Int J Infect. 2018 October; 5(4):e83194. doi: 10.5812/iji.83194.

Published online 2018 October 14. **Research Article**

First Sequence Analysis of Genes Mediating Extended-Spectrum Beta-Lactamase (ESBL) *bla-TEM*, *SHV*- and *CTX-M* Production in Isolates of Enterobacteriaceae in Southern Benin

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Received 2018 August 11; **Accepted** 2018 October 08.

Abstract

The production of extended-spectrum beta-lactamase (ESBL) by Enterobacteriaceae is a global public health problem. The present study was carried out on 156 strains of enteric bacteria isolated from urinary and cervicovaginal fluid samples. Identification of the strains was performed using MALDI-TOF MS and antibiotic susceptibility tests by disk diffusion method on Mueller Hinton agar in accordance with the recommendations of the Antibiogram Committee of the French Society for Microbiology. ESBL genes were sought by real time-polymerase chain reaction (RT-PCR) and by gel-based PCR. Gel-based PCR products were used for sequencing of the resistance genes, which were analyzed in the NCBI and Arg Annot databases. Results showed a predominance of *Escherichia coli* both in the urinary and cervicovaginal fluid samples. *Klebsiella pneumoniae* was the second most isolated bacterium in the specimens. Sensitivity to antibiotics revealed high levels of cephalosporin resistance but low resistance to carbapenem. No resistance was noted to colistine. The *bla-TEM* gene was present in *Escherichia coli*, while *bla-SHV* was found in *Klebsiella pneumoniae* and *bla-CTX-M* was recovered in both strains. Analysis of the sequences revealed that *bla-Tem1* was predominant in *bla-TEM* and *bla-CTX-M-15* was most represented by *bla-CTX-M*. This study confirms the presence of ESBL-producing Enterobacteria in Benin. This was an epidemiological study aimed at detecting cephalosporin resistance in gram-negative *Bacillus* isolated from urinary tract and genital infections developed by women. Since the advent of molecular biology techniques for the identification of resistance in bacteria including determination of ESBL resistance genes (i.e., *TEM*, *SHV*, *CTX-M*), no study has been conducted to identify the different variants that circulate in Benin by sequencing these resistance genes. This sequencing is essential in order to differentiate the non-ESBL parental enzymes, which is not possible with the commonly used PCR techniques that do not permit differentiation of the point generating different variants of the ESBL genes. The present study then helped to identify those variants, in particular *Tem1*, *SHV1*, and *CTX-M15*, which are most encountered in Benin and around the world.

Keywords: Sequence, Analysis, ESBL, Benin, Antimicrobial Resistance

1. Background

Antibiotic resistance of enteric bacteria remains a major public health concern worldwide (1). Many mechanisms of resistance have been described in Enterobacteriaceae and gram-negative non-fermentative bacilli (2-4). The oldest and most persistent is the production of ESBL by these bacteria, conferring resistance to beta-lactams mainly against third-generation cephalosporin (5, 6). ESBL are enzymes produced by bacteria that hydrolyze the beta-lactam ring common to beta-lactam antibiotics. At the beginning of these resistances, only some genes were described, namely *Tem-1*, *Tem2*, and *SHV1* (7, 8). However, many other types have been reported recently. Three genes are mostly involved in this resistance, including *Tem*, *VHS*, and CTX-M, which have appeared in the 2000s (5, 9). This resistance usually occurs in *Escherichia coli* and *Klebsiella pneumoniae* and rarely in other enteric bacteria such as *Enterobacter cloacae* (10).

The present study sought to detect cephalosporin resistance in gram-negative bacilli isolated from urinary tract and genital infections in women. Since the advent of molecular biology techniques for the identification of resistance to bacteria including the determination of ESBL

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resistance genes, no study has been conducted to detect the different variants that circulate in Benin by sequencing these resistance genes. This sequencing is essential in order to differentiate the non-ESBL parental enzymes, which is not possible with the commonly used PCR techniques that do not allow the differentiation of the point mutations generating different variants of the ESBL genes. The present study aimed to identify those variants, in particular *Tem1*, *SHV1*, and *CTX-M15*.

