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First Detection of Resistance Genes Encoding Extended Spectrum β -Lactamase Producing *Escherichia coli* at Lomé, Togo

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Abstract

Background: Extended Spectrum β -lactamases (ESBLs) are enzymes produced by some bacteria that inactivate oxyimino-cephalosporins, a class of β -lactam antibiotics family. They are mainly encoded by the β -lactamase gene families TEM, SHV and CTX-M. The *Enterobacteriaceae* expressing these genes, in particular *Escherichia coli* are widely expanded in the world with different prevalence rate from one region to another. The objective of this study, carried out from strains isolated at the National Institute of Hygiene (INH) in Togo, was to (i) detect and characterize the genes involving in the production of β -lactamase among *E. coli* strains and (ii) evaluate their prevalence.

Methodology: From May 2013 to July 2015, 91 *E. coli* strains resistant to third generation cephalosporins (C3G) were collected from several specimens and analyzed for the detection of β -lactamase resistance genes, bla_{TEM}, bla_{SHV} and bla_{CTX-M} by simplex and multiplex PCR.

Results: The *E. coli* strains resistant at least to one third generation cephalosporin, were isolated from urine 58/91 (63.74%), vaginal samples 17/91 (17.68%), wound swabs 7/91 (7.69%), 4/91 semen samples (4.40%), urethral curettage CU 2/91 (2.20%), sputum 1/91 (1.1%), stool 1/91 (1.1%) and joint fluid 1/91 (1.1%). Resistance to third generation cephalosporins was associated with a resistance to quinolone (96.67%), trimethoprim/sulfamethoxazole (94.44%), gentamicin (75.82%) and chloramphenicol (59.09%). ESBL genes were present in all *E. coli* strains with the following proportions: TEM/CTX-M1 52/91 (57.14%), TEM/SHV/CTX-M1 19/91 (20.88%), CTX-M 18/91 (19.78%), TEM 1/91 (1.10%) and TEM/SHV 1/91 (1.10%).

Conclusion: The presence of ESBL producing *E. coli* is a reality in Togo and their wide dissemination compromises the treatment of classical infections such as urinary tract infection, bacterial pneumonia, sepsis or meningitis. The acquisition of molecular data on bacterial resistance to antibiotics is necessary for better therapeutic management of these infections. This is a first determination of the β -lactamase genes in Togo.

Keywords: *E. coli*, ESBL, bla_{TEM}, bla_{SHV}, bla_{CTX-M1}, Togo

Background

Infectious diseases are still a major public health issue, especially in Africa where they are responsible for more than 70% of morbidity and mortality [1]. They are dominated by bacterial infections responsible of most of nosocomial and community infections. The β -lactam antibiotic family including penicillins and cephalosporins is more often used to treat these bacterial infections because of their broad-spectrum, low toxicity, efficiency and low cost [2]. Unfortunately, during the last decade, a significant increase in bacterial resistance to these antibiotics was observed. Indeed, according to the World Health Organization (WHO) 2014 report on the global surveillance of antimicrobial resistance in the world, antibiotic resistance has become a serious threat to public health [3]. The emergence and spread of bacteria resistant to β -lactam usually occur by mechanisms of acquisition of exogenous genes of resistance, as plasmid. These genes can inhibit the action of β -lactam antibiotics through production of cephalosporinase (AmpC), Extended Spectrum β -lactamase (ESBL) or carbapenemase, a consequence of the inappropriate use of β -lactam antibiotics in human, animal and in agriculture [4].

Discovered in the early 1980s, ESBLs are a large family of plasmid borne bacterial enzymes belonging to the class A or D of the Ambler classification and found mainly in *Enterobacteriaceae* [5,6]. Since their discovery and until 1990, most of ESBL detected were TEM and SHV types that disseminated mostly within hospital clones of *Klebsiella pneumoniae* and *Enterobacter spp* and associated with nosocomial outbreaks in intensive care unit. These recent years a new type of plasmid encoded β -lactamase CTX-M which spread in *Escherichia coli* strains and cause urinary tract infections in the communities has been discovered. The bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes, respectively encoded the production of ESBL TEM, SHV and CTX-M, have been described in several epidemiological studies in Europe, Asia, USA, and South America [7-10]. The bacterial species expressing these genes are widespread in the world but some geographical areas have a significantly higher prevalence rates such as South America, Asia and Europe [11].

