : Primers used for detection

Genes	Primers	**Squences 5'-3'**	**References**	**Amplicon size (bp)**
***bla* TEM**	F	ATGAGTATTCAACATTTCCGTG	Essack *et al.,* 2001	**840**
	R	TTACCAATGCTTAATCAGTGAG		
***bla* CTX**	F	TTTGCGATGTGCAGTACCAGTAA	Birkett *et al*., 2007	**544**
	R	CGATATCGTTGGTGGTGCCATA		
***bla* SHV**	F	TTTATGGCGTTACCTTTGACC	Yagi *et al*., 2000	**1051**
	R	ATTTGTCGCTTCTTTACTCGC		
***qnr*A**	F	TTCTCACGCCAGGATTTGAG	Seyed *et al*., 2014	**571**
	R	TGCCAGGCACAGATCTTGAC		
***qnr*B**	F	TGGCGAAAAAATTG AAC AGAA	Seyed *et al*., 2014	**594**
	R	GAGC AAC GATCGCCTGGTAG		
qnrS	F	GACGTGCT AAC TTGCGTGAT	Seyed *et al*., 2014	**388**
	R	AAC ACCTCGACTTAAGTCTGA		
***intI*1**	F	CCTCCCGCACGATGATC		**280**
	R	TCCACGCATCGTCAGGC		
***intI*2**	F	TTATTGCTGGGATTAGGC	Goldstein *et al*., 2001	**233**
	R	ACGGCTACCCTCTGTTATC		
***intI*3**	F	AGTGGGTGGCGAATGAGTG		**600**
	R	TGTTCTTGTATCGGCAGGTG		

bp : base pair

Table III : Amplification conditions for the study genes

Amplification steps	Conditions		
	genes *bla (TEM, SHV, CTX-M)	****genes *qnr*** (A, B, S)	*****Integrons *Int1, Int2, Int3*
Initial denaturation	95 °C / 15 min	95 °C / 5 min	95 °C / 5 min
Cyclic denaturation	94 °C / 1 min	94 °C / 1 min	94 °C / 1 min
Hybridation	55 °C / 50 s	57 °C / 1 min	60 °C / 1 min
Cyclic elongation	72 °C / 90 s	72 °C / 1 min	72 °C / 1 min
final elongation	72 °C / 7 min	72 °C / 10 min	72 °C / 10 min
Number of cycles	35	30	35

*Group 1, **Group 2, ***Group 3

2.5- Sequencing

Purification of the positive PCR products was performed with the Gene JET PCR Purification kit from Thermo Scientific® according to the manufacturer's recommendations. Sequencing

was performed on an ABI PRISM 3730 (Applied Biosystems). The objective of this method was to identify the resistance gene variants in the beta-lactam family that are involved.

2.6- Statistical analysis

The variables analysed were: gender, age, and antibiotic resistance rates. The rate of multi-drug resistant isolates was calculated. The comparison between the infection rate in men and women was performed by Monte Carlo test. Comparison of resistance rates of *Klebseilla pneumoniea* and *Echerichia coli* isolates was performed by Chi-square test or Fisher's Cochran's rule. The collected data and statistical tests were analysed using XLSTAT 2016 software. Graphs were made using Excel 2013 and differences were considered significant when *P-value* ≤ 0.05.

3- RESULTS

3.1- Strain collection and distribution

A total of 266 clinical strains collected over the period March to July 2016 were selected from the biological collection of the Institut Pasteur de Côte d'Ivoire. Epidemiological data revealed that 94 bacteria were from males and 172 from females. Of the strains collected, more were isolated from females (63%) than males (37%) (Figure 1). A significant difference in the infection rate between males and females was observed according to the two-sided Monte Carlo test with *p-value* = 0.0001 and $\alpha = 0.05$.

