Antimicrobial resistance patterns and genes of β-lactamase involved in extended spectrum β-lactamase producing enterobacteria isolated from human, animal and environment.

Abstract

Objective: The aim of this study is to determine the antibiotic resistance pattern and beta-lactamase gene profile of Extended Spectrum β-Lactamases (ESBL) producing enterobacteria strains isolated from human biological products, fecal matter of animals and the environment.

Material and methods: Enterobacteria producing ESBL strains were isolated from human products, fecal matter of healthy animals (cattle, sheep and pigs) intended for human consumption and environment (hospital effluents and municipal sewage) using homemade medium (Drigalski supplemented with 2 mg/L of ceftazidime). Resistance to beta-lactams has been evaluated by the diffusion method was carried out as recommended by NCCLS. Characterization of Beta-Lactamase resistance genes (*blaCTXM*, *blaSHV*, *blaTEM*, *blaGES*, *blaPER* and *blaVEB*) was performed by simplex and multiplex PCR.

Results: The strains were resistant to antibiotics from beta-lactamins family (penicillin with inhibitor, monobactam, cephalosporin) but no resistant was observed to carbapenem (imipénème, méropénème). All resistance genes were identified in environment strains.

Conclusion: This study showed the presence of common beta-lactam resistance genes (blaTEM, blaSHV and blaCTX-M) to human, animal and environment. The risk of dissemination and circulation of ESBL enterobacteria between animals, humans and the environment exists in Ivory Coast because of the absence of a barrier between them.

Mots clés: Enterobacteria ESBL, Human, Animal, Environment, Ivory Coast

1. Introduction

Antibiotics are widely used not only to treat human and animal infections but also in farms and aquacultures, as food additives to promote animal growth and prevent diseases. Unfortunately, the intensive use and misuse of antibiotics in these different domains have resulted in antibiotic resistance among bacteria such as enterobacteria producing extended-spectrum β-lactamase **[1, 2]**. Antibiotic resistance is not confined to clinically important bacteria but is also present in bacteria in the aquatic environment and animal production, which might be an important contributory factor in the spread of resistance **[3, 4].** There are

two main mechanisms involved in the development of antibiotic resistance, namely mutation **[5]** and acquisition of resistance genes **[6]** by horizontal gene transfer (HGT).

The most abundant ESBL types are represented by SHV, TEM and CTX-M. However, a variety of other enzymes such as VEB, GES/IBC, PER, BEL, and oxacillinases with ESBL activity have been described worldwide **[7].** Plasmid-encoded Extended-spectrum b-lactamases (ESBL) are increasingly spreading among Enterobacteriaceae from human, animal and environment isolates throughout the world due mostly to their presence on highly conjugative plasmid **[8]**.

Originally, ESBLs were mainly demonstrated in hospital environment but now high frequencies of antimicrobial resistance have been found in enterobacteria, in fecal flora as well as in clinical isolates **[9]**. Also, the release of antibiotics in large amounts into natural ecosystems through hospital and municipal waswaters untreated can impact the structure and activity of environmental microbial populations. Acquired resistance to β-lactamin antibiotics in gram-negative bacteria is mainly mediated by bacterial β-lactamases and the emergence of extended-spectrum β-lactamases (ESBLs) is of great clinical importance. The increase of these bacteria and their spreading in the hospital has been well documented in the world **[10, 11]** and in Ivory Coast **[12, 13]**. Very patchy data on enterobacteria producing extended spectrum β-lactamases in the fecal flora of animal and environment (hospital effluents and municipal wastewater) in Ivory Coast have been published, and thus we know surprisingly little about the enterobacteria ESBL and their resistance genes outside environment clinical. It is important, therefore, to document the occurrence and types of antibiotic resistance genes in the environment and animal.

The aim of this study is to determine the antibiotic resistance pattern and beta-lactamase gene profile of Extended Spectrum β-Lactamases (ESBL) producing enterobacteria strains isolated from human biological products, fecal matter of animals and the environment.