In Africa, the prevalence of ESBL in *Enterobacteriaceae* was recently estimated at less than 15% [12], with a predominance of CTX-M-15, and also the AmpC cephalosporinase and carbapenemase [13].

In Togo, an analysis of hospital data on the resistance of blood culture bacteria in the Sylvanus Olympio Teaching Hospital of Lomé conducted in 2009 revealed a prevalence rate of 17.6% of ESBL producing *Enterobacteriaceae* [14]. However, no information was available about the different types of β -lactamase genes carried by these *Enterobacteriaceae*.

This study aimed to detect and characterize the resistance genes harbored by ESBL producing *E. coli* strains isolated from different biological specimens at the National Institute of Hygiene (INH) at Lomé, Togo.

Methods

Collection and identification of *E. coli* strains, antibiotic susceptibility and detection of ESBL phenotype

The *E. coli* strains were collected during a descriptive study from May 2013 to July 2015 at the National Institute of Hygiene, Lomé in Togo. INH is the national reference laboratory for the biomedical analysis, epidemiological surveillance, water and food quality control in the country. The strains were isolated from various biological specimen including urine, vaginal samples, semen, urethral curettage (CU), wound swabs and stool. They have been then purified on culture media (MacConkey or Eosin Methylene Blue agar) and identified by API 20E identification system (Biomerieux, Marcy-Etoile, France).

The antibiotic susceptibility testing was performed using the disc diffusion method of Kirby and Bauer [15] on Mueller-Hinton agar (MH) and interpreted using the criteria of the 2014 Antibiogram Committee of the French Society of Microbiology [16]. The antibiotics (Bio-Rad, Marnes-la-Coquette, France) tested were amoxicillin+clavulanic acid (AMC, 20/10 μ g),

piperacillin+tazobactam (TZP, 75/10 μ g), cefoxitin (FOX, 30 μ g), ceftriaxon (CRO, 30 μ g), ceftazidim (CAZ, 30 μ g), cefotaxim (CTX, 30 μ g), cefepim (FEP, 30 μ g), aztreonam (ATM, 30 μ g), imipenem (IMP, 10 μ g), amikacin (AN, 30 μ g) gentamicin (GM, 15 μ g), nalidixic acid (NA, 30 μ g) ciprofloxacin (CIP, 5 μ g), trimethoprim/sulfamethoxazole (SXT 1.25/23.75 μ g), fosfomycin (FOS, 50 μ g), chloramphenicol (C, 30 μ g).

All isolates were subjected to the double disc synergy test for ESBL detection [17]. The presence of ESBL is detected by a synergy between ceftazidim and cefotaxim or ceftriaxon discs and amoxicillin+clavulanic acid disc. *Escherichia coli* ATCC 25922 was used as control strain for antibiotic susceptibility testing.

E. coli strains resistant to at least one third generation cephalosporin tested, ceftazidim, cefotaxim or ceftriaxon were collected and conserved at -80°C in trypticase soy broth (TCS). They were then sended (in triple packaging boxes with ice packs) to the Biomolecular and Genetic Laboratory (LABIOGENE); Pietro Annigoni Biomolecular Research Center (CERBA), Ouagadougou, Burkina Faso.

Extraction of bacterial DNA

From TCS broth, strains were reactivated on TCS agar for 18-24 h and then inoculated in Luria Bertani (LB, 2 ml). After 18-24 h of overnight culture, LB broth were centrifuged at 10285 g for 10 min and the pellet suspended in 500 μ l of phosphate buffer (100 mM, pH 7) to weaken the membranes. The mix was heated at 100°C for 15 min in a water bath to release the genetic material. The DNA is then precipitated in 250 μ l of absolute ethanol, washed twice in 1000 μ l of ethanol 75%, dried and re-suspended in 100 μ l of sterile water.

Detection of ESBL genes

The thermocycler GeneAmp PCR System 9700 (Applied Biosystems, California, USA) was used for the amplification of ESBL genes bla_{TEM}, (TEM1, TEM2), bla_{SHV} (SHV1), bla_{CTX-M-G1} (CTX-M-1, 3 or 15), bla_{CTX-M-G2} and bla_{CTX-M-G9} (CTX-M-9 and CTX-M-14). Primers used were purchased from Applied Biosystems (California, USA) (Table 1). Three PCR were performed: two multiplex for TEM/SHV, CTX-M-G2 and CTX-M-G9 group [18] and a simplex for CTX-M-G1 [19].