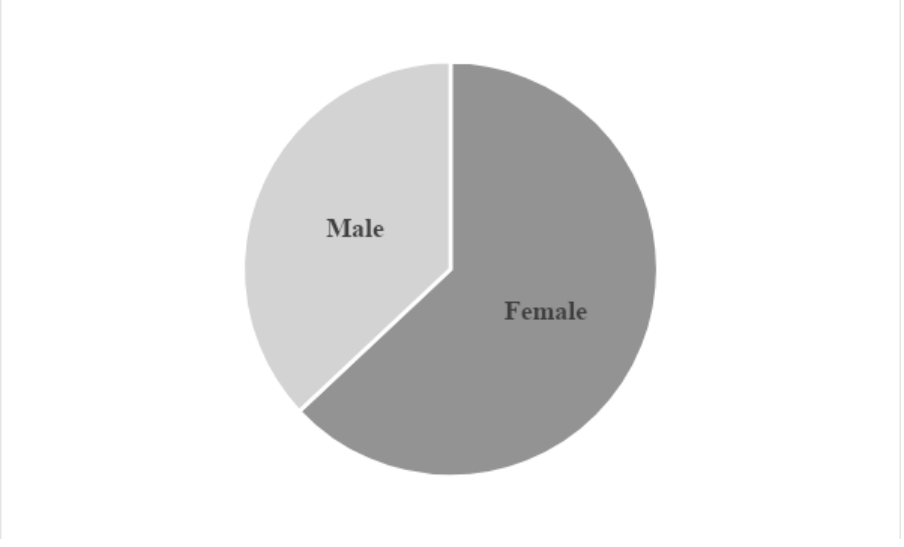

Figure 1 : Distribution of isolated species by genus

Maldi-Tof (Vitek MS) identified several uropathogenic enterobacteria. The most isolated were *Escherichia coli* and *Klebsiella pneumoniae* with 53 (39.25%) and 36 (26.66%) respectively. All the species identified are shown in Figure 2. In males, *E. coli* accounts for 42% followed by *Klebsiella pneumoniae* with 26%. In females, *E. coli* was isolated in 54.1% and the second strain, *Klebsiella pneumoniae* in 30.5%.

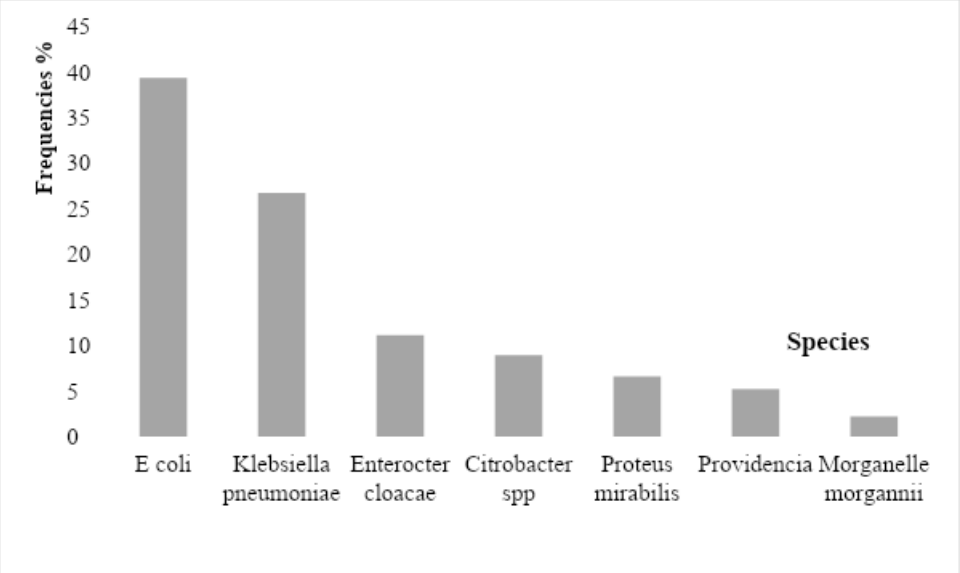

Figure 2 : Overall distribution of Enterobacteria by specie

3.2- Antibiotic resistance of isolated strains

Antibiotic resistance concerned *Escherichia coli* and *Klebsiella pneumoniae* strains, which were the most isolated. The other germs were therefore not considered in this study because of their low isolation rate. Thus, the values obtained revealed that the *E. coli* strains tested expressed resistance to the antibiotic molecules used. Resistances above 50% were observed

for gentamicin, norfloxacin, third generation cephalosporins and tobramicin. Only imipenem had a low resistance rate of 5.6% (Table IV). However, apart from norfloxacin, the *Klebsiella pneumoniae* strains tested expressed resistance to aztreonam and second generation cephalosporins in excess of 50%. The rate of resistance to aztreonam and cefotaxin was statistically different between *E. coli* and *Klebsiella pneumoniae* strains (Table IV).

