

Full Length Research Paper

First detection of *bla* TEM, SHV and CTX-M among Gram negative bacilli exhibiting extended spectrum β -lactamase phenotype isolated at University Hospital Center, Yalgado Ouedraogo, Ouagadougou, Burkina Faso

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Resistance to a wide variety of common antimicrobials is observed among clinical strains designed as extended spectrum β -lactamase (ESBL) producers. They produce enzymatic protein which inactivates efficiently oxyimino cephalosporin and constitutes a serious global health concern that has complicated treatment strategies. Many studies report high prevalence of ESBL producers among Gram negative bacilli. The aim of this work was to identify the presence of TEM, SHV and CTX-M families in these strains which were initially screened by phenotypic method. Gram negative bacilli resisting third or four generation cephalosporin were isolated during anti-biogram study. The presence of ESBL positivity was detected using the double disk synergy test. Minimal inhibitory concentrations (MICs) of ceftriaxon for any strain were determined using E-test manufacturing protocol. Polymerase chain reaction (PCR) analysis for β -lactamase (*bla*) genes of TEM, SHV and CTX-M family was carried out using designed primers in 171 ESBL isolates producers. Among 259 Gram negative bacilli collected, 171 (66, 02%) exhibited ESBL producers' profile. Urine samples constitute major source of ESBL producers. The highest prevalence of ESBL was observed in *Escherichia coli* (75, 50%). Among ESBL isolates producers, gene prevalence of *bla*-CTX-M (65, 49%) was highest, followed by *bla*-TEM (25, 73%) and *bla*-SHV (18, 71%) in the present study. The frequency of ESBL producing strains among clinical isolates has been steadily increased. Continual drug resistance surveillance and molecular characteristics of ESBL isolates are necessary to guide the appropriate and judicious antibiotic use.

Key words: Extended spectrum β -lactamase (ESBL), double disk synergy test, *bla* TEM, *bla* SHV, *bla* CTX-M, PCR.

INTRODUCTION

Loss of antibacterial proprieties is now established for many antibiotics substances, particularly those of β -lactam

class with regard to therapy failure observed in clinical offices. For Gram negative bacilli, the most important

mechanism of resistance is based on the production of β -lactamases, enzymatic proteins, which hydrolyze β -lactam ring (Webb, 1984) described several decades later (Abraham and Chain, 1940). In Gram negative bacilli, β -lactamases are produced and stocked in periplasmic compartment (Frère, 1998). According to catalytic mechanism, two families of β -lactamases are distinguished (Frère, 1995; Matagne et al, 1999). The serine β -lactamases carrying an amino-acid residue-seryl in the catalytic site and metallo- β -lactamases are needed for their catalytic activity in the presence of metallic ion in active site. According to their molecular structure, β -lactamases are organized in class A, C, D and B enzymes (Ambler, 1975, 1978, 1980). Serine β -lactamases consist of class A, C and D enzymes while metallo- β -lactamases consist of class B enzymes. Our study concerns class A enzymes which consist of the following groups: Temoneira (TEM), sulfidrhyl-variable (SHV), cefotaximase (CTX-M), pseudomonas extended resistance (PER). Those chromosomal or plasmid-mediated enzymes are either penicillinases (TEM-1/2 and SHV-1) hydrolyzing penicillins, first and second generation cephalosporins or extended-spectrum- β -lactamases (ESBL). Extended-spectrum- β -lactamases (TEM-3, SHV-2 and CTX-M), a form of β -lactamases, hydrolyzing third generation of cephalosporins (like oxyiminocephalosporins); detected since 1983 (Knothe et al., 1983) are now worldwide reported. ESBL, detected at first in *Klebsiella pneumoniae* and *Escherichia coli* (Medeiros, 1984; Bradford, 2001), is now fully spread in a number of enterobacterial species and other kind of bacterial isolates, often responsible for nosocomial epidemic outbreak. Therapy failure due to these fastidious strains makes clinicians to prescribe more and more carbapenem antibiotics for fighting resistant strains resisting all class of antibiotic (Queenan and Bush, 2007; Poirel et al., 2007). Therefore, a research on how to control ESBL epidemiology is a noble undertaking. Molecular studies leading in many countries have allowed getting more information on genetic matrix and molecular types. In Turkey, a survey of *Klebsiella* spp. from intensive care units from eight hospitals showed that 58% of 193 isolates harbored ESBLs (Gunseren et al., 1999). In Chicago, infections with TEM-type ESBLs particularly TEM-10, TEM-12, and TEM-26 were reported since 1996 (Schiappa et al., 1996). Boyd et al. (2004) in their study, described CTX-M-type ESBLs in United States and Canada. CTX-M-12 was first detected in Kenya by Kartali et al. (2002) (Kartali et al., 2002). Characterization of ESBLs from South Africa revealed the types of TEM and SHV (especially SHV-2 and SHV-5) (Hanson et al., 2001). In Burkina Faso, *bla* SHV and *bla* TEM were first reported in 2004 in community medical center (Zeba et