2. Material and methods

Sampling collection: This study was carried out from December 2012 to November 2013 in Abidjan (Ivory Coast). Human enterobacteria strains producing extended spectrum beta-lactamases (ESBL) were obtained from the clinical bacteriology unit (CBU) of the Institut Pasteur of Ivory Coast. These strains were isolated from biological products (urine, blood and pus) of hospitalized and nonhospitalized patients. In the same period, ESBL enterobacteria strains were isolated from the fecal matter of healthy animals (cattle, sheep and pigs) intended

for human consumption. In the environment, ESBL enterobacteria strains were isolated from hospital effluents and municipal sewage.

Isolation and identification of ESBL enterobacteria strains: All ESBL producing enterobacteria strains were isolated on Drigalski supplemented with 2 mg/ml of ceftazidime **[14]** and were identified using the API 20E galerie (bioMérieux, Marcy l'Etoile, France).

Antibiotic Susceptibility testing: The antimicrobial susceptibility of the extended spectrum enterobacteria β-lactamase isolates was determined by the Bauer-Kirby disk diffusion test using antibiotic disks (Bio-Rad, France) **[15]**. Of these, sixteen antimicrobial agents from four antibiotic families (β-lactams, quinolones, aminosides and cyclins) were tested. Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms (*E. coli* ATCC 25922). Isolates were screened for the ESBL-producing phenotype by the standard double-disc synergy test, as described previously **[16]**. Antimicrobial discs (concentration of antibacterial in µg) used were amoxycillin/clavulanic acid (10/20), ceftazidime (30), ceftriaxone (30), cefotaxime (30), cefepime (30), cefoxitin (30), imipenam (10), meropenam (30), aztreonam (30), nalidixic acid (30), ciprofloxacine (5), amikacin (30), gentamycin (15), tetracycline (30), minocycline (30) and tigecycline (30). All the antibiotics were procured from Bio-rad (France). Only included in the study, were ESBL enterobacteria showing resistance to beta-lactamins, quinolones, aminoglycosides and cyclins.

PCR amplification of beta-lactamase genes: Plasmid DNA was used for detection of β-lactamases and was extracted using Mini prep K0502 kit (Fermentas, Vilnius, Lithuania). The ESBL gene was characterized by polymerase chain reaction as described by **[12].** PCR amplification was performed in a final reaction volume of 50 µl. Primers used in this study is given in Table 1. The reaction mixture contained a PCR Reaction Buffer, 10x concentrated with 20 mM MgCl2, PCR Grade Nucleotide Mix (2.5 mM each), specific primers for each target (20 pmol) and a FastStart Taq DNA Polymerase, 5 U/µl (Roche). The PCR conditions were carried out in a thermalcycler UNOII (BIOMETRA®). Amplification products were analyzed by electrophoresis in a 2% agarose gel (Invitrogen) stained with ethidium bromide and visualized under Ultra Violet light.

The cycling conditions for amplification were as follows: for blaTEM, initial denaturation at 94°C for 1 min and 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, followed by 7 min at 72°C; for blaSHV, PER, VEB, GES et CTXM gene, initial denaturation of 1 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by 7 min at 72°C.

Table 1. Primers used in the study

Genes *bla*	Primers	Sequence (5'->3')	Position	PCR Product size (pb)	Accession Number
TEM	a216 (+)	ATAAAATTCTTGAAGACGAAA	1-21	1079	AB282997
	a217 (-)	GACAGTTACCAATGCTTAATCA	1080-1059		
SHV	os-5 (+)	TTATCTCCCTGTTAGCCACC	23-42	795	X98098
	os-6 (-)	GATTTGCTGATTTCGCTCGG	818-799		
PER	per (+)	CCTGACGATCTGGAACCTTT	465-485	716	721957
	per (-)	GCAACCTGCGCAAT(GA)ATAGC	1181-1161		
VEB	veb (+)	ATTTCCCGATGCAAAGCGT	351-370	542	AF010416
	veb (-)	TTATTCCGGAAGTCCCTGT	893-875		
GES	ges (+)	ATGCGCTTCATTCACGCAC	1332-1350	863	AF156486
	ges (-)	CTATTTGTCCGTGCTCAGGA	2195-2176		
CTXM-1	ctxM1(+)	GGTTAAAAAATCACTGCGTC	65-84	863	X92506
	ctxM1(-)	TTGGTGACGATTTTAGCCGC	928-909		
CTXM-2	ctxM2(+)	ATGATGACTCAGAGCATTCG	6-25	865	X92507
	ctxM2(-)	TGGGTTACGATTTTCGCCGC	871-852		
CTXM-8	CtxM8(+)	GCGGCGCTGGAGAAAAGCAG	712-731	608	AF189721
	CtxM8(-)	GCTGCCGGTTTTATCCCGA	6336-6355		
CTXM-9	ctxM9(+)	ATGGTGACAAAGAGAGTGCA	6336-6355	869	AF174129
	ctxM9(-)	CCCTTCGGCGATGATTCTC	7205-7187		

