Molecular characterization and Antibiotic resistance profiles of *Escherichia coli* extended-spectrum β-lactamases producer strains isolated from urine samples in Benin

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Abstract

Urinary tract infections are the second common reason of medical consultations and antibiotics prescription. *Escherichia coli* is known to cause most urinary tract infections. The aim of this study was to characterize and determine the antibiotic resistance profile of *E. coli* extended-spectrum β-lactamases (ESBL) producer strains isolated from urine samples. The urine samples collected came from hospitalized and non-hospitalized patient

referred to Hubert Koutoukou Manga (HKM), National and University Hospital Center (Cotonou, Benin). The resistance to antibiotics was determined according to the disk diffusion method. The production of penicillinase and ESBLs was researched respectively by the acidimetric test and double disk synergy method. The presences of genes encoding β-lactamases were detected by Polymerase Chain Reaction (PCR). Our data revealed that 60 % of *E. coli* strains (101) were isolated from female patients. Also, 69.31 % of the strains were isolated from non-hospitalized patients. The high resistance levels were recorded with amoxicillin (96.04 %) and amoxicillin + clavulanic acid (66.34 %). Twenty percent (20%) of strains were ESBLs. Among ESBLs strains, 70% comes from non-hospitalized patients. Eighty percent of *E. coli* strains produced penicillinase among which 25 % were ESBL producers. All the ESBL producers strains carried bla_{TEM} gene whereas only 30 % carried the bla_{SHV} gene. This study updates the data on the prevalence to antibiotic resistance of *E. coli* ESBL producers strains for better management of urinary tract infections.

Keywords: Urinary Infections, Antibiotic, Resistance, ESBL, Benin

Introduction

Infectious diseases are world widespread causing over 50 000 deaths daily (Ahmad et Beg, 2001). Many bacteria, such as *Escherichia coli*, were involved in those infections. *E. coli*, part of the human commensal Flore, is known to be the first bacteria to colonize humans gut. Indeed, *E. coli* belonging to the family of *Enterobacteriaceae* is an important opportunist human pathogen associated with infections such as urinary tract, surgical site, skin, soft tissues, bacteremia, and pneumonia infections (Pitout, 2012; Carvalho et al., 2012). Among those infections, urinary tract infections are considered as a public health problem (Fatna et al., 2009) because the urine of healthy person must be exempt of any microorganism (Foxman, 2002). Thus, the identification of the cause and severity of the urinary tract infection are usually established through biochemical and microbiological analysis of urines. Urinary tract infections having *E. coli* as etiological agent are common infections with an estimated annual global incidence of at least 250 million cases (Chauhan et al., 2015).

To treat infections induced by *E. coli* strains, antibiotics such as those β-lactam groups are currently used. This group includes the penicillin's cephalosporins, carbapenems, clavams and monobactams (Singleton, 1999). Often, the β-lactam ring is cleaved by a bacterial enzyme named β-lactamase (Edwards et Greenwood, 1992). But population density, uncontrolled use of antibiotics, lack of clean water supply and lack of proper treatment for sewage has resulted in the selection of multidrug resistant bacterial strains. This

becomes a serious challenge for drug prescription in infectious diseases management (Carlet et al., 2012). For example, in developed countries, it was reported that the economic and human damage caused by antibiotic-resistance is increasing at an alarming rate (Bush et al., 2011). Infections with multidrug-resistant organisms have linked with poorer clinical outcomes and prolonged hospitalization on average compared to infectionss with susceptible pathogens (Maina et al., 2017).

