Research Article

Detection of High Prevalence of TEM/SHV/CTX-M Genes in ESBL Producing and Multidrug Resistant *Klebsiella pneumoniae* and *Klebsiella oxytoca*

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Abstract

Background: *Klebsiella* spp. are Enterobacteriaceae frequently isolated from pathological specimens during urinary tract infections, bloodstream infection, and pus. They are becoming more and more resistant to antibiotics and challenging treatment options. β-lactamases are a great variety of enzymes capable of inducing resistance to β-lactams. The objective of this study was to identify extended-spectrum-β-lactamase (ESBL) genes in *Klebsiella* spp. strains isolated from various specimens in Lomé, Togo.

Methods: Sixty-four strains of *Klebsiella* spp. were isolated from different pathological specimens. They were then further characterized and tested against 3rd generation cephalosporin (ceftazidime, ceftriaxone, cefotaxime) and aztreonam. The detection of bla_{TEM}, bla_{SHV} and bla_{CTX-M} was performed on these strains using simplex and multiplex PCR techniques.

Results: Fifty five (85.94%) *Klebsiella pneumoniae* and 9 (14.06%) *Klebsiella oxytoca* were isolated. These strains derived from urine (n=33; 51.56%), vaginal swabs (n=21; 32.81%), pus (n=8; 12.5%) and sperm samples (n=2; 3.13%). All strains were resistant to cefepime. The resistance rate to other β-lactams was 29.69% (19/64) for piperacillin-tazobactam, 23.08% (12/52) for cefoxitin and 1.56% (1/64) for imipenem. Other inactive antibiotics were trimethoprim-sulfamethoxazole 96.72% (59/61), doxycycline 92.06% (58/63), ciprofloxacin 90.63% (58/64), nalidixic acid 80.95% (51/63), chloramphenicol 77.42% (48/62) and gentamicin 76.69% (51/64). Amikacin and fosfomycin remained the most active antibiotics with 1.56% (1/64) and 4.69% (3/64) resistance rates respectively. ESBL genes were detected in 63/64 (98.44%) strains. TEM/SHV/CTX-M was predominant 61.90% (39/63) followed by TEM/SHV 20.63% (13/63), SHV/CTX-M 11.11% (7/63), TEM/SHV 4.76% (3/63) and TEM 1.59% (1/63).

Conclusion: ESBL genes occur more by combination 96.88% (62/64) than singularly 1.59% (1/63). These strains were also very resistant to quinolones and trimethoprim-sulfamethoxazole. These findings are of high importance in a medical and scientific perspective and may motivate decision makers towards a better monitoring and control of antimicrobial resistance in Togo.

Keywords: *Klebsiella* spp; Antimicrobial resistance; ESBL; TEM; SHV; CTX-M; Togo

Introduction

β-lactamases are serine enzymes (class A, C or D of Ambler classification) or metalloenzyme (Class B of Ambler classification) that confer resistance to β-lactams by hydrolyzing their β-lactam ring [1]. The discovery and use of new classes of β-lactams have immediately been followed by the emergence of new β-lactamases. The first reported β-lactamases TEM-1/2 and SHV-1, described since 1960 from *E. coli* and *Klebsiella pneumoniae* were usually chromosomal. They are able to inactivate penicillins (amoxicillin, ampicillin, ticarcillin) hence the name penicillinases narrow spectrum [2]. From the 80s, plasmid mediated extended-spectrum-β-lactamases (ESBLs) have been described and confer resistance to penicillins, oxyimino

cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefuroxime, cefepime) and monobactams (aztreonam) [3-5]. These ESBLs are divided into several groups; the main ones are the derivated of TEM and SHV groups and CTX-M [6]. In recent years, the CTX-M ESBL spread around the world and have been described in the commensal flora and also in hospital-acquired and community infections with a tendency to supplant the first ESBL groups TEM and SHV [7-14].