The PCR were performed in a total volume of 50 μ l containing to 2 μ l of DNA, 25 μ l of 1X AmpliTaq Gold (Master Mix), 5 μ l Enhancer, 2 μ l of each primer at different concentration (0.2 to 0.4 pmol/ μ l) and sterile water. The cycling conditions were as follow:

- Bla_{TEM/SHV} and bla_{CTX-G2-M/G9}: initial denaturation 94°C for 10 min. 30 cycles of denaturation 94°C for 40s, annealing 60°C for 40s and extension 72°C for 1 min. Final extension 72 °C for 7 min.
- Bla_{CTX-M-G1}: initial denaturation 96°C for 10 min. 35 cycles of denaturation 94°C for 1 min, annealing 50°C for 1 min and extension 72°C for 1 min. Final extension 72°C for 10 min.
- The PCR products were separated on 2% agarose gel staining with ethidium bromide and visualized under UV illumination.

A 100 bp DNA ladder (Promega, USA) was used as a marker size.

Table 1 Primers used for PCR amplification of bla genes identification.

PCR target	Sequence (5'–3')	Fragment size (pb)	Reference
TEM	For: CATTCCGTGTCGCCCTTATTC	800	[18]
	Rev: CGTTCATCCATAGTTGCCTGAC		
SHV	For: AGCCGCTTGAGCAAATTAAC	713	[18]
	Rev: ATCCCGCAGATAAATCACCAC		
CTX-M-G1	For: GTTACAATGTGTGAGAAGCAG	1000	[19]
	Rev: CCGTTTCCGCTATTACAAAC		
CTX-M-G2	For: CGTTAACGGCACGATGAC	404	[18]
	Rev: CGATATCGTTGGTGGTRCCA*		
CTX-M-G9	For: TCAAGCCTGCCGATCTGGT	561	[18]
	Rev: TGATTCTCGCCGCTGAAG		

*R=A ou G

Statistical analysis

Statistical significance was determined by Fisher's exact test used for small samples size. Analyses were performed using Epi Info Version 7.1.1.14 and a p-value <0.05 was considered to be statistically significant.

Results

Bacterial strains

From May 2013 to July 2015, 91 *E. coli* strains, resistant at least to one third generation cephalosporin, ceftazidim, cefotaxim, ceftriaxon, were isolated from urine 58/91 (63.74%), vaginal samples 17/91 (17.68%), wound swabs 7/91 (7.69%), 4/91 semen samples (4.40%), urethral curettage (CU) 2/91 (2.20%), sputum 1/91 (1.1%), stool 1/91 (1.1%) and joint fluid 1/91 (1.1%).

Antibiotic susceptibility profile

All *E. coli* strains were resistant to ceftriaxon and cefotaxim and 98% to ceftazidim. The resistance rate to other β -lactam antibiotics was respectively 2.20%, 17.59% and 25.35% for imipenem (very low levels), piperacillin-tazobactam and

cefoxitin. Quinolones, nalidixic acid and ciprofloxacin, trimethoprim/sulfamethoxazole and chloramphenicol were very resistant with rates of 96.67%, 94.50%, 94.44% and 59.09% respectively. Among the aminoglycosides, gentamicin was the most resistant antibiotic (75.82%) in contrast to amikacin (3.30%) which showed also very low levels of resistance. The results are shown in (Table 2).

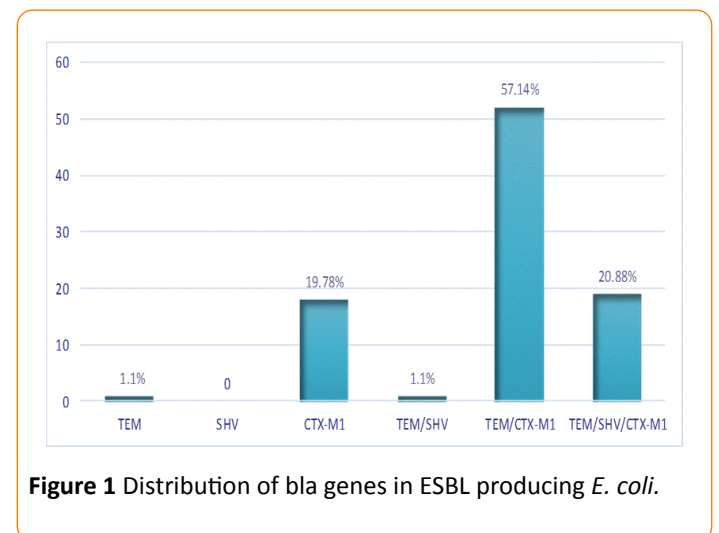


Figure 1 Distribution of bla genes in ESBL producing *E. coli*.