3. Results

3.1 Enterobacteria ESBL strains

The human strains consist of 70 species of ESBL dominated predominantly by the species *Escherichia coli* (37,1 %), *Klebsiella pneumoniae* (30,6 %) and *Enterobacter cloacae* (17,7 %). The animal strains (239 species of ESBL) were distributed as follows: 81 strains from cattle, 60 from sheep, and 98 from pigs. These strains consist primarily of *Escherichia coli* strains with over 87,4 %. Environment ESBL strains, 130 and 127 species were isolated respectively from hospital effluents and municipal wastewater. The main species were *Escherichia coli* (36,9 %), *Klebsiella pneumoniae* (15,2 %) and *Enterobacter aerogenes* (14,9 %).

Table 2: Diversity of ESBL strain isolated from various origins

	Origins		
ESBL species	**Human (%)**	**Animal (%)**	**Environment (%)**
Escherichia coli	37,1	87,4	36,9
Escherichia vulneris	0,0	1,7	2,0
Klebsiella pneumoniae	30,6	2,0	15,2
Klebsiella oxytoca	3,2	0,0	9,7
Enterobacter aerogenes	1,6	3,7	14,9
Enterobacter cloacae	17,7	0,0	3,0
Enterobacter amnigenes	0,0	0,0	1,2
Citrobacter freundii	1,6	0,0	7,0
Citrobacter koseri	0,0	6,2	2,6
Proteus mirabilis	0,0	0,0	3,2
Proteus vulgaris	0,0	0,0	0,4
Citrobacter amalonaticus	0,0	0,0	0,4
Serratia marcescens	0,0	0,0	3,0
Levinea sp	1,7	0,0	1,5

3.2 Enterobacteria ESBL Resistance rates according to their origins

The average levels of resistance to third generation and fourth generation (CRO, CTX, CAZ, and FEP) cephalosporins for all strains of human, animal and environmental origin ranged from 97.2% to 100%. There was no significant difference ($P > 0.05$) exist between these levels regardless of the origin of the ESBL strains. Mean resistance levels for amoxicillin + clavulanic acid (AMC) were higher in strains of human origin (87.1%) followed by strains of environmental origin (64.5%). A significant difference ($P < 0.05$) was observed between the mean strain resistance of AMC strains. However, the mean levels of resistance to cephamycins were 36.1% and 24.6% for strains of environmental and human origin respectively. No resistance was observed for carbapenems (imipenem and meropenem).

Table 3: Enterobacteria ESBL Resistance rates according to their origins

Antibiotics (load in µg)	Mean resistance (%) according to their origins		
	Human	Animal	Environment
AMC (20/10)	87,1^{d}	49,3^{c}	64,5^{e}
CRO (30)	100^{a}	100^{a}	100^{a}
CTX (30)	100^{a}	99,2^{a}	99,6^{a}
FEP (30)	100^{a}	100^{a}	97,2^{a}
CAZ (30)	100^{a}	100^{a}	100^{a}
ATM (30)	100^{a}	100^{a}	99,6^{a}
FOX (30)	36,1^{c}	3,6^{b}	24,6^{c}
IPM (10)	0^{b}	0^{b}	0^{b}
MEM (10)	0^{b}	0^{b}	0^{b}

AMC = amoxicillin + clavulanic acid; **FEP** = cefepime ; **FOX** = cefoxitine ; **CAZ** = ceftazidime ; **CTX** = cefotaxime ; **CRO** = ceftriaxone ; **ATM** = aztreonam ; **IPM** = imipenem ; **MEM** = meropenem

In line and column, the values assigned to the same letter are not significantly different at the 5% threshold according to the Newmann-Keuls test

3.3 Search beta-lactamases (*bla*) genes of ESBL strains according to their origin

All resistance genes and their different associations were detected in environmental strains. In animal strains, however, only a few of these genes and their associations have been detected. No association of resistance genes was detected in human strains during the study.