Table IV : Antibiotic resistance rate of the most isolated strains

Antibiotics **Dose (µg)**	**Bacteria species**		**Chi² test or Fischer's test**
	E. coli	***K. pneumoniae***	***P-value***

	N= 53 (%)	N= 36 (%)	
Amikacin 10 µg	22 (41,5 %)	13 (36,3 %)[a]	0,954
Gentamicin 10 µg	29 (54,7 %)	10 (27,8 %)[a]	0,138
Imipeneme 10 µg	3 (5,6 %)	5 (13,9 %)[a]	0,127
Ciprofloxacin 5 µg	33 (62,3 %)	17 (47 %)[a]	0,679
Nalidixic Acid 30 µg	27 (50,9 %)	16 (44,4 %)[a]	0,941
Norfloxacin 5 µg	39 (73,6 %)	20 (55,5 %)[a]	0,638
Cefotaxin 30 µg	38 (71,7 %)	11 (30,5 %)[b]	**0,032**
Ceftazidime 30 µg	30 (56,6 %)	13 (36,3 %)[a]	0,360
Ceftriaxone 30 µg	24 (45,2 %)	8 (22,2 %)[a]	0,158
Aztreoname 5 µg	19 (36 %)	24 (66,7 %)[b]	**0,008**
Cefalotin 30 µg	20 (38 %)	19 (53 %)[a]	0,106
Cefoxitin 30 µg	23 (43,4 %)	23 (64 %)[a]	0,051
Tobramicyne 10 µg	29 (54,7 %)	14 (39 %)[a]	0,558
Amoxicillin-clavulanic acid 20/10 µg	16 (30,2 %)	11 (30,5 %)[a]	0,654

N : Number, **%** : percentage

3.3- Research for antibiotic resistance genes

The PCR technique allowed the detection of various resistance genes in the tested strains. Concerning the beta-lactam resistance genes, *blaCTX-M*, *blaSHV*, and *blaTEM* were observed with respective sizes of 544, 1051 and 840 bp. Compared to the resistance observed in the quinolone family, only the *qnr* B gene at 594 bp was detected. A class I integron was found in

this strain with a size of 280 bp (Figure 3). The distribution of the genes detected according to the strains tested is summarised in Table V.