In hospital area, the extended spectrum β-lactamases (ESBL) strains are one of the barriers to efficient treatment of *E. coli* infections (Lucet et al., 1996). The selection of *E. coli* strains producing ESBL is increased by the large prescription of third cephalosporins generation. We should notice that ESBL are enzymes that confer resistance to aztreonam, cefotaxime, ceftazidime, and like to relate oxyimino-β-lactams as well as other penicillin and cephalosporins (Bush et al., 1995). The most common mechanism of the resistance to β-lactam antibiotics is the production of β-lactamase. The genes encoding for β-lactam production are generally harbored by plasmids (Paterson et Bonomo, 2005). Although there are several type genes encoding for ESBL, the first and most founded mutants are bla_{TEM} and bla_{SHV} (Bois et al., 1995; Medeiros, 1997). The mutants with those genes can deflect the oxyimino group and then induce the resistant to all oxyimino-cephalosporins (except α-methoxy cephalosporins) (Livermore et Woodford, 2006).

In Benin, few papers report on the urinary tract infection focusing on the *E. coli* strains characterization. To fill this gap, it will be useful to update *E. coli* ESBLs information. Thus, the aim of this study was to characterize and determine the antibiotic resistance profile of *E. coli* ESBL producer strains isolated from urine samples.

Material and methods:

Samples collection

A total of 979 urines samples were collected from both hospitalized patient and non- hospitalized patient referred to National and University Hospital Center Hubert Koutoukou Manga (Cotonou, Benin). The urine samples collected from January to April 2015. Sample of patient hospitalized in the Hospital Center was considered as hospitalized patient. If the patient wasn't hospitalized in Hospital Center, he was considered as no hospitalized patient. Among the urine samples sent to the Microbiology laboratory of CNHU for analysis, only those that were analyzed for the purpose of diagnosing urinary tract infection and whose *Escherichia coli* strains were isolated mainly were taken into account in this study.

Isolation and identification of *E. coli* strains

The collected samples were cultured on Eosin Methylene Blue (EMB) agar (Oxoid, UK) media. On the EMB agar, *E. coli* gives purple colonies in the dark center, semi-convex 2 to 3 mm in diameter and sometimes have a metallic sheen After overnight incubation in aerobic condition (18-24 h) at 37 °C, the colonies suspected to be *E. coli* were picked and purified for *E. coli* identification according to standard biochemical tests (gram coloration, oxidase test and citrate utilization) (Riegel et al., 2005), before a confirmation with API 20E (Bio Mérieux, France) gallery.

Antibiotics resistance of isolated strains

The susceptibility of the isolated *E. coli* strains to 16 antimicrobial agents was evaluated by the disk diffusion method on Mueller-Hinton agar according to the French Society for Microbiology guidelines (CASFM, 2015).

The antimicrobial agents (BioMérieux, France) used in this study are: amoxicillin (30 µg), amoxicillin/clavulanic acid (20/10 µg), cefoxitin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), gentamicin (15 µg), netilmicin (10 µg), chloramphenicol (30 µg), nalidixic acid, (30 µg), norfloxacin (5 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg) and nitrofurantoin (300 µg). These antibiotics have been chosen because they are the most used in the treatment of urinary tract infections au Benin.

Detection of Penicillinase production

The production of penicillinase was researched on all *E. coli* strains isolated according to tube acidimetric method as describe by Catlin in 1975. Benzyl-penicillin (600 mg) was diluted in 400 µL of distilled water before adding 300 µL of aqueous phenol red solution (1 %, w/v). The pH of the precedent solution was adjusted to 8 with NaOH solution (1 M). The final 1 mL reaction volume was composed of a young *E. coli* colonies in suspension and about 150 µl of benzyl-penicillin solution. The *E. coli* ATCC 25922 strains was used as a control. The yellow color apparition within one hour indicates penicillinase production.

Phenotypic detection of Extended Spectrum β-Lactamase (ESBL) production

The production of Extended Spectrum β-Lactamase was performed on the all isolated *E. coli* strains by the double disk synergy test as described by Jarlier et al. (1988). Agar Muller Hinton medium was seeded with bacteria suspension (10^6 CFU /mL). Amoxicillin + clavulanic acid disk was deposited in the Centre of agar. About 20 mm away from two disks external, cefotaxime

and ceftazidime disks were added. After 18-24 h of incubation at 37 °C, the presence of ESBLs was indicated by a champagne cork aspect between the central and the external disks.