Antibiotic resistance thus is a public health concern because of the difficulty to find treatment options and the fatal outcome to which they may lead [15-19]. *Klebsiella* spp. is one of the most frequently isolated Gram-negative bacteria in hospital and community infections with ESBL [20-22]. These ubiquitous bacteria, commensal of nasopharynx and gastrointestinal tract are responsible of community-acquired pneumonia, urinary tract infections, nosocomial infections, rhinoscleroma, ozena, chronic ulceration of the genital tract and colonization [23,24]. *Klebsiella pneumoniae* and *Klebsiella oxytoca* are

the most medically important species, often isolated from severe infections in hospitalized or ambulatory patients [25-29]. *Klebsiella* spp. are naturally resistant to penicillins (amoxicillin, ampicillin, piperacillin and ticarcillin) due to a low production of a class A chromosomal β-lactamase (penicillinase). The severity of *Klebsiella* spp. infections is increased when the clinical strains acquire resistance genes, such as ESBLs genes, following the misuse of antibiotics and other risk factors such as a long hospitalization associated with regular antibiotics, poor adherence, and low socioeconomic conditions [30-32]. Previous studies have shown high frequency of ESBL in hospitals among *Klebsiella* spp. with variation from one continent to another. The global multi-center surveillance study Tigecycline Evaluation and Surveillance Trial (TEST) reported from 2004 to 2009 an overall prevalence of ESBL producing *Klebsiella pneumoniae* isolated from intensive care units (ICUs), ranging from 12.8% in North America to 26.6% in Europe, 33.8% in the Middle East, 35.6% in the Asia-Pacific region, 45.5% in Latin America an up to 54.9% in Africa [33]. In addition, Hoban et al. reported a prevalence of ESBL of 8.8% and 38.9% in North America and Europe respectively with a predominance of CTX-M15 subgroup [34] on *Klebsiella pneumoniae* strains isolated from urinary tract infections.

In Togo, the prevalence of ESBL was previously reported [35]; however, their molecular characterization was not yet described. A recent study focused on the molecular characterization of ESBL producing *E. coli* (Salah et al. submitted for publication). The present study aimed to characterize ESBL genes in *Klebsiella* spp. isolated from various pathological specimens received at the National Institute of Hygiene in Lomé, Togo.

Material and methods

Collection and identification of *Klebsiella* spp. strains, antimicrobial susceptibility testing, and detection of ESBL phenotype

Klebsiella spp. strains were collected during a prospective study from May 2013 to July 2015 in the bacteriology laboratory of the National Institute of Hygiene (INH) in Lomé, Togo. This institute is a public health service specialized in biomedical analysis, epidemiological surveillance, immunization, water, and food quality control. The strains were isolated from various pathological specimens including urine, vaginal swabs, pus, and sperm samples. The standard microbiological methods were used to isolate and purify bacterial strains on Mac-Conkey or eosin methylene blue (EMB) media. The strains were identified by the API 20E identification system (Biomérieux, Marcy-Etoile, France).

Antimicrobial susceptibility testing was performed and interpreted according to the 2014 recommendations of Antibiogram Committee of the French Society of Microbiology [36]. Antibiotics were purchased from BioRad (Marnes-la-Coquette, France) and included amoxicillin +clavulanate (AMC, 20/10 μg), piperacillin-tazobactam (TZP, 75/10 μg), cefoxitin (FOX, 30 μg), ceftriaxone (CRO, 30 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), cefepime (FEP, 30 μg), aztreonam (ATM, 30 μg), imipenem (IPM, 10 μg), amikacin (AMK, 30 μg), gentamicin (GEN, 15 μg), nalidixic acid (NAL, 30 μg) ciprofloxacin (CIP, 5 μg), trimethoprim-sulfamethoxazole (SXT 1.25/23,75 μg), fosfomycin (FOF, 50 μg), doxycycline (DOX, 30 μg) and chloramphenicol (CHL, 30 μg). The double disk synergy test was used to detect ESBL production [37]. *E. coli* ATCC 25922 was used as a control strain for antimicrobial susceptibility testing.