Table 4: ESBL genes and theirs associations in the different strains

Beta-lactamases genes	Human	Animal	Environment
bla $_{TEM}$	+	+	+
bla $_{SHV}$	+	+	+
bla $_{CTX\text{-}M1}$	+	+	+
bla $_{CTX\text{-}M2}$	+	+	+
bla $_{CTX\text{-}M8}$	-	-	+
bla $_{CTX\text{-}M9}$	-	+	+
bla $_{GES}$	-	+	+
bla $_{PER}$	-	-	+
bla $_{VEB}$	-	-	+
Beta-lactamases genes associations			
bla $_{TEM\,/\,SHV}$	-	+	+
bla $_{TEM\,/\,CTX\text{-}M}$	-	+	+
bla $_{SHV\,/\,CTX\text{-}M}$	-	+	+
bla $_{TEM\,/\,SHV\,/\,CTX\text{-}M}$	-	-	+
bla $_{TEM\,/\,SHV\,/\,CTX\text{-}M\,/\,GES}$	-	-	+

(+): detected
(-): not detected

4. Discussion

This study presents the diversity antimicrobial resistance profile and betalactamase gene of Enterobacteria producing broad-spectrum.

BlaCTXM -1 and blaTEM genes were the most identified, followed by the blaSHV genes, whereas genes association such as blaTEM / blaSHV / blaCTX-M-1, blaTEM / blaCTX -M-1, blaSHV / blaCTX-M-1 and blaTEM / blaSHV were observed in low frequency. The proportions found are lower than those of Guessennd *and al.* **[12]** in human Enterobacteriaceae producing broad-spectrum beta-lactamases in Abidjan. Their proportions were 65.6%, 64.9% and 48.3% for blaCTX- M-1, blaTEM and blaSHV respectively. The results of this study are also lower than those of Mohammad *and al.* **[17]** who reported high proportions of blaCTX-M-1 genes (46.5%), blaTEM (54%) and blaSHV (67.4%) in their study of *Klebsiella pneumoniae* strains in Teheran. They also demonstrated beta-lactamases genes associations with 23.2% and 19.2% for blaTEM / blaCTX-M and blaTEM / blaSHV / blaCTX-M respectively.

Several studies have shown an alarming increase of blaCTX-M of ESBLs with a strong predominance type **[10, 18]**. In other studies in Tunisia on *Escherchia coli* ESBL strains isolated from children, Réjiba *and al.* **[19]** showed a high proportion of 97% blaCTX-M gene. The majority of the blaCTX-M genes belonged to group 1. The blaSHV had an occurrence of 6% while the blaTEM gene was not detected. High levels of blaCTX-M genes, low blaSHV and blaTEM gene frequency have also been reported in Algeria **[20]**, Thailand **[21]**, Switzerland **[22]** and in Saudi Arabia **[23]** in EBLSE strains isolated from clinical specimens.

The blaTEM, blaSHV and blaCTX-M genes of the ESBL family have also been identified in animal enterobacteria ESBL strains. Unlike to human strains with the blaCTX-M gene (blaCTX-M-1) was predominance, the genes blaSHV and blaTEM were most identified in animal strains. These two genes are responsible for resistance to beta-lactam of more than 50% of the EBLSE strains in cattle, sheep and pigs. However, a new group of blaCTX-M, blaCTX-M-9 has been identified in some animal's EBLSE strains. These results could be explained by a naturally high prevalence of certain plasmid types and subtypes harbored by *E. coli* isolated from animal, which in fact constituted a preferred host of ESBL genes. Indeed, according to Haenni *and al.* **[24]** (Plasmid IncI1 / ST3, for example) are often found in bacteria without epidemiological links and belonging to many animal species (dog, cat, cow, horse, goat, Hen, sheep).