Genomic detection of genes coding to Extended Spectrum β-Lactamase (ESBL) production

Detection of the ESBL genes encoding bla_{TEM} and bla_{SHV} was performed with total DNA. The DNA template was prepared from freshly cultured bacterial isolates by suspending 2-3 colonies in 500 µL of molecular grade water and then vortexed to get a uniform suspension. Bacterial cells were lysed by heating at 95 °C for 10 min. Cellular debris was removed by centrifugation at 12,000 rpm for 2 min and the supernatant was directly used as DNA template to amplify bla_{TEM} and bla_{SHV} genes by Polymerase Chain Reaction (PCR). The PCR amplification was carried out in volume of 30 µl containing 5 µL of DNA, 0.5 µM of each primer (F and R), 1.5 mM $MgCl_2$, 250 µM dNTPs, 1X PCR buffer (Invitrogen) and 1U *Taq* DNA polymerase (Invitrogen). The primer sequences and the generated fragments are presented in the table 1.

Table 1: Primers used to research for genes bla_{TEM} and bla_{SHV}.

Genes	Names	Sequences	Size (base pairs)
bla_{TEM}	TEM OT-1-F	5'-TTGGGTGCACGAGTGGGTTA-3'	465
	TEM OT-2-R	5'-TAATTGTTGCCGGGAAGCTA-3'	
bla_{SHV}	SHV A	5'-CACTCAAGGATGTATTGTG-3'	885
	SHV B	5'-TTAGCGTTGCCAGTGCTCG-3'	

Amplification was carried out on a DNA thermal cycler (Multigene Labnet International, Inc.) as follows: i- for bla_{TEM} (initial denaturation 94 °C for 5 min followed by 30 cycles 94 °C for 30 s, 52 °C for 30 sec, 72 °C for 1 min and a final elongation step 10 min at 72 °C) and ii- for bla_{SHV} (initial denaturation was performed at 96 °C for 5 min, 30 cycles of 96 °C for 15 s, 50 °C for 15 sec, 72 °C for 1 min and a final elongation step 10 min at 72 °C). The amplicons were visualized by a trans-illuminator (Euromedex, Mundosheim, France) after electrophoresis at 150 V for 30 min on an agarose gel (1.2 %, w/v) containing ethidium bromide.

Data analysis

The results of antimicrobial susceptibility tests were interpreted with the standards of the French Society of Microbiology. Microsoft Excel 2013 Spreadsheet has been used for data processing and the statistical analysis was conducted using GraphPad Prism 5. The statistical analysis was run at 95 % confidence limit; two tailed test and $p < 0.05$ were considered as significant.

Results

E. coli strains isolated and penicillinase detection

About 10.32 % of the 979 urine samples analyzed were contaminated by *E. coli* strains. Our data reveals that 60 % of patients with urinary tract infection due to *E. coli* were female. Among the patients with urinary infection due *E. coli*, non-hospitalized patients represented 69.31 %. But the difference of proportion between the two groups of patients (hospitalized patients and non-hospitalized patients) was significant (p>0.05). Among the eighty *E. coli* strains (79.21%) producing penicillinase, 54 (67.5%) were isolated from non-hospitalized patients. The statistical analysis reveals that the presence of *E. coli* strains producing penicillinase is not function of the patient (in or out) origin (p >0.05).

Extended Spectrum β-Lactamase (ESBL) production by *E. coli* strains isolated

The extended-spectrum β-lactamase (ESBL) was produced by 20 % of isolated strains. Seventy percent (70 %) of these strains were isolated from non-hospitalized patients. Twenty (20) of 80 strains producer of penicillinase were ESBL. About 80 % of these ESBL were isolated from non-hospitalized patients against 20 % isolated from hospitalized patients. The data indicate that no hospitalized patients carried more ESBL than hospitalized patients (p <0.05).