Klebsiella pneumoniae and *Klebsiella oxytoca* strains resistant to at least one 3rd generation cephalosporin, ceftazidime, ceftriaxone, cefotaxime, or to aztreonam were collected, stored in a storage medium (tryptic soy broth TSB) at -80°C. They were then transferred to the Molecular Biology Laboratory of CERBA/LABIOGENE in Ouagadougou, Burkina Faso for ESBL genes detection.

Extraction of bacterial DNA

The bacterial chromosomal and plasmid DNA was extracted by a boiling method. Briefly, from the TSB, strains were reactivated on tryptic soy agar (TSA) for 18-24 h and then inoculated in Luria Bertani broth (LB, 2 ml). After 18-24 h culture, LB broth were centrifuged (10 000 RPM/min for 10 min) and bacterial cells were suspended in 500 μl of phosphate buffer (100 mM, pH 7) to weaken the membranes. An immersion in a boiling water bath (100°C for 15 min) releases the genetic material. The DNA is then precipitated with 250 μl of absolute alcohol, washed twice in 1000 μl of 70% alcohol (stored at -20°C), dried and re-suspended in 100 μl of sterile water.

Detection of ESBL genes

The ESBL genes, bla$_{TEM}$ (TEM-1/2), bla$_{SHV}$ (SHV-1), bla$_{CTX-M}$-G1 (CTX-M1, 3 and 15), blaCTX-M-G2 and bla$_{CTX-M}$-G9 (CTX-M9 and CTX-M14) were detected by PCR using the thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, California, USA). The sequences of the different primers provided by Applied Biosystems (California, USA) are presented in Table 1. Three PCR were performed: two multiplex for bla$_{TEM}$, bla$_{SHV}$, bla$_{CTX-M}$-G2 and bla$_{CTX-M}$-G9 [38] and one simplex for bla$_{CTX-M}$-G1 [39].

Bla genes	Sequence (5'-3')		Fragments (pb)	References
TEM	For : CATTTCCGTGTCGCCCTTATTC		800	[38]
	Rev : CGTTCATCCATAGTTGCCTGAC			
SHV	For: AGCCGCTTGAGCAAATTAAAC		713	[38]
	Rev: ATCCCGCAGATAAATCACCAC			
CTX-M-G1	For: GTTACAATGTGTGAGAAGCAG		1000	[39]
	Rev: CCGTTTCCGCTATTACAAAC			
CTX-M-G2	For: CGTTAACGGCACGATGAC		404	[38]
	Rev: CGATATCGTTGGTGGTRCCA*			
CTX-M-G9	For: TCAAGCCTGCCGATCTGGT		561	[38]
	Rev: TGATTCTCGCCGCTGAG			
*: R=A ou G				

Table 1: Sequences of targeted *bla* genes.

The PCR final volume of 50 μl, consisted of 2 μl of DNA, 25 μl of 1X AmpliTaq Gold (used as Master Mix), 5 μl Enhancer, 2 μl of each primer at different concentration (0.2 to 0.4 pmol/μl) and sterile water (qs). The amplification programs were:

Bla$_{TEM}$/SHV and bla$_{CTX-M}$-G2/G9: initial denaturation 94°C for 10 min. 30 cycles of denaturation 94°C for 40 s, annealing 60°C for 40 s and elongation 72°C for 1 min with a final elongation step at 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 elongation 72°C for 1 min with a final elongation step at 72°C for 10 min.

An Electrophoretic migration at 100 volts for 1 hour was performed on a 2% agarose gel stained with ethidium bromide to separate PCR products. A marker of 100 bp was used as reference. After migration, the various bands were observed under UV illumination and pictures recorded.

Statistical analysis

Statistical analysis were performed with the software Epi Info Version 7.1.1.14. Fisher's exact test with a significance level of 5% (p<0.05) which was used to interpret results.

Ethical consideration

This study received the INH approval for the transfer of *Klebsiella* spp. strains, to the molecular biology laboratory of CERBA/ LABIOGENE, University Ouaga I, Professor Joseph Ki-Zerbo, Burkina Faso. The institutional ethic committee of CERBA/LABIOGENE reviewed and approved the study protocol.