al., 2004). Microbial drug resistances based on ESBL were reported (Karou et al., 2009), but there is no study on determining the different types of gene encoding. Therefore, this study aimed to contribute to clear bacterial resistance epidemiology by establishing genetic profile of Gram negative bacilli that resist oxyiminocephalosporins and exhibit extended-spectrum β -lactamase at University Hospital Center Yalgado, Ouedraogo.

MATERIALS AND METHODS

Clinical specimens and strains

Bacterial strains collected during a prospective study from July 2009 to March 2012 were Gram negative bacilli that resist third generation of cephalosporin. They were isolated and diagnosis analysis of biological specimens was done. Different clinical specimens such as blood, urine, pus, ascitic fluid, peritoneal fluid, stool and rachis fluid samples were collected from different hospitalized patients. Samples were taken from infected patients who presented infectious evident symptoms like fever and purulent urine. Isolates were identified using conventional method (Kelly et al., 1985). Identification of isolates was achieved using API 20E test trips (BioMerieux S.A., Marcy l'Etoile, France).

Antibiotic susceptibility testing and ESBL detection

Antibiotic susceptibility was tested by disk diffusion method (Bauer, 1966), with antibiotic disks used to test Gram negative bacilli particularly monobactam: Aztreonam (30 μ g), third generation cephalosporin like cefotaxim (30 μ g), ceftriaxon (30 μ g), ceftazidim (30 μ g), and fourth generation cephalosporins: cefepim (30 μ g). Antibiotics were tested on Petri plates containing Muller Hinton agar. Measurements of inhibition area determine the clinical categories (CA-SFM, 2010; 2011; 2012). Isolates that were resistant to at least one of the antibiotics in clinical test, using NCCLS methods (NCCLS, 2000) were collected, purified and conserved at -80°C for further analysis. In order to screen ESBL phenotypical profile, isolates were submitted for synergy test (Jarlier et al., 1988) between third generation of cephalosporins disks (cefotaxime or ceftazidime) and amoxicillin plus clavulanic acid.

In addition, MICs of ceftriaxone, antibiotic frequently used in clinical routine in our sanitary centers was performed as recommended by guide E-test AB BIODISK.

Molecular characterization of ESBL producing-isolates

All isolates which were positive for synergy test were screened in order to detect β -lactamase encoding genes for *bla* TEM, SHV and CTX-M families by previously described PCR protocols (Olivier et al., 2002; Pagani et al., 2003). DNA template was prepared from purified bacteria grown overnight at 37°C on Muller Hinton agar plates. Crude DNA extracts were obtained by suspending a colony in 100 μ l of purified water and boiling at 95°C for 10 mn (Munday et al., 2004). Mixed PCR for one reaction has the following components: 5 μ l of Green buffer 5X; 1 μ l of each Primer 10 μ M; 0.65 μ l of dNTPs 10 mM; 0.12 μ l of Gotaq 0.5 U/ μ l and 15.25 μ l of purified water. Reaction volume is completed to 25 μ l with 2 μ l of

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Table 1. Primers used for amplification of *BlaTEM*, *SHV* and *CTXM* genes.