Antibiotics resistance of isolated strains

Our susceptibility test data displays a variability of isolated *E. coli* strains according to tested antibiotic (Table 2). More than 50% of the *E. coli* strains were resistant to new antibiotics (amoxicillin, amoxicillin+clavulanic acid, cefuroxime, nalidixic, ciprofloxacin, ofloxacin, norfloxacin, chloramphenicol and trimethoprim sulfa). In addition, high resistance was observed with amoxicillin (96.75 %) and trimethoprim sulfa (93.37 %), while the most effective antibiotic was Imipenem with less than 1 % of resistant strains.

Among *E. coli* strains, there was a variation of the resistance depending on the antibiotic tested considering their ability to produce or not penicillinase (Table 2). However, the recorded differences of resistance proportions were not significant independently to the tested molecule (p> 0.05). It should be noted that the high amoxicillin resistance was recorded on both penicillinase producing strains (92.96 %) than non-producing (100 %). Only strains resistant to imipenem were penicillinase producers and not extended spectrum β-lactamases producers.

The strain's resistance profile is highly variable according to antibiotics when we consider the extended spectrum β-lactamase production

character (p <0.0001). Globally, the highest proportions of the strains resistant to β-lactam antibiotics (cefotaxime, amoxicillin, and ceftriaxone) and quinolones (ciprofloxacin, norfloxacin and ofloxacin) were observed with the strains of ESBL-producing *E. coli* (Table 2).

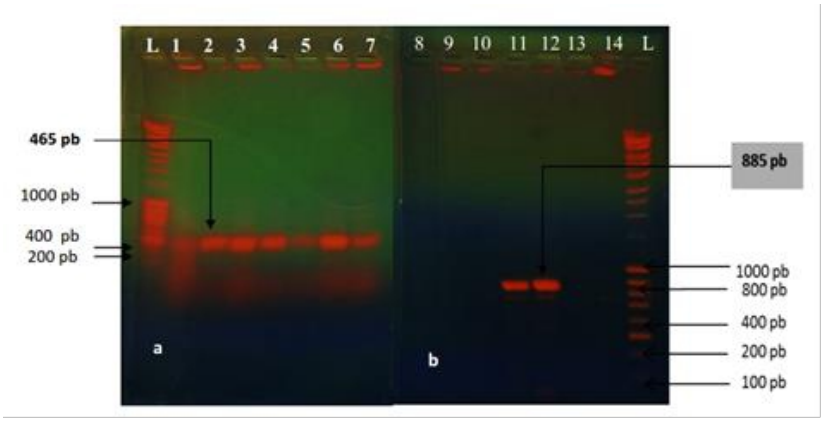
Table 2: Resistance to antibiotics profile of *Escherichia coli* strains isolated from urinary tract infections.

	Resistance rate (%)				
	EC (n=101)	EC- ESBL + (n=20)	EC- ESBL - (n=81)	EC-Péni + (n=80)	EC-Péni - (n=21)
Net	44	100	30	44	50
NA	69.75	100	64.81	60.61	70
AMX	96.75	100	93.85	92.96	100
AMC	67.33	84.21	63.23	74.32	23.07
FOX	25.58	31.25	21.15	22.41	30
CTX	32	100	12.86	30.67	35.71
CXM	53.72	100	43.55	56.52	50
CIP	51.03	94.74	40	54.28	50
OFX	67.79	100	59.18	69.09	80
GM	41.65	84.21	28.78	40.84	42.86
NOR	60.37	94.12	53.45	64.61	45.45
IPM	1	0	1.43	1.33	0
CRO	33	100	14.28	33.33	28.57
FT	15.15	26.31	13.04	14.67	21.43
SXT	93.37	100	88.09	86.05	71.43
C	66.89	66.67	61.4	58.73	77.78

Amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), cefoxitin (FOX), cefuroxime (CXM), cefotaxime (CTX), ceftriaxone (CRO), imipenem (IPM), gentamicin (GM), netilmicin (NET), chloramphenicol (C), nalidixic acid, (NA), norfloxacin (NOR), ofloxacin (OFX), ciprofloxacin (CIP), trimethoprim/ sulfamethoxazole (SXT) and nitrofurantoin (FT) , EC (*Echerichia coli* strains), EC- ESBL + (ESBLs producer *Echerichia coli* strains), EC-ESBL- (not ESBLs producer *Echerichia coli* strains), EC Péni + (Penicillinase producer *Echerichia coli* strains), EC Péni- (not penicillinase producer *Echerichia coli* strains).