In Benin, several studies have been conducted on beta-lactam resistance (11, 12), but none has examined the sequences of ESBL in order to identify the different types of ESBL that circulate in the country. The present study was initiated to bridge this molecular epidemiology gap of the ESBLs isolated in Southern Benin.

2. Methods

2.1. Bacterial Species Identification and Antibiotics Susceptibility

A total of 154 isolates of enteric bacteria (gram-negative *Bacillus*, Oxidase -) were recovered from 508 urinary and cervicovaginal fluid samples collected from three hospitals in Southern Benin, namely Bethesda Hospital, Zonal Hospital of Menontin, and the Regional Hospital of Oueme-Plateau from July to September 2015. The identification of the strains was carried out by MALDI-TOF MS (13). The susceptibility of the strains to antibiotics was examined by disk diffusion method on Mueller Hinton-2 agar (14). The antibiotic discs used were amoxicillin (AX 25), fosfomycin (FF50), ciprofloxacin (CIP5), amoxicillin + clavulanic acid (AMC30), ertapenem (ERT10), trimethoprim + sulphamethoxazol (SXT25), imipinem (IMP10), amilkacin (AK30), gentamycin (CN15), ceftriazone (CRO30), cefotaxime (CTX30), ticarcillin + sulphamethoxazol (TIM85), cefoxitin (FOX 30), rifampicin (RA 30), and aztreonam (ATM 30). Inhibition diameters were compared to those recommended by CA-SFM, 2013.

2.2. Resistance Genes Detection

The *TEM*, *SHV*, *CTX-M* genes were sought using real time and conventional polymerase chain reaction (PCR). The primers and probes as well as the positive controls used are shown in Table 1. The reaction medium was composed of 10 μL QuantiTec Master Mix, 1 μL Primer F, 1 μL Primer R, 2 μL DNase free water, 1 μL probe, and 5 μL template DNA for qPCR. The reaction mixture for conventional PCR was made of 12.5 μL of QuantiTec, 0.5 μL Primer F, 0.5 μL Primer R, 6.5 μL DNase free water, and 5 μL DNA.

2.3. Sequencing

PCR products were purified and BigDye PCR was performed with the same primers. For each sample, the primers were used differently in two reactions. The reaction medium for the BigDye PCR was 3 μL buffer BigDye, 2 μL BigDye, 1 μL primers, and 10 μL DNase free water. The product of the BigDye PCR was then filtered on sephadex and subjected to gene sequencing by the Sanger method by ABI 3730 (Applied Biosystems, Foster City, CA, USA). The obtained sequences were aligned according to the primers and blasted in GenBank NCBI (http://blast.ncbi.nlm.nih.gov/) and Arg-Annot databases.

3. Results

Table 2 exhibits the distribution of the strains identified in MALDI-TOF. *Escherichia coli* was the most isolated organism in both urinary (48.7%) and cervicovaginal fluid (11.0%) samples, followed by *Klebsiella pneumoniae* (23.4% in urinary and 3.2% in cervicovaginal fluid samples). *Enterobacter cloacae* was the third most isolated bacterium found only in urinary samples (9%).

The antibiotic susceptibility pattern of the isolated enteric bacteria is depicted in Table 3. High levels of resistance were observed with beta-lactams. *Proteus mirabilis*, *Citrobacter koseri*, and *Enterobacter asburiae* showed low resistance compared to *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*.

Bla-TEM was found in high proportion (57%) followed by *bla-CTX-M* (16%) and *bla-SHV* (18%). Only the three bacterial species of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* produced ESBL. *Escherichia coli* produced more ESBL than the other bacteria and a single strain of *Escherichia coli* produced *bla-SHV*. On the other hand, the strains of *Klebsiella pneumoniae* produced more *bla-SHV*. About 6% of the strains carried the three ESBL genes. The carriage of CTX-M was most often associated with the presence of *bla-TEM* gene (Table 4).