Gene	Primer	Sequence (5'-3')	Amplicons size (bp)	References
blaTEM	TEM-F	ATAAAATTCTTGAAGACGAAA	1080	Olivier et al. (2002)
	TEM-R	GACAGTTACCAATGCTTAATCA		
blaSHV	SHV-F	ATGCGTTATATTCGCTGTG	1030	Olivier et al. (2002)
	SHV-R	TTAGCGTTGCCAGTGCTC		
BlaCTX-M	CTX-F	GTTACAATGTGTGAGAAGCAG	1041	Pagani et al. (2003)
	CTX-R	CCGTTTCCGCTATTACAAAC		

**Figure 1.** Detection of ESBL *Escherichia coli* producer on plate. a) Pure colonies of *Escherichia coli*. b) Antibigram of *Escherichia coli*. c) Synergy test showing ESBL *E. coli* producer on Petri plate.

DNA template. Primers supplied by Promega according to each β -lactamase gene type are in Table 1. PCR was carried out under the following conditions on SensoQest Labcycler, GmbH, Germany: initial denaturation step at 96°C for 5 min, followed by 35 cycles consisting of denaturation at 96°C for 1 min, annealing at 58°C, 60°C and 50°C for TEM, SHV and CTX-M at 1 min, primer extension at 72°C for 1 min and final extension for 10 min.

Migration of PCR products watched by Green buffer (Eugentec) during 30 min at 80 V was performed on agar gel of 1%, prepared with BET at 0.25% as final concentration.

RESULTS

Bacterial strains and antibiotic susceptibility testing

During this study, 259 Gram negatives bacilli which resist at least one- third or fourth generation of cephalosporins or monobactam were collected from different samples (Figure 2). For all the strains collected, diameters of inhibiting area around oxy-iminocephalosporins disc were less than 16 mm. Resisting bacterial species isolated were about quantitative importance: 132 *Escherichia coli*, 43 *Klebsiella pneumoniae*, 34 *Pseudomonas aeruginosa*, 24 *Enterobacter* sp., 11 *Citrobacter* spp 7 *Accinetobacter baumannii*, 6 *Proteus mirabilis*, and 1 *Salmonella typhi*. Extended-spectrum β -lactamase phenotype screening showed that 171 strains (66, 02%) were positive for

synergy test, suggesting that they were extended-spectrum- β -lactamases-producers (Figure 1). The highest proportion (62.57%) of their strains was isolated from urine samples.

The MICs of ceftriaxone determined by E-test were less than 50, 100 and 256 μ g/ml for 81, 57, 55.26 and 39.74% isolates (Figure 2). MICs of ceftriaxone for 73.33% of *E. coli* (22/30) and 80% of *K. pneumoniae* were less than 64 μ g/ml. Among the positive isolates for synergy test, bacterial species were spread as follows: 99 *E. coli* (that is, 75% of *E. coli*), 28 *K. pneumoniae* (that is, 65.11% of *K. pneumoniae*), 19 *P. aeruginosa* (55.88%) 15 *enterobacter* sp. (62,50%), 4 *citrobacter* sp(36,36%), 2 *acinetobacter baumannii*, 3 *Proteus mirabilis*, 1 *Salmonella typhi*. Except *P. aeruginosa* and *Accinetobacter baumannii*, the rest isolates belong to enterobacterial family. The study focused on bacterial resistance and no process determined community or hospital origin of isolates.