In this study, distributions of the different types of ESBL genes identified in enterobacteria producing broad-spectrum beta-lactamases strains of animal origin corroborates distributions reported in Europe **[25, 26].** The blaTEM-52, blaSHV-12, and blaCTX-M-1 genes are the most frequently reported types in order of importance in non-human reservoirs such as live animals or in the processing chain of these animals **[27].**

However, Felix *and al.* **[28]** showed a predominance of the blaCTX-M-1 gene in *Escherichia coli* strains isolated from carcass and caecum from healthy broiler chickens. Horton *and al.* **[29]** also reported the prevalence of *Escherichia coli* ESBL with blaCTX-M-1 gene in the stool of cattle, pigs and chickens in the UK with higher isolation rates than other animal species intended Consumption.

Furthermore, there is evidence that for *Salmonella sp*. and enteropathogenic *Escherichia coli*, producing ESBL enzyme and responsible for food infections, is an example of direct transmission of these genes from animals to humans **[10, 30].**

As far as transmission to humans is concerned, the scientific evidence supports the existence of two distinct bacterial reservoirs, human and animal. However, some identical ESBL plasmids such as those carrying the blaCTX-M-15 gene have been described in humans and in

cattle **[31]**. Comparison between human and animal strains established that these are more plasmids (IncI1 / ST3) than the bacterial populations that are found identical between humans and animals **[32].**

The problem of antibiotic resistance genes is not limited to hospital and animal strains, resistance is also present in bacteria of environmental (hospital effluents and domestic wastewater) origin. This work has shown that municipal wastewater and hospital effluents represent a source or reservoir of antibiotics resistant bacteria and antibiotic resistance genes that could be transmissible to humans. Thus, the aquatic environment could be an important factor in the spread of resistance as indicated by Zhang *and al.* **[4]**. In addition, contamination of wastewater with antibiotic residues can also lead to selective pressure on antibiotic-resistant bacteria and resistant genes that can pose a risk to human and even animal health **[33].**

In this study, all the genes found in EBLSE strains of human and animal were also identified in the environment in addition to new resistance genes, including blaGES, blaVEB and blaPER genes called "new ESBLs". Gene combinations were much higher in hospital effluent strains than in municipal effluents. The high proportion of association of resistance genes in hospital effluents could be explained by large sizes of plasmids harbored by these strains. The plasmids sizes can often reach up to 104 base pairs and would allow bacteria to survive in hostile environments (hospital and municipal effluents). Indeed, according to Reinthaler *and al.* **[34]**, the plasmids of ESBL enterobacteria in the environment and especially in hospital effluents are known to contain within them several plasmids harboring numerous antibiotic resistance genes. In addition, these plasmids are able to autotransfer from one bacteria to another and replicate independently in hosts **[4].** All these facts make hospital effluents a reservoir of resistance genes.

Some authors have already noted the presence of a wide variety of resistance genes in entero-bacteria producing broad-spectrum beta-lactamase, including *Escherichia coli*. These authors have demonstrated all resistance genes including the genes of new ESBLs that may be involved in antibiotic resistance **[35, 36].**

5. Conclusion

The genotypic profile of isolated enterobacteria producing broad-spectrum beta-lactamases from human, animal and environment strains showed the presence of common beta-lactam

resistance genes (*blaTEM*, *blaSHV* and *blaCTX-M*) and specific resistance genes (*blaPER*, *blaVEB* and *blaGES*). Hospital effluents appear to be an important reservoir of strains with resistance genes. Some genes not detected in humans and animals, are present in these effluents. These hospital effluents discharged without treatment into surface water can be a source of dissemination of potentially pathogenic enterobacteria ESBL that can cause public health problems. The risk of dissemination and circulation of ESBL enterobacteria between animals, humans and the environment exists in Côte d'Ivoire because of the absence of a barrier between them. The dissemination and circulation of ESBL enterobacteria is a public health problem.

Conflict of interest

None

6. References

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