Table 2 Antibiotic susceptibility profile in ESBL producing *E. coli*.

ATB	CAZ	CRO	CTX	FOX	FEP	ATM	TZP	IPM	GM	AN	NA	CIP	C	FOS	SXT
R(n/N)	79/91	91/91	91/91	18/71	90/91	90/91	16/91	20/91	69/91	30/91	87/90	86/91	52/88	40/91	85/90
R(%)	86.81	100	100	25.35	98.9	99	17.58	2.2	75.8	3.3	96.7	94.5	59.09	4.44	94.44

ATB: antibiotic, R resistant, CAZ ceftazidim, CRO ceftriaxon, CTX cefotaxim, FOX cefoxitin, FEP cefepim, ATM aztreonam, TZP piperacillin-tazobactam, IPM imipenem, GM gentamicin, AN amikacin, NA nalidixic acid, CIP ciprofloxacin, C chloramphenicol, FOS fosfomycin, SXT trimethoprim/sulfamethoxazole.

In short, resistance to third generation cephalosporins in *E. coli* has been associated with a resistance to quinolone, trimethoprim/sulfamethoxazole, gentamicin and chloramphenicol. The double disc synergy test was positive in 85/91 strains giving an ESBL presumption rate of 93.40%.

Detection of ESBL genes

PCR was performed to determine the presence of ESBL genes and all *E. coli* strains, positive or not to the double disc synergy test, carried at least one genes bla_{TEM}, bla_{SHV} and bla_{CTX-M-G1}. These results are shown in (Figure 1). The findings revealed a low presence of ESBL genes alone 19/91 (20.88%) in contrast to percentage of associations, 72/91 (79.21%) *E. coli* strains harbored more than one ESBL resistance gene. SHV was absent, TEM present in one strain (1.10%) and CTX-M1, in 18/91 (19.78%) strains. The proportion of combinations TEM/SHV, TEM/CTX-M1, were respectively 1/91 (1.10%) and 52/91 (57.14%) and the triple combination TEM/SHV/CTX-M1 was found in 19/91 (20.88%) strains. Bla_{CTX-M-G2} and bla_{CTX-M-G9} were not found in our study.

Antibiotic resistance profile and distribution of resistance genes in ESBL producing *E. coli* isolated from urine and non-urine specimens

Table 3 Antibiotic susceptibility profile and bla genes distribution in ESBL producing *E. coli* from urinary specimen versus non urinary specimen.

Antibiotics % (n/N)	Urinary specimen 63.74% (58/91)	Non-Urinary specimen 36.26% (33/91)	p-value
Cefoxitin	25.35 (12/47)	25 (6/24)	1.00
Piperacillin-tazobactam	22.41 (13/58)	9.09 (3/33)	0.154
Imipenem	1.72 (1/58)	3.03 (1/33)	1.00
Gentamicin	77.59 (45/58)	72.72 (24/33)	0.619
Amikacin	3.45 (2/58)	3.03 (1/33)	1.00
Nalidixic acid	96.55 (56/58)	96.88 (31/32)	1.00
Ciprofloxacin	94.83 (55/58)	93.94 (31/33)	1.00
Chloramphenicol	55.65 (34/57)	58.06 (18/31)	1.00
Fosfomicyn	6.90 (4/58)	0 (0/32)	0.293
Trimethoprim/sulfamethoxazole	94.84 (55/58)	93.75 (30/32)	1.00
TEM	0	3.03 (1/33)	-
TEM/SHV	0	3.03 (1/33)	-
CTX-M	22.41 (13/58)	15.15 (5/33)	0.585
TEM/CTX-M	58.62 (34/58)	54.55 (18/33)	0.826
TEM/SHV/CTX-M	18.97 (11/58)	24.24 (8/33)	0.598

The antibiotic resistance profile in ESBL producing *E. coli* isolated from urine is similar to that of non-urinary specimens

(vaginal samples, wound swabs, semen samples, urethral curettage, sputum, stool, joint fluid) for cefoxitin, imipenem, gentamicin, amikacin, ciprofloxacin, chloramphenicol, and trimethoprim/sulfamethoxazole. The differences of prevalence observed for piperacillin-tazobactam and fosfomicin resistance and for the distribution of ESBL genes were not statistically significant (Table 3).