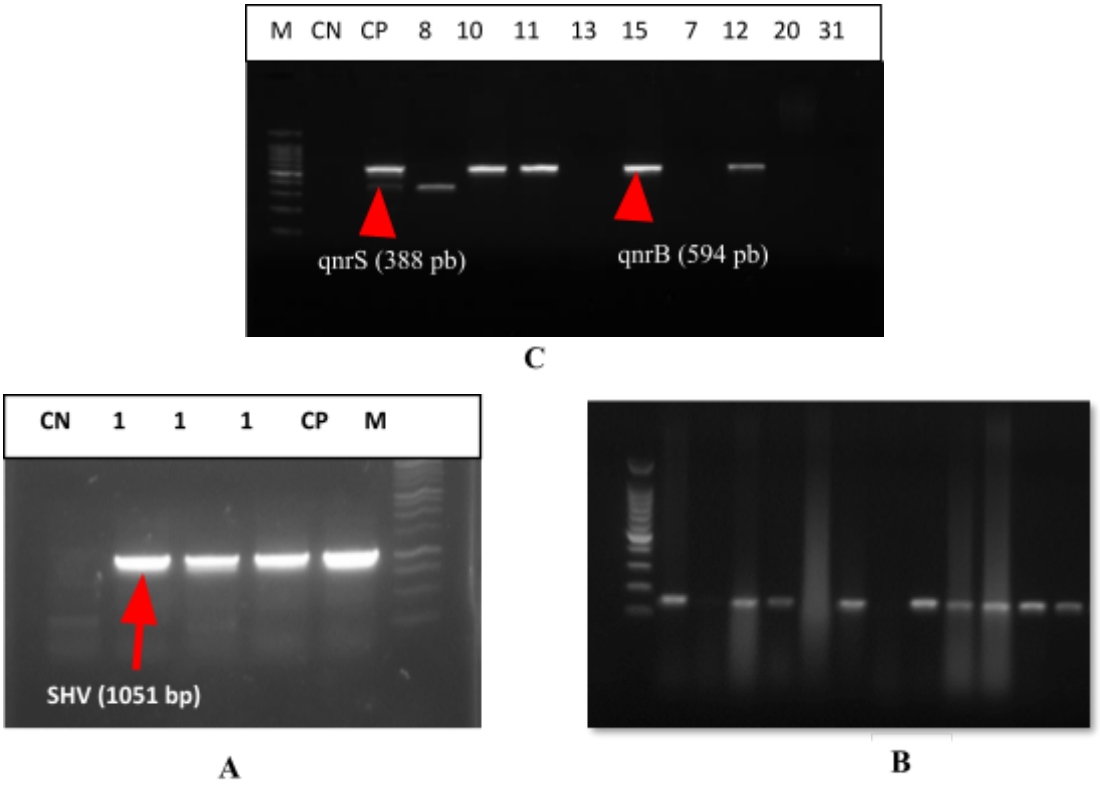

Figure 3: 1.5% agarose gel electrophoresis showing PCR for detection of *blaSHV* (A) and int 1 (B) genes, duplex for detection of *qnr* B and S genes (C)

Lane M: Molecular weight marker (Invitrogen, DNA Ladder 1kb); Lane CN: Negative control; Lane CP: (A) SHV positive control (1051 bp), Lane 1: *blaSHV* positive sample, (B); Lane CP: int 1 positive control (280 bp); Lane 1: int 1 positive sample

Table V : Distribution of genes detected according to the germs tested

Tested germs	tested genes				
	bla_{CTX-M} N (%)	bla_{TEM} N (%)	bla_{SHV} N (%)	**int1** N (%)	***Qnr*B** N (%)

Escherichia coli **N=53**	34 (64,2)	21 (39,6)	16 (30,2)	39 (73,6)	25 (47,2)
Klebsiella pneumoniae **N=36**	12 (33,3)	13 (36,1)	24 (66,6)	25 (69,4)	18 (50)

N : Number, **%** : percentage

3.4- Variants identified after sequencing

Sequencing of the PCR product of the *blaSHV* gene allowed the identification of mutations that could explain the observed resistance phenotype. Thus, the analysis of the SHV gene sequence showed the presence of three types of variants: SHV-1, SHV-28 and SHV-187. These variants have the reference sequences of Genbank accession number NG_050053.1, NG_050016.1 and DQ219473.1 respectively. The PCR product of the *blaCTX*-M gene conferring cefotaxin resistance was also sequenced. The resulting sequence was compared to the sequence of the reference strain of Genbank accession number KT986227.1 and was similar to CTX-M-15. The result of the nucleotide sequence analysis of the *blaTEM* gene identified the TEM-1 variant. However, after alignment of the reference sequence of Genbank accession number KR632748.1 with the neoformed sequences, no mutations were detected.

4- DISCUSSSION

The spread of multidrug resistance in different ecosystems has become a major problem in the treatment of infections caused by enterobacteria [10]. *E. coli* and *Klebsiella sp*. are the most common species encountered in UTIs, according to [11] and [12]. In this study, these two germs were isolated from urine with 39.25% and 26.66% respectively. This predominance of isolation of these pathogens from urine was reported by [13] in Iran with 69.3% and [14] with 64.7% in Nigeria.

The results in this study indicate that the prevalence of UTIs is higher in women than in men. These results were also obtained by [15] who showed that the prevalence of UTIs was higher in females with 38.5% than in males with 19.3%. Comparison of the rate of infection between the two sexes showed a significant difference indicating that women are more infected with enterobacteria than men. The predominance of infection in the female sex could be due to the proximity of the terminal digestive tract and the urogenital tract associated with a short urethra [16]. In addition, the commensal flora located in the vagina could explain the frequent contamination of urine in women [16]. According to these authors, this could be justified by factors such as hormonal changes during pregnancy and the anatomical difference between the male and female urethra. In addition to these, the proximity of the male and female urethra to the opening of the anus, poor personal hygiene, certain cultural practices in women and the absence of prostate secretion in women could be underlying factors for the moderately high prevalence of UTIs observed in women compared to their male counterparts.