Results

Characteristics of *Klebsiella spp.* strains

A total of 64 strains of *Klebsiella spp.* resistant to at least one 3rd generation cephalosporin (ceftazidime, ceftriaxone, cefotaxime) or aztreonam were collected during the study period. They were isolated from urine 51.56% (33/64), vaginal swabs 32.81% (21/64), pus 12.5% (8/64) and sperm samples 3.13% (2/64). They included *Klebsiella pneumoniae* 85.94% (55/64) and *Klebsiella oxytoca* 14.06% (9/64).

All *Klebsiella pneumoniae* strains were resistant to ceftazidime, cefepime and aztreonam. The resistance to ceftriaxone and cefotaxime was 98.18% (54/55). All (100%) *Klebsiella oxytoca* strains were resistant to ceftriaxone and cefepime and 88.89% (8/9) were resistant to ceftazidime, cefotaxime and aztreonam. The resistance profile to other β-lactams and other antibiotics is presented in Figure 1. Only imipenem, amikacin and fosfomycin are very active on *Klebsiella* spp. strains with a low resistance rate (below 5%). Ciprofloxacin, doxycycline and trimethoprim-sulfamethoxazole are the most inactive antibiotics with at least a resistance rate of 90%.
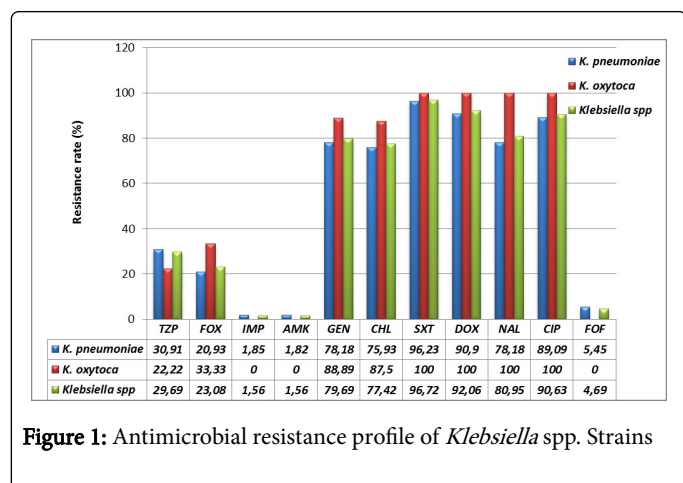

	TZP	FOX	IMP	AMK	GEN	CHL	SXT	DOX	NAL	CIP	FOF
K. pneumoniae	30,91	20,93	1,85	1,82	78,18	75,93	96,23	90,9	78,18	89,09	5,45
K. oxytoca	22,22	33,33	0	0	88,89	87,5	100	100	100	100	0
Klebsiella spp	29,69	23,08	1,56	1,56	79,69	77,42	96,72	92,06	80,95	90,63	4,69

Figure 1: Antimicrobial resistance profile of *Klebsiella* spp. Strains

ESBL genes distribution within *Klebsiella* spp. strains

With regards to the PCR results, bla$_{TEM}$, bla$_{SHV}$ and bla$_{CTX-M}$ group 1 (including bla$_{CTX-M}$1, bla$_{CTX-M}$3 and bla$_{CTX-M}$15) were found in 98.44% (63/64) *Klebsiella* spp. strains. Of these, 98.41% (62/63) strains harbored a combination of bla$_{TEM}$, bla$_{SHV}$ and bla$_{CTX-M}$ with predominance for the triple combination of TEM/SHV/CTX-M estimated at 61.90% (39/63) (Figure 2). Only one strain of *Klebsiella oxytoca* carried TEM gene. This strain was negative to the double disk test synergy for the phenotypically detection of ESBL. We did not identify any gene in one *Klebsiella pneumoniae* strain that did not show any ESBL phenotype.
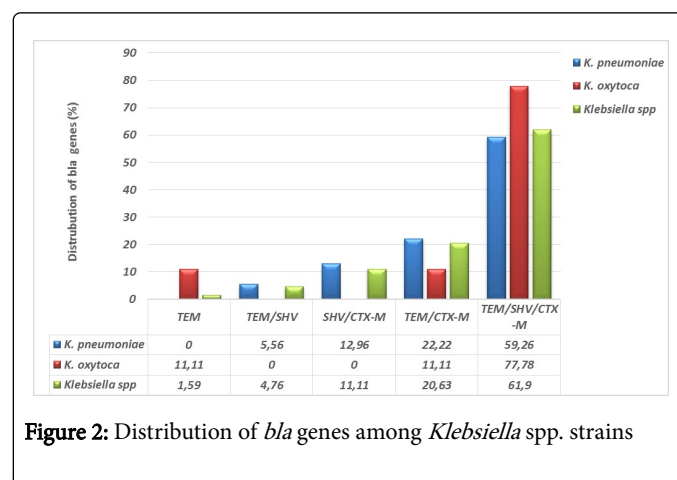