The analysis of the sequences obtained shows a predominance of the type *bla-TEM-1* for *TEM*, *bla-SHV-1* for *SHV*, and *bla-CTX-M-15* for *CTX-M* (Table 5).

4. Discussion

The production of ESBL by enteric bacteria is one of the most widespread forms of antibiotic resistance in the world. Out of 154 enteric bacteria isolated in three hospitals in Southern Benin, identification with MALDI-TOF revealed a high presence of *Escherichia coli* in both urinary (48.7%) and cervicovaginal fluid (11%) samples. Several studies have reported the strong involvement of *Escherichia coli* in urinary (15-17) and cervicovaginal infections (18-20).

Table 1. Primers and Probes Used for the Polymerase Chain Reactions

Genes (T +)	Type of PCR	Primer or Probe	Oligonucleotide Sequences	References
TEM (Kpnasey)	qPCR	ALLTEM-F	TTCTGCTATGTGGTGCGGTA	KJ939560.1
		ALLTEM-R	GTCCTCCGATCGTTGTCAGA	
		ALLTEM-Probe	AACTCGGTCGCCGCATACACTATTCTCAGA	
	PCR-Std	ALLTEM-F	ATGAGTATTCAACATTTCCGTG	
		ALLTEM-R	TTACCAATGCTTAATCAGTGAG	
SHV (Kpnasey)	qPCR	SHV-F	TCCCATGATGAGCACCTTTAAA	AF124984.1
		SHV-R	TCCTGCTGGCGATAGTGGAT	
		SHV-Probe	TGCCGGTGACGAACAGCTGGAG	
	PCR-Std	SHV-F	ATTTGTCGCTTCTTTACTCGC	
		SHV-R	TTTATGGCGTTACCTTTGACC	
CTX-M (Kpnasey)	qPCR CTX-M A	CTX-M-A-F	CGGGCRATGGCGCARAC	JQ397665.1
		CTX-M-A-R	TGCRCCGGTSGTATTGCC	
		CTX-M-A-Probe	CCARCGGGCGCAGYTGGTGAC	
	qPCR CTX-M A	CTX-M-B-RT-F	ACCGAGCCSACGCTCAA	
		CTX-M-B-RT-R	CCGCTGCCGGTTTTATC	
		CTX-M- B-Probe	CCCGCGYGATACCACCACGC	
	PCR-Std CTX-M 1	CTX-M-1-F	CCCATGGTTAAAAAATCACTGC	
		CTX-M-1-R	CAGCGCTTTTGCCGTCTAAG	
	PCR-Std CTX-M 9	CTX-M-9-F	GCGCATGGTGACAAAGAGAGTGCAA	
		CTX-M-9-R	GTTACAGCCCTTCGGCGATGATTC	

Table 2. Distribution of Isolates Per Specimens[a]

	Urinary	CVS	Total
Escherichia coli	75 (48.7)	17 (11.0)	92 (59.7)
Klebsiella pneumoniae	36 (23.4)	5 (3.2)	41 (26.6)
Enterobacter cloacae	14 (9.0)	0 (0)	14 (9.0)
Proteus mirabilis	4 (2.6)	0 (0)	4 (2.6)
Enterobacter asburiae	2 (1.3)	0 (0)	2 (1.3)
Citrobacter koseri	1 (0.6)	0 (0)	1 (0.6)
Total	132 (85.7)	6 (14.3)	154 (100)

Abbreviation: CVS, Cervico-vaginal secretions.
[a]Values are expressed as No. (%)

Klebsiella pneumoniae was the second most isolated bacterium in our specimens, which has also been reported in urinary and vaginal infections (21-23). A high resistance to cephalosporins was recorded in the present study. Nevertheless, a very low proportion of carbapenem resistance was observed. In the aminoglycoside family, we observed a high proportion of gentamicin resistance compared to a low resistance to amikacin. This discrepancy in the aminoglycoside family is attributable to the fact that amikacin has a higher minimum inhibitory concentration (MIC) and therefore, it is less used (24). This demonstrates the implication of inappropriate and non-moderate use of antibiotics in the emergence of resistances (25).