Polymerase chain reaction: Amplification of bla genes

In the electrophoresis of PCR products realized with DNA ladder, positive and negative control showed bands as previously around 875 bp for *bla* SHV and 1000 bp for *bla* TEM CTX-M (Figure 3). Molecular characterization of our

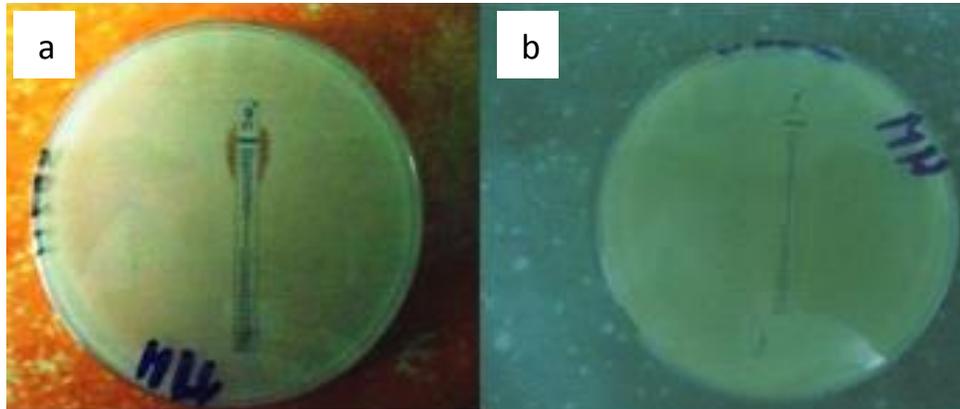


Figure 2. a. Sensitive pattern of *Escherichia coli* Ceftriaxon, CMI = 96 µg/ml. b) Sensitive pattern of *Klebsiella pneumoniae* Ceftriaxon, CMI = 256 µg/ml.

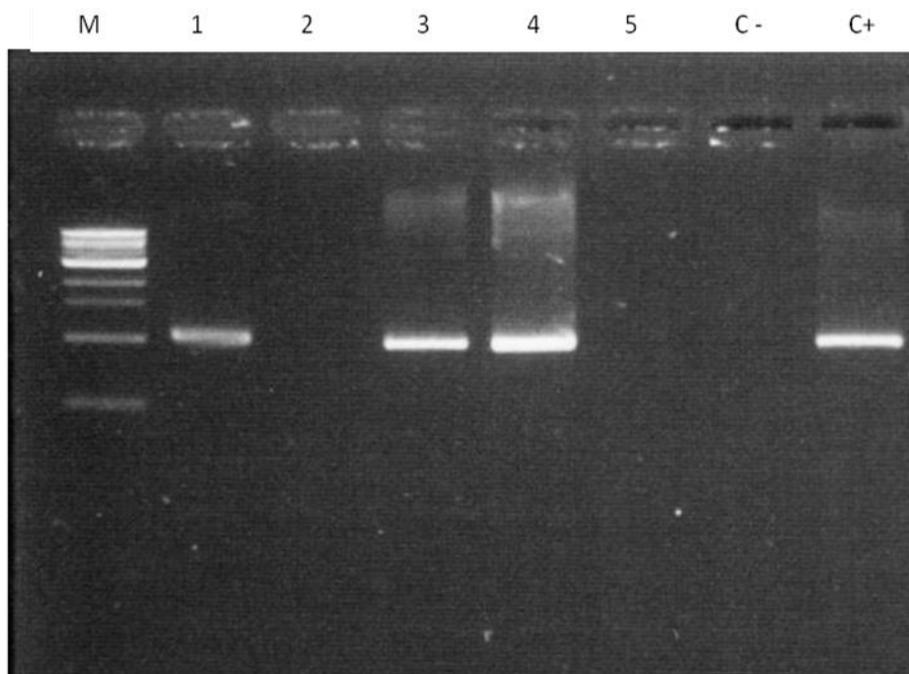


Figure 3. Agarose gel of CTX-M PCR products. M, Marker of molecular weight (GeneRuler 100 bp DNA ladder). Number 1-5 correspond to samples: 1 = 45H, 2 = 219P, 3 = 310P, 4 = 315 Uro, 5 = 225H. C+ = Positive control and C- = negative control.

isolates (exhibiting extended β -spectrum β -lactamase phenotype) showed that 112 strains (65.49%) were positive for CTX-M screening; 44 stains (25.73%) for TEM screening and 32 strains (18.71%) for SHV. In all the bacterial species, some associations were found between the three types of *bla* genes. Eleven (11) isolates carried *bla* TEM, SHV, CTX-M at the same time. Finally, 134 isolates (78, 36%) were positive for β -lactamase gene screening and 37 strains did not carry any of their β -lactamase genes. Repartition of β -lactamase gene type among bacterial species was established.