	TEM	TEM/SHV	SHV/CTX-M	TEM/CTX-M	TEM/SHV/CTX-M
K. pneumoniae	0	5,56	12,96	22,22	59,26
K. oxytoca	11,11	0	0	11,11	77,78
Klebsiella spp	1,59	4,76	11,11	20,63	61,9

Figure 2: Distribution of *bla* genes among *Klebsiella* spp. strains

Resistance to antibiotics according to the distribution of ESBL genes

Antibiotic resistance rate according to the distribution of genes in *Klebsiella pneumoniae* and *Klebsiella oxytoca* strains are summarized in Table 2. Results show that 100% *Klebsiella pneumoniae* strains (3/3) which expressed the first reported ESBL genes, TEM and SHV were resistant to ciprofloxacin and trimethoprim-sulfamethoxazole, 66.67% (2/3) were also resistant to piperacillin-tazobactam, gentamicin, chloramphenicol and nalidixic acid. All *Klebsiella pneumoniae* strains (7/7) carrying SHV and CTX-M genes were resistant to chloramphenicol and trimethoprim-sulfamethoxazole. Six (85.71%) of them were resistant to ciprofloxacin and gentamicin, 5 (71.43%) of them to doxycycline and nalidixic acid. All *Klebsiella pneumoniae* strains (12/12) carrying TEM and CTX-M genes were resistant to ciprofloxacin, doxycycline and trimethoprim-sulfamethoxazole while 90.91% (10/11) were resistant to chloramphenicol, 75% (9/12) to gentamicin and 66.67% (8/12) to nalidixic acid. Strikingly, the majority of studied strains, 32 *Klebsiella pneumoniae* and 7 *Klebsiella oxytoca* expressed simultaneously the three genes TEM, SHV and CTX-M. Among *Klebsiella pneumoniae* strains, 96.88% (31/32) were resistant to doxycycline, 96.67% (29/30) to trimethoprim-sulfamethoxazole, 87.5% (28/32) to nalidixic acid and ciprofloxacin, 81.25% (26/32) to gentamicin and 65.63% (21/32) to chloramphenicol. All *Klebsiella oxytoca* were resistant to gentamicin, nalidixic acid, ciprofloxacin, doxycycline and trimethoprim-sulfamethoxazole, while 83.33% (5/6) were resistant to chloramphenicol. Imipenem, amikacin and fosfomycin remained the most active antibiotics according to the ESBL genes distribution whereas one third (1/3) of strains expressing TEM and SHV genes are resistant to imipenem. The resistance rate to piperacillin-tazobactam ranged from 28.13% for the triple

combination TEM/SHV/CTX-M to 66.67% for the double combination TEM/SHV in *Klebsiella* pneumoniae. Similarly, the resistance rate to cefoxitin ranged from 17.39% for the triple combination TEM/SHV/CTX-M to 33.33% for the double combination TEM/SHV. We did not find statistically significant differences for these two antibiotics (p=0.472 and p=0.426>0.05) between the groups expressing three ESBL genes (TEM/SHV/CTX-M) or two ESBL genes (TEM/SHV, SHV/CTX-M, TEM/CTX-M).