Discussion

Enterobacteriaceae are among the most frequently isolated pathogens in biological specimens and involved in bacterial infections with a predominance of *E. coli* [20]. Antibiotics, β -lactams, aminoglycosides, quinolones, frequently used to cure in these infections are becoming increasingly less and less effective [4]. Indeed, the acquisition of antibiotic resistance factors is due along others, to the excessive and uncontrolled use of antibiotics especially in poor hygiene conditions in developing countries [21,22]. So, the accurate identification of the pathogen and its antibiotic susceptibility profile are necessary for appropriate and effective use of antibiotics and also for a resistance surveillance strategy. The threat of antibiotic resistance in the world is real and each country according to WHO recommendations must develop an antimicrobial resistance surveillance system. In Togo, the prevalence of ESBL producing *Enterobacteriaceae* was estimated at 17% in laboratory blood cultures [14]. The presence of hospital-acquired ESBL producing *Enterobacteriaceae* is therefore a reality in Togo and these resistance genes can spread rapidly within the community [23].

In this study, the low frequency of isolation of *E. coli* resistant at least to a third generation cephalosporin (approximately 6 strains per month) in a non-hospital laboratory could predict their low prevalence in the community. On the other hand, the prevalence of ESBL phenotype among *E. coli* resistant at least to one third generation cephalosporin was high at 93.4%. This prevalence is higher than 75% observed in Burkina Faso on *E. coli* also resistant to third generation cephalosporin [24]. This difference could be explained by an hyperproduction of β -lactamase in strains isolated at Lomé or a poor phenotypic detection due to the limited sensitivity of the double disc synergy test as indicated by Livermore [2].

The presence of ESBL was also associated with a resistance to quinolone, trimethoprim-sulfamethoxazole, gentamicin and chloramphenicol leading these bacteria to become multiresistant. This co-resistance was also observed in a study in Benin, on clinical ESBL producing *E. coli* from various biological specimens with 100% resistance rate to ciprofloxacin and trimethoprim/sulfamethoxazole and 82.7% for gentamicin [25]. The same trend is observed in other African countries such as Sudan and the Democratic Republic of Congo with resistance rates of 81.4% and 96.5% for ciprofloxacin and 98.6% and 96.5% for trimethoprim/sulfamethoxazole [26,27]. If the resistance to quinolones and trimethoprim/sulfamethoxazole remains generally high above 90%, that of gentamicin is variable with a rate of 48.6% in Sudan [26] and 48.1% in Tanzania on ESBL producing *E. coli* from clinical urinary specimens [28]. This co-resistance, frequently observed in ESBL producing *E. coli* is due to the existence of other mechanisms of resistance in addition to

the β -lactamase gene on the same plasmid, such as the *aac(6')*-*lb-cr* gene which induces resistance to both aminoglycosides and fluoroquinolones [5].

In this study, a decrease in susceptibility to piperacillin-tazobactam and cefoxitin was observed with the respective resistance rate 17% and 25%. With carbapenems, these antibiotics are among those used for the treatment of infections caused by ESBL producing *Enterobacteriaceae* including *E. coli* [29,30]. Resistance to cefoxitin and piperacillin-tazobactam might be explained by the loss or alteration of porin outer membrane or the coexistence of AmpC cephalosporinase with β -lactamase [5]. The resistance of ESBL producing *E. coli* to cefoxitin and piperacillin-tazobactam is highly variable. On clinical urinary ESBL producing *E. coli* strains resistance to piperacillin-tazobactam in India, China and Japan was respectively 10.1%, 6.3% and 13.7% [31-33]. Resistance to cefoxitin was 41% in Cambodia [34], and 0% in Morocco [35]. The presence of this co-resistance could limit the choice of antibiotics to be used in case of ESBL producing *E. coli* infection. Imipenem and amikacin, with a low resistance rate at 2.20% and 3.30% respectively remained the antibiotics of choice for non-urinary infection with ESBL producing strains [30,36]. Fosfomycin, with a resistance rate at 4.40% for ESBL producing *E. coli* still remains one of the efficient treatment in case of urinary tract infection [37] caused by *E. coli* [38]. Nevertheless, the emergence of resistance to these antibiotics, advocates the establishment of a more enhanced surveillance strategy.