Among 99 *E. coli* investigated, 11.11% carried single *bla* TEM; 7.07% carried *bla* SHV and 48.48% carried single *bla* CTX-M. 5.05% harbored *bla* TEM linked to *bla* SHV; 5.05% carried *bla* TEM linked to *bla* CTX-M; 5.05% carried *bla* SHV with *bla* CTX-M and 6.06% carried both *bla* TEM, *bla* SHV and *bla* CTX-M. 70.70% of *E. coli* carried at least *bla* CTX-M or CTX associated with other *bla* genes. Among 28 *K. pneumoniae* screened for *bla* genes, 14.28% carried single *bla* TEM; 10.71% carried *bla* SHV; 25% carried single *bla* CTX-M and 10.71% carried *bla* TEM associated with *bla* SHV. 3.57% strains

Table 2. Spread of *bla*-genes among bacterial species.

Bacterial species	Number	TEM	SHV	CTX-M	TEM-SHV-CTXM
<i>Escherichia coli</i>	99	25	20	70	6
<i>Klebsiella pneumoniae</i>	28	9	4	12	1
<i>Enterobacter</i> sp.	15	3	5	10	2
<i>Pseudomonas aeruginosa</i>	19	5	2	12	2
<i>Citrobacter</i> sp.	4	1	1	3	0
<i>Acinetobacter baumannii</i>	2	1	0	2	0
<i>Proteus mirabilis</i>	3	0	0	2	0
<i>Salmonella typhi</i>	1	0	0	1	0
Total	171	44	32	112	11

carried *bla* TEM, *bla* SHV and *bla* CTX-M. 42.85% carried at least *bla* CTX-M.

From 15 *Enterobacter* sp. screened, 13.33% of strains carried *bla* SHV, 20% carried *bla* TEM associated with other genes, 20% carried *bla* SHV in association with *bla* CTX-M. 66.66% carried either *bla* CTX-M or CTX-M linked to other genes. Among 19 strains of *P. aeruginosa*, 10.52% harbored *bla* TEM, 31.57% carried *bla* CTX-M. 21.05% of strains carried *bla* TEM associated with *bla* CTX-M, while 5.26% carried *bla* CTX-M associated with *bla* SHV and 10.52% of strains harbored *bla* TEM, *bla* CTX-M and *bla* SHV. At least, *bla* CTX-M was found in 52.63% of *P. aeruginosa* screened. The distribution of *bla* genes among the bacterial species is shown in Table 2.

Our study has noted that patients of all age were affected by *bla* gene and ESBL (Table 3). From the results, 11.36, 9.37% and 5.35% of *bla* TEM, SHV and CTX-M, respectively were found on patients of less than 1 year old. For children of less than five years old, 22, 28.12 and 14.28% of *bla* TEM, SHV and CTX-M were found. According to sexual repartition, 40,90%, 50% and 44,64% of *bla* TEM, SHV and CTX-M were found in female group, while 65.90, 50 and 55.35% of *bla* TEM, SHV and CTX-M were found in male group.

DISCUSSION

The most important part of our strains was resistant to cefotaxim, ceftriaxon and aztreonam. The rate of resistance to those antibiotics was globally greater than 50% of isolates. Bacteriological and susceptibility study at Tunis University Hospital reported rates of resistance to β -lactam by enterobacterial at 57.90% (Larabi et al., 2003). PCR results have shown that most proportion of strains carried *bla* CTX-M which are ESBLs (Bonnet, 2004). There is high possibility that *bla* TEM and *bla* SHV are found in ESBL because producing stains were resistant to oxi-iminocephalosporin (Livermore, 2008). ESBLs have become a widespread serious problem. These enzymes are becoming increasingly expressed by many strains of pathogenic bacteria with a potential for dissemination. The presence of ESBL compromises the

activity of wide-spectrum antibiotics creating major therapeutic difficulties with a significant impact on the outcome of patients. The continuous emergence of ESBLs presents diagnostic challenges to the clinical microbiology laboratories.