ATB	TEM	TEM/SHV	SHV/CTX-M	TEM/CTX-M		TEM/SHV/CTX-M	
	Ko# (n/N)	Kp (%, n/N)	Kp (%, n/N)	Kp (%,n/N)	Ko# (n/N)	Kp (%,n/N)	Ko (%,n/N)
TZP	0/1	66.67 2/3	14.29 1/7	41.67 5/12	0/1	28.13 9/32	28.57 2/7
FOX	1/1	33.33 1/3	14.29 1/7	22.22 2/9	0/1	17.39 4/23	28.57 2/7
IPM	0/1	33.33 1/3	0 0/7	0 0/12	0/1	0 0/32	0 0/7
AMK	0/1	0 0/3	0 0/7	0 0/12	0/1	3.13 1/32	0 0/7
GEN	0/1	66.67 2/3	85.71 6/7	75 9/12	1/1	81.25 26/32	100 7/7
CHL	1/1	66.67 2/3	100 7/7	90.91 10/11	1/1	65.63 21/32	83.33 5/6
FOF	0/1	0 0/3	0 0/7	8.33 1/12	0/1	3.13 1/32	0 0/7
NAL	1/1	66.67 2/3	71.4 5/7	66.67 8/12	1/1	87.5 28/32	100 6/6
CIP	1/1	100 3/3	85.71 6/7	100 12/12	1/1	87.5 28/32	100 7/7
DOX	1/1	66.67 2/3	71.43 5/7	100 12/12	1/1	96.88 31/32	100 6/6
SXT	1/1	100 3/3	100 7/7	100 12/12	1/1	96.67 29/30	100 6/6

Table 2: Antimicrobial resistance profile of *Klebsiella* spp. strains according to the distribution of *bla* genes. ATB: Antibiotic, Kp: *Klebsiella* pneumoniae, Ko: *Klebsiella* oxytoca, TZP: piperacillin-tazobactam, FOX; cefoxitin, IPM: imipenem, AMK: amikacin, GEN: gentamicin, CHL: chloramphenicol, FOF: fosfomycin, NAL: nalidixic acid, CIP: ciprofloxacine, DOX: doxycycline, SXT: trimetroprim-sulfamethoxazole; #The proportions was not indicated for a number equal to one strain.

Distribution of the combination TEM/SHV/CTX-M according to the biological specimen

The combination of TEM, SHV and CTX-M genes was carried by 57.14% (12/21), 45.45% (15/33) and 37.5% (3/8) of *Klebsiella* spp. strains isolated from vaginal swabs, urine and pus respectively. *Klebsiella pneumoniae* from the two sperm samples expressed simultaneously these three genes, TEM, SHV and CTX-M.

Discussion

Nowadays, resistance to antibiotics is a real public health concern worldwide. It has been clearly demonstrated that the production of β-lactamases is the most important mechanism of antibiotic resistance in Gram negative bacteria. The description of first β-lactamases TEM-1/2, SHV-1 and their derivatives was followed by the characterization of new ones dominated by CTX-M group which is now, the most widespread. This study was carried out to characterize ESBL genes in *Klebsiella* spp. strains resistant to at least one 3rd generation cephalosporins or aztreonam, isolated from different specimen received at INH Lomé, Togo. It has been reported that *Klebsiella* spp. present a high medical interest among other Enterobacteriaceae as expressing ESBL resistance mechanism and are frequently isolated in hospital and community infections.

According to our expectation, all *Klebsiella* spp. strains were highly resistant (over 98%) to 3rd generation cephalosporins (ceftazidime, ceftriaxone, cefotaxime), 4th generation (cefepime) and aztreonam. However, their resistance rate is higher than those found in previous studies conducted in North America. Indeed, through the international monitoring program for antibiotic resistance SENTRY involving four geographic regions, Asia Pacific, Europe, Latin America, North