Presence of gene encoding for Extended Spectrum β-Lactamase (ESBL)

The genes (bla_{TEM} and bla_{SHV}) encoding for ESBL production by *E. coli* strains isolated from urinary tract infections were found in various proportions. Thus, our data reveal that all phenotypically ESBL-producer strains had the gene bla_{TEM} (Figure 1a) while 30 % of these strains harbored the bla_{SHV} gene (Figure 1b).



Legend: - lane 1-7: bla_{TEM} positive samples;

- lane 8: negative control;
- lane 9, 10, 13 and 14: negative samples bla_{SHV};
- lane 11, 12 positive samples bla_{SHV};
- lane L: ladder.

Figure 1: Agarose gel showing polymerase chain reaction amplified product of bla_{TEM} (a) and bla_{SHV} (b) genes.

Discussion

We found a prevalence of 10.32 % for the strains of *E. coli* isolated from urine samples collected from both hospitalized patient and non-hospitalized patient referred to National and University Hospital Center Hubert Koutoukou Manga. The frequency of urinary *E. coli* infections is higher in female patients (59.41 %) than in men. The same tendency was observed in Morocco though their recorded prevalence (75 %) is higher than ours (Fatna et al., 2009). This is explained the small distance of the female urethra and the promoting sex on the growth of bacteria in the bladder urethral (Beutin et al., 1997).

The phenotypic detection of Extended Spectrum β-Lactamase (ESBL) showed that the prevalence of *E. coli* ESBL producer isolated from urinary tract infections was 20 %. Majority of those strains (70 %) were isolated from non-hospitalized patients. Our results are relatively higher than the 10 % isolated from urinary tract infections in developed country such as America (Galles et al., 2002). This different observed can be due to the fact that in developing countries, the health care system is more organized than low incoming countries such as Benin and the use of drugs such as antimicrobials under medical prescriptions. Even in developing countries such as Yaoundé in Cameroon (Gangoué-Piéboji et al., 2005) and Departmental Hospital Center of Zou-Collines in Benin (Ahoyo et al., 2007), a rate of 14.3 % and 22 % were respectively reported. Although, our results are similar to those reported in North Africa (Winokur et al., 2001). Indeed, the work of Abbassi et al. (2010) in Tunisia founded 67.7 % *E. coli* ESBL from community

patients. The *Enterobacteriaceae* strains producing ESBL were more often founded in hospitals (Winokur et al., 2001). But we nowadays mostly recorded an increase of *E. coli* ESBL strains isolated from outpatient (Younes et al., 2011; Lonchel et al., 2012).