No resistance was noted to colistine, which is the last resort against infections caused by *Pseudomona aeruginosa*, *Acinetobacter baumanii*, and multidrug-resistant Enterobacteriaceae (26). It is therefore necessary to monitor the use of this antibiotic in both human and veterinary health in order to avoid resistance to this antibiotic. The bacteria that showed higher levels of resistance were *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. The same observation was previously made in Burkina Faso (27, 28).

About 65% of the enteric bacterial strains carried an ESBL gene. This prevalence of ESBL is so far the highest reported in Benin and shows high dissemination of this resistance in the country. Previous studies in Benin were based on the ESBL phenotype, which has shown deficiencies over time because bacteria that produce β-lactamases are regarded as susceptible to 3rd and 4th-generation cephalosporins (29). In addition, most of these studies are biased with the poor quality of antibiotic discs marketed

Table 3. Antibiotic Susceptibility of the Isolates

	AMX	AMC	TIM	CTX	CRO	FOX	ATM	ERT	IPM	CS	AK	GEN	CIP	FF	SXT
Escherichia coli	78/92 (85%)	69/92 (74%)	72/92 (78%)	23/92 (25%)	25/92 (27%)	37/92 (40%)	26/92 (28%)	7/92 (8%)	0/92 (0%)	0/92 (0%)	2/92 (2%)	28/92 (30%)	47/92 (51%)	3/92 (3%)	74/92 (80%)
Klebsiella pneumoniae	41/41 (100%)	24/41 (59%)	22/41 (54%)	11/41 (27%)	11/41 (27%)	2/41 (5%)	13/41 (32%)	1/41 (2%)	0/41 (0%)	0/41 (0%)	1/41 (2%)	11/41 (27%)	12/41 (29%)	0/41 (0%)	22/41 (54%)
Enterobacter cloacae	14/14 (100%)	14/14 (100%)	11/14 (79%)	3/14 (21%)	3/14 (21%)	14/14 (100%)	6/14 (43%)	4/14 (29%)	1/14 (7%)	0/14 (0%)	2/14 (14%)	4/14 (29%)	3/14 (21%)	1/14 (7%)	3/14 (21%)
Proteus mirabilis	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	4/4 (100%)	0/4 (0%)	0/4 (0%)	2/4 (50%)	1/4 (25%)	3/4 (75%)
Enterobacter asburiae	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	0/2 (0%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)
Citrobacter koseri	1/1 (100%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)
Total	138/154 (90%)	111/154 (72%)	105/154 (68%)	37/154 (24%)	39/154 (25%)	57/154 (37%)	45/154 (29%)	14/154 (9%)	1/154 (0.6%)	0/154 (0%)	5/154 (3%)	43/154 (28%)	79/154 (51%)	5/154 (3%)	104/154 (67%)

Table 4. Repartition of Detected Extended-Spectrum Beta-Lactamase Genes

	ESBL	TEM	SHV	CTX-M	TEM + SHV	TEM + CTX-M	TEM + CTX-M + SHV
Escherichia coli	68/92 (74%)	54/92 (59%)	0/92 (0%)	1/92 (1.1%)	0/92 (0%)	14/92 (15%)	1/92 (1.1%)
Klebsiella pneumoniae	28/41 (68%)	4/41 (10%)	11/41 (27%)	1/41 (2.5%)	2/41 (5%)	3/41 (7.5%)	8/41 (20%)
Enterobacter cloacae	4/16 (25%)	1/16 (6%)	2/16 (12%)	0/16 (0%)	1/16 (6%)	0/16 (0%)	0/16 (0%)
Total	100/154 (64%)	59/154 (38%)	13/154 (8%)	2/154 (1%)	3/154 (2%)	17/154 (11%)	9/154 (6%)