America (United States of America and Canada), Sader et al. found a resistance rate of 80.3% for ceftazidime, 62.1% for ceftriaxone, 83.9% for aztreonam and 10.6% for cefepime [40]. Nevertheless, our results are in line with those of Irenge et al., showing 100% prevalence of resistance for ceftriaxone and ceftazidime on ESBL producing *Klebsiella* spp. strains isolated from urine in Congo [41]. For piperacillin-tazobactam and cefoxitin two β-lactams subject of comparative study on their efficiency on ESBL producing Enterobacteriaceae [42-44], we found a prevalence of resistance at 30.91% and 20.93% for *Klebsiella pneumoniae* and 22.22%, 33.33% for *Klebsiella oxytoca* respectively. These prevalence rates seem low compared to those reported in North America, 47.4% for piperacillin-tazobactam, and 44.2% for cefoxitin [40]. The resistance to cefoxitin can be induced by a production of cephalosporinase AmpC, a class A enzyme that inactivates other 3rd generation cephalosporins. AmpC decreases the intracellular concentration of the antibiotic by the efflux pump mechanism and mediates a loss of outer membrane porins [45]. Fortunately, *Klebsiella* spp. isolated in this study have remained relatively sensitive to imipenem, amikacin and fosfomycin which are among other, recommended antibiotics in case of infection by ESBL producing bacteria [46-48]. The prevalence of resistance to these antibiotics ranged from 0 to 5% as in a series of ESBL producing *Klebsiella* spp. strains isolated in Tunisia, where the prevalence of resistance to imipenem was zero [49]. However, the resistance rates for amikacin and fosfomycin were high, 10% and 17.5%, respectively [49]. Higher resistance rates were found in non-intensive care units in the United States of America and Europe for imipenem (22% and 6.9%) and amikacin (18.9% and 19.1%), respectively [50].

In addition, *Klebsiella* spp. isolated in the present study showed a strong resistance rate to quinolones (nalidixic acid and ciprofloxacin) ranging from 78.18% (43/55), 89.09% (49/55) for *Klebsiella pneumoniae* to 100% for *Klebsiella* oxytoca. Similar results were found in Congo, in Tunisia and in Bolivia. In Congo, 83.3% of *Klebsiella* spp. strains were resistant to ciprofloxacin [41], in Tunisia, 80% (32/40) of *Klebsiella* spp. strains were resistant to nalidixic acid and 67.5% (27/40) to ciprofloxacin [49]. Results in Bolivia showed that 100% (6/6) of *Klebsiella* spp. strains were resistant to ciprofloxacin and nalidixic acid [51].

The analysis of the PCR product showed the simultaneous presence of the three genes, TEM, SHV and CTX-M in 61.90% of the studied strains, 59.26% (32/54) in *Klebsiella* pneumoniae, 77.78% (7/9) in *Klebsiella* oxytoca. TEM was detected in 1.59% (1/9) *Klebsiella oxytoca* strain, SHV gene alone was not found in this study. In Burkina Faso, on 28 ESBL producing *Klebsiella pneumoniae* screened for the detection of *bla* genes, 14.28% expressed TEM, 10.71% SHV, 25% CTX-M, 10.71% TEM and SHV and 3.57% of strains exhibited the triple combination of TEM, SHV and CTX-M [52]. In another study conducted in Burkina Faso, CTX-M15 was found in 17/17 *Klebsiella* spp. isolated from urine in a pediatric hospital in Ouagadougou [53] suggesting that CTX-M15 was present in West Africa. Our results are consistent with those found by Alibi et al. in Tunisia, on a large series of 118 ESBL producing *Klebsiella* spp. where the triple combination TEM/SHV/CTX-M represented 44.91% (53/118), and the double combinations were SHV/CTX-M represented 28.81% (34/118) of cases, TEM/CTX-M represented 4.23% (5/118) and TEM/SHV represented 3.39% (4/118) of cases [54]. The presence of TEM, SHV and CTX-M genes alone in the study of Alibi et al. in Tunisia was high compared to our results with 3.39% (4/118), 4.23% (5/118) and 11% (13/118) respectively [54]. In Mozambique, on 19 ESBL producing *Klebsiella pneumoniae* isolated from urine and blood culture, Pons et