Overall, the strains tested were resistant to both beta-lactams, aminoglycosides and quinolones. These results confirm the presence of multidrug-resistant bacteria (MRB), as shown in the previous work of [17]. This finding could be the consequence of the selection pressure due to the abusive use of broad-spectrum antibiotics in hospitals, as well as the cross-transmission of acquired resistance with plasmid determinism [18], [19]. Thus, it is clear that the spread of such bacteria constitutes a public health threat.

The genetic profile of ESBL enzymes in the 89 strains, 53 of which were *Escherichia coli* and 36 of which were *Klebsiella pneumoniae*, was variable, as several resistance genes were observed. These are the CTX-M and TEM types, which are in the majority in *E. coli* with 64.2 and 39.6% respectively. As for the SHV type, it is in the majority in *Klebsiella pneumoniae*. These rates obtained are much lower than those observed by [20] in Algeria with 92.5, 95 and 91.25% for TEM, CTX-M and SHV, respectively. [21], obtained 57.89% for SHV, 26.31% for

TEM and 18.42% for CTX-M. These differences in results could be due to the numbers of strains tested. Indeed, these authors conducted their study on 100 and 53 strains, respectively. The strains tested also harboured 47.2% of the qnrB gene in *E. coli* and 50% of this gene in *Klebsiella pneumoniae*. The presence and expression of these genes in these microorganisms could lead to therapeutic failures [22]. Co-resistance is therefore the result of the dissemination of various resistance genes via conjugative plasmids or transposons between bacteria of the same or different species [23].

Integrons play an important role in the spread of antibiotic resistance genes in bacteria [24]. In this study, only class 1 integrons were identified with a rate of 73.9% in *E. coli* and 69.4% in *Klebsiella pneumoniae*. [25], [26] also detected the presence of class 1 integrons with rates of 25.8 and 47% of the strains tested respectively. The presence of class 1 integrons in the Ivory Coast ecosystem could be attributed to horizontal transfer in which conjugative plasmids and transposons are involved [27]. This observed phenomenon leads to the emergence of multi-drug resistant bacteria in several ecosystems [28]. In addition, the multi-disposition of gene cassettes through specific recombination mechanisms allows bacteria to develop diverse resistance to antibiotics [29], a consequence of the adaptation of bacteria to their environment according to [30].

Identification of the blaTEM gene after sequencing showed the unique presence of TEM-1. It is responsible for more than 90% of the ampicillin resistance observed in *Escherichia coli*. This enzyme is also able of hydrolysing first generation cephalosporins according to [31]. Also, the TEM-1 gene is widely distributed in various ecosystems hence its presence in different species of enterobacteria [32]. According to [33], this gene is predominant in Africa with prevalence rates up to 100%. Furthermore, TEM-1 was also identified in Côte d'Ivoire with a rate of 63.4% by [34].

CTX-M enzymes, like TEM and SHV, emerged in the late 1980s after the introduction of cefotaxime in infectious therapeutics [35]. This study also showed the presence of the CTX-M-15 variant. The latter is one of the most frequent ESBL types in ESBL-producing bacteria causing human infections [36]. It has been shown that the successful spread of the CTX-M-15 enzyme has been attributed to the spread of genetic elements, through horizontal gene transfer, and clonal expansion of a pandemic *E. coli* clone: *E. coli* ST131 [37].

Sequencing results revealed genetic diversity of the blaSHV gene with several variants, including SHV-1, SHV-28 and SHV-187 types. Of these variants, SHV-1 and SHV-28 have already been identified in Côte d'Ivoire by [38]. In addition, the SHV-28 variant was previously reported in Burkina Faso by [39]. The dissemination of these genes in these countries would be due to mobile genetic elements such as integrons, insertions and transposons. Furthermore, according to the work of [40], the SHV gene has a broad spectrum hydrolytic activity. This makes it epidemiologically relevant as it is able to hydrolyse ceftazidime, cefotaxime and aztreonam.

5- CONCLUSION

At the end of this study, the impact of antibiotic therapy in human health reveals that health actors and the population play a major role in the emergence of multidrug-resistant pathogenic bacteria. The prevalence of UTIs was higher in women than in men. Several resistance genes were detected including the CTX-M15 variant which has been the subject of several concerns and publications worldwide. The study detected integrons in Klebsielle pneumoniae and Escherichia coli. The prevalence of mobile genetic elements such as integrons showed considerable rates that raise concerns in the management of bacterial antimicrobial resistance.

Ethical approval

Not required.

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