Globally, *E. coli* strains isolated from urinary tract infections were highly (96.75 %) resistant to amoxicillin. This percentage is higher than those obtains in developed countries like France (Golstein, 2000) and United State of America (Mathai et al., 2001). In developing countries like Dakar, high resistance level to antibiotics was observed (Seck, 2005). The drug resulting from the association amoxicillin+clavulanic acid was not efficient on *E. coli* strains isolated from urinary tract infections (61.33 % of resistance). These observations remain bigger than the 40 % of resistant strains recorded in France (Golstein, 2000). The resistance rate (33 %) obtained in this study with ceftriaxone is higher than the 5 % observed in Morocco (Fatna et al., 2009) and lower than the 43.38 % previously founded in Benin (Ahoyo et al., 2007). Thirty-two percent (32 %) of strains isolated were resistance to cefotaxime. This resistance percentage is higher than the 3.7 % to 8.8 % obtained respectively in United States of America (Mathai et al., 2001) and Senegal (Lemort et al., 2006). Relatively, there are high proportions of resistance with nalidixic acid (69.75 %), ofloxacin (67.79 %) ciprofloxacin (51.03 %), trimethoprim/sulphamid (93.37 %) and chloramphenicol (66.89 %). The increase of these resistance levels in developing countries can be attributed to many factors such as the poor quality of drugs and/or inadequate dosage, long-term treatment, misuse of antibiotics by health professionals and poor sanitation (Tandé et al., 2012). The misuse and often uncontrolled antibiotic self-medication are addition selection pressure to select resistance strains in bacteria. In a previous study, Van der Starre et al. (2010) had found that all *E. coli* strains isolated from urinary tract infections patients who were treated inappropriately with ciprofloxacin, were resistant to ciprofloxacin. In addition, Wang et al. (2009) have found that the *E. coli* strains isolated from Respiratory Tract Infections samples were resistant to ciprofloxacin (71.8 %) and ofloxacin (63.8 %). In fact, many cases of multidrug-resistant bacteria have been reported in Benin (Sina et al., 2011), Ivory Coast (Akoua-Koffi et al., 2004) and in other sub-Saharan Africa countries (Akinyemi et al., 2005). All *E. coli* ESBL strains isolated in this study were resistant to amoxicillin, cefotaxime and ceftriaxone. The emergence of producing *Enterobacteria* ESBL is reported to be a major problem in the treatment of nosocomial infections (Camara et al., 2013).

Among the 16 tested antibiotics, the most efficient was imipenem and more than 99 % of *E. coli* strains isolated were sensitive. These results can be explained by the fact that this antibiotic is a β-lactam class of carbapenem. This class is known to be resistant to β-lactamases (carbapenemases)

hydrolysis (Therrien, 1998). In addition, imipenem is the most sensitive carbapenem for the detection of carbapenemases strains producers (Dahmen et al., 2012). However, some cases of resistance to imipenem had recently been described (Lee et al., 2012; Dash et al., 2013). The strains resistance to nitofurantoïn was low (15.15 %). This result is higher than 9.8 % obtained at India (Ferron, 1994) on *E. coli* strains isolated from urinary tract infections. These results confirm the effectiveness of this antibiotic in urinary infections treatment (McOsker et Fitzpatrick, 1994). The Nitrofurantoin is indicated only for the treatment of acute cystitis. The therapeutic doses have a bactericidal activity against *E. coli* isolated from urine (Hooper, 2000; Calbo et al., 2006). The resistances observe in our study may be due to the increases production of penicillinase and extended-spectrum β-lactamases.

The research of genes encoding for ESBL production on *E. coli* strains reveals that bla_{TEM} gene was carried by all strains but only 30 % harbored bla_{SHV} gene. Our results are higher than 60 % of bla_{TEM} reported in Spain (Sanders et Sanders, 1992). These results confirm those of the susceptibility testing displaying that all *E. coli* strains were resistant to penicillinase and third generation cephalosporins (cefoxitin, cefuroxime, cefotaxime and ceftriaxone) (Livermore, 1995). In other cases, the results of Ahoyo et al. (2007) displays 25 % strains carrying the gene bla_{TEM} and 56.25 % also harbored the bla_{SHV} gene. African strains possess bla_{SHV} gene (Gangoué-Piéboji et al., 2005) and the strains producing ESBL currently have genetic mutation of β-lactamases (TEM and SHV) (Rakotonirina et al., 2013; Philippon, 2013).

Conclusion

Urinary tract infections are major public health problem. The study updates the information about *E. coli* strains isolated from urinary tract infections in Benin. Our data reveals high prevalence of multidrug-resistant *E. coli* strains producing ESBL among no hospitalized patients. Thus, it is necessary to avoid prescribing antimicrobial agent treatment to patients before pathogen agent identification. The study took into account only strains isolated from urinary tract infections. It will be important to extend future studies on *E. coli* strains isolated from other samples.

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