al., found 15.79% (3/19) expressed only CTX-M15, 63.16% (12/19) TEM/CTX-M15 and 21.05% (4/19) the triple combination TEM/SHV/CTX-M15 [55]. In Tanzania, on 92 ESBL producing *Klebsiella* pneumoniae, Mshana et al. found 53.26% (49/92) carried TEM/CTX-M15, 11.96% (11/92) SHV/CTX-M15 and 10.87% (10/92) CTX-M15 alone [56]. Similarly, in Brazil, Jaskulski et al. found that 16.67% (2/12) of ESBL producing *Klebsiella pneumoniae* carried TEM, CTX-M, TEM/SHV genes, while 8.33% (8/12) carried SHV alone. The double TEM/CTX-M and triple TEM/SHV/CTX-M combinations were 25% (3/12) and 16.67% (2/12) respectively [57]. It is observed that the expression of bla$_{CTX-M}$ is currently more prevalent and as we report for the first time in Togo, more frequent in a triple combination with bla$_{TEM}$ and bla$_{SHV}$. The explanation of this triple combination should be more investigated.

The antimicrobial susceptibility profile of ESBL producing *Klebsiella* spp. strains shows that the different ESBL genes do not significantly influence the activity of the antibiotics. As expected, resistance to imipenem, amikacin and fosfomycin was low. On the contrary, the resistance rates to trimethoprim-sulfamethoxazole, ciprofloxacin, nalidixic acid, chloramphenicol and gentamicin were very high ranging from 65.63% for chloramphenicol in the triple combination TEM/SHV/CTX-M to 100% for most of them in all double combinations. These antimicrobial resistances associated with ESBL genes are in line with our expectation and are consistent with the results found in previous studies [1–6]. Indeed, it has been described that ESBL genes and some antibiotic resistance genes are often found on the same mobile genetic elements and are thereby transmitted together by horizontal transfer [1–6]. In Tanzania, on ESBL producing *Klebsiella pneumoniae* causing sepsis in neonates, Mshana et al. showed that bla$_{CTX-M}$15 was localized on a plasmid of 25 to 485KB and that ST14 and ST48 clones carried also resistance genes to gentamicin and trimethoprim-sulfamethoxazole [56]. More recently, in a study in Central African Republic, Rafai et al. showed that all ESBL producing *Klebsiella pneumoniae* isolated from surgical wounds, expressed bla$_{CTX-M}$15 and aac(6 H)-Ib-cr [58]. The last one has been described to be associated with a resistance to ciprofloxacin, norfloxacin and aminoglycosides [59]. This is probably due to the same mechanisms that might explain the results we observed.

In this study, the combination of TEM, SHV and CTX-M genes was found on strains isolated mostly from urine and sperm. This result is also consistent with previous findings in Tunisia where 86.44% of ESBL producing *Klebsiella* spp. were isolated from urine and 44.91% (53/118) of all studied strains expressed the triple combination TEM/SHV/CTX-M [54]. The observation of this combination based on the pathological sample needs to be analyzed more thoroughly.

Conclusion

In this study, we observed that the majority of *Klebsiella* spp. resistant to 3rd generation cephalosporins were producing ESBL. The major groups of ESBL genes, TEM, SHV and CTX-M were present. We report for the first time, that the triple combination of TEM, SHV and CTX-M genes was predominant. Stressfully, all ESBL producing *Klebsiella oxytoca* were resistant to quinolones (nalidixic acid and ciprofloxacin), doxycycline and trimethoprim-sulfamethoxazole and also to a lesser extent to gentamicin and chloramphenicol. The same trend is observed for ESBL producing *Klebsiella pneumoniae*. Antibiotics that remained active, including fosfomycin, amikacin and imipenem are unfortunately a little affordable to patients in limited resources countries. It is therefore important to these countries to

strengthen public health policies in order to prevent, monitor and control antimicrobial resistance. In addition, other studies have been done for screening of medicinal plants with potential antimicrobial activities. In a public health point of view, it is now important for political, economic and medical sectors to work together towards the selection of those plants which could be active against clinical resistant strains.

Author's Contribution

SD, FS, SK and JS designed the study, FS, SS, AO, AS, AK, 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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