For the presence of resistance genes, PCR was positive for *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M-G1} in all *E. coli* strains and negative for *bla*_{CTX-M-G2} and *bla*_{CTX-M-G9} genes. These results showed that the combination TEM/CTX-M1 (57%) dominated the resistance profile in ESBL producing *E. coli* followed by the combinations TEM/SHV/CTX-M1 (20.8%) and CTX-M1 (19.7%). The *bla*_{TEM} gene and the combination *bla*_{TEM/SHV} were less frequent with a prevalence of 1.1% each. The worldwide expansion of the community acquired ESBL gene, CTX-M [39] is thus confirmed in this study and also its association with TEM and SHV genes, previously reported in studies on *E. coli* from urinary specimens in Europe [40], Asia [41,42], America [43,44] and North Africa [35].

In West Africa, recent studies have also reported the prevalence of CTX-M gene and its association with the TEM and SHV in ESBL producing *E. coli* and isolated from various biological specimens. Thus in Burkina Faso, Zongo et al. had reported on 96 *E. coli* the following rates of these genes: TEM 25/96, SHV 20/96, CTX-M 70/96 and TEM/SHV/CTX-M 6/96 [24]. In Senegal, Dia et al., found on 32 *E. coli*: TEM 9/32, SHV 1/32, CTX-M15 29/32 and CTX-M15/TEM 13/32 [45]. In Benin, Anago et al. had found 74.2% for TEM, 24.1% for SHV and 17.4% for TEM/SHV combination, CTX-M was not determined [25]. The combination of ESBL genes is more common in this study 72/91, 79.12% than in Burkina Faso, with 6/96, 6.96% [24], in Senegal, 13/32, 40.63% [45] and in Morocco, 2/7, 28.57% [35].

It is especially noted that the resistance genes are most often in combination (79.12% of associated genes versus 20.88% of genes alone). The absence of the first genes BLSE (TEM and SHV) solo as opposed to the new CTX-M-G1 gene found alone in 18

strains suggests a progressive consolidation of resistance genes on a single mobile genetic element (plasmids, Integron, etc.)

The CTX-M-G1 includes CTX-M-1, CTX-M-3 or CTX-M-15 types. The CTX-M β -lactamase belong to the class A of β -lactamase enzymes described for the first time in Western Europe and therefore found worldwide [6,46]. The CTX-M-15 type that differs from CTX-M-3 by Asp240Gly substitution conferring a resistance to ceftazidim [5,46] is the most widespread [46]. In the absence of more detailed results on the characterization of CTX-M1 gene and face to a high level of resistance to ceftazidim (100%) in this study, we can suppose that they are likely to carry the CTX-M15 gene. The prevalence of this gene described in some studies in West Africa, Senegal [45,47], Niger [48], Nigeria [49-51] would suggest its wide distribution in that part of Africa.

In this study, the prevalence of ESBL genes and the different antibiotic susceptibility profiles distribution is independent of biological specimen (urinary specimen versus non urinary specimen) in which ESBL producing *E. coli* was isolated. The small sample size could explain this finding.

Conclusions

This study showed that *E. coli* strains isolated at the National Institute of Hygiene in Lomé and resistant to third generation cephalosporins generally produce extended spectrum beta-lactamase (ESBL). The results revealed the high prevalence of CTX-M-G1 alone or in combination with TEM and SHV genes. ESBL production in these strains was associated with a resistance to quinolones, trimethoprim/sulfamethoxazole, gentamicin and chloramphenicol. But they remain largely susceptible to imipenem, amikacin and fosfomycin allowing an alternative therapy for infections by ESBL producing *E. coli* strain.

Antibiotic resistance by ESBL production is a reality worldwide and particularly in Togo. Their wide dissemination jeopardize the treatment of common infections in the community and in hospitals. The need of molecular characterization of these resistance mechanism to antibiotics is necessary for better therapeutic management of infections and also to contribute to the establishment of an antibiotic resistance surveillance system.

This is a first determination of the β -lactamase genes in Togo.

Declarations

Ethical approval

This study has received an approval for the transfer of *E. coli* strains from INH Lomé, Togo to the Biomolecular and Genetic Laboratory (Labiogene); Pietro Annigoni Biomolecular Research Center (Cerba), University of Ouaga I, Prof. Joseph Ki Zerbo, Burkina Faso and have been accepted by the institutional review board of CERBA/LABIOGENE.

Availability of data

The database analyzed during the study is available on reasonable request from the corresponding author.

Author's contribution

FS, SD and JS designed the study, FS, AS, SS, AO, TZ and AD performed the experiments, FS, CN and SD analyzed the data and wrote the manuscript which was approved by all the other co-authors.

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Conflict of Interest

The authors declare no conflict of interest.

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