Table 5. Repartition of the Detected Extended-Spectrum Beta-Lactamase Genes

	Types	No. (%)	*E. coli*	*K. pneumoniae*	*E. cloacea*
Bla Tem	Tem 1	83 (94)	65/88 (72%)	17/88 (20%)	1/88 (1%)
	Tem 2	3 (3)	3/88 (3%)	0/88 (0%)	0/88 (0%)
	Tem 54	2 (2)	1/88 (1%)	0/88 (0%)	1/88 (1%)
Bla CTX-M	CTX-M 15	24 (89)	12/28 (43%)	12/28 (43%)	0/28 (0%)
	Others	4 (11)	4/28 (14%)	0/28 (0%)	0/28 (0%)
Bla SHV	SHV-1	15 (60)	0/25 (0%)	11/25 (44%)	2/25 (8%)
	SHV-12	8 (32)	1/25 (4%)	8/25 (32%)	1/25 (4%)
	SHV-2	2 (8)	0/25 (0%)	2/25 (8%)	0/25 (0%)

in Benin (30).

This study also revealed the presence of *bla-TEM* in *Escherichia coli* and *bla-SHV* in *Klebsiella pneumoniae*. Anago et al. (11) also reported a high presence of *bla-Tem* in *Escherichia coli* strains isolated from nosocomial infections in Southern Benin. Hou et al. (31) reported high proportions of *bla-SHV* in *Klebsiella pneumoniae* strains compared to *bla-Tem* in China. The natural production of penicillinase by *Klebsiella pneumoniae* strains encoding a gene with SHV, LEN, and OKP variants explains the presence of *SHV* in *Klebsiella pneumoniae* strains (32). Kamga et al. (33) observed a strong presence of TRI (inhibitor-resistant TEM) in *Escherichia coli* strains, which may justify the high presence of *TEM* in the *Escherichia coli* strains isolated in the present study. Zongo et al. (28) in Burkina Faso, Salah (34), and Diagbouga et al. (35) in Togo also found that the *bla-Tem* gene was the most widespread in ESBL-producing *Escherichia coli* strains, whereas *Bla-SHV* was the predominant gene in ESBL-producing *Klebsiella pneumonia* strains. However, the high proportion of *bla-CTX-M* in Togo (95.7%) and Burkina Faso (65.49%) is not the same as in our study because this gene came second after *bla-Tem* and represented only 18%.

The molecular typing of the various resistance genes revealed the presence of three types of *Bla-TEM* including *bla-TEM1* (83/88), *bla-TEM2* (3/88), and *bla-TEM54* (2/88). *Bla-CTX-M15* was the most represented type of *bla-CTX-M*. The strong presence of this type has been reported in many studies in Africa and worldwide (27, 36-41). *SHV-1* was the predominant *VHS* in our study followed by *SHV-12* and *SHV-2*. These types have also been reported in (42-45) as well.

4.1. Conclusion

The present study revealed the strong presence of ESBL-producing Enterobacteriaceae in Benin. It remains the most dominant resistance to beta-lactam and evolves towards resistance to carbapenems. The types of ESBL gene reported in the study are widely replicated in Africa and are implicated in many infectious pathologies. There is an urgent need to devise policies towards reducing antimicrobial resistance in Africa in order to reduce mortality and the high cost of treatment associated with ESBL-producing Enterobacteriaceae.

Acknowledgments

We thank Edgarthe Priscilla Ngaiganam for technical assistance, Stephanie Lepage for her expertise in antimicrobial susceptibility, and the URMAPha team for sample collection.

Footnote

Authors' Contribution: All authors equally contribute to the study.

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