In this study, urine represent samples indexed as extended-spectrum β -lactamase isolates producing source. Similar observations are reported in others studies (Shanthi and Sekar, 2010; Iraj and Nilufar, 2011; Saba et al., 2012). Invasive treatment using catheter at hospital centers often explains high prevalence of urinary infections (Cantón et al., 2008). In addition, in community, people practice auto medication with antibiotics that can select ESBLs isolates which may be carried at hospital notably in emergencies units and disseminated in other clinical offices (Mbutiwi et al., 2013). In this study, it was found that *E.coli* is frequently identified as ESBL producing isolate (Shanthi and Sekar, 2010; Abhilash et al., 2010; Umadevi et al., 2011). It is bacterial species, most adapted to and represented in digestive stratus and consequently receives plasmid-mediated gene from other bacteria.

Phenotypic tests for ESBL detection only confirm if an ESBL is produced but cannot detect the ESBL subtype. Some ESBLs may fail to reach a level to be detected by disk diffusion tests but result in treatment failure in the infected patient. Nuesch-Inderbinen and Hachler (1996) reported that molecular methods appear sensitive, are expensive, time consuming and require specialized equipment and expertise. However, definitive identification is possible only by molecular detection methods.

There are so many types of β -lactamase like TEM, SHV, CTX, OXA, AmpC, etc, but majority of the ESBLs are derivatives of TEM or SHV or CTX-M enzymes and these enzymes are most often found in *E. coli* and *K. pneumoniae* (Paterson and Bonomo, 2005). Keeping in view this fact, the current study investigated Gram negative bacilli among which *E. coli* and *K. pneumoniae* were most represented. The study aimed to look for the presence of *TEM*, *SHV* or *CTX-M* gene.

From the results of the study, the three families of β -lactamase genes screened were found with high prevalence of CTX-M, followed by TEM and SHV. PCR

Table 3. Spread (number) of *bla* genes by sex/age on infected patients.

Age (years)	<1		1-		5-14		15 -25		≥26	
	Sex		Sex		Sex		Sex		Sex	
<i>Bla</i>	M	F	M	F	M	F	M	F	M	F
TEM	03	02	03	02	02	03	04	02	17	09
SHV	01	02	02	04	03	00	02	03	08	07
CTX	03	03	04	06	03	02	05	08	47	31

screening of 99 *E. coli* has shown that 25.25% harbored TEM gene, 20.20% harbored SHV gene and 70.70% harbored CTX-M gene. Of 28 *K. pneumoniae*, 14.28% harbored TEM gene, 32.14% harbored SHV gene and 42.85% harbored CTX-M gene. 20% of *Enterobacter* sp. harbored TEM gene, 33.33% harbored SHV gene and 66.66% harbored CTX-M gene. 26.31% *Pseudomonas aeruginosa* harbored TEM gene, 10.52% harbored SHV gene and 63.15% harbored CTX-M gene. Livermore et al., stated in separate studies that the CTX-M gene is the most prevalent ESBL-encoding gene worldwide and is replacing TEM and SHV types as the predominant ESBL in many European and Asian countries (Livermore et al., 2007; Bali et al., 2010). The high prevalence of CTX-M gene in our study is in concordance with the study of other authors (Vaida et al., 2010) who reported CTX-M-encoding genes in the majority of *E. coli* (96%) and *K. pneumoniae* (71%) isolates showing ESBL phenotype. In our study, major part of the isolates carried more than one type of gene. Among *E. coli*, *bla* TEM, *bla* SHV and *bla* CTX-M matched with each other in the same proportion (5.05%). Among *Klebsiella pneumoniae*, *bla* TEM associated with *bla* SHV in 10, 71% of strains. 6, 43% isolates harbored all the three β lactamase genes. CTX-M is frequently associated with other β lactamase genes. According to Goyal et al., majority of strains (57.3%) harbored two or more ESBL genes. Bali et al. have observed in their study that 19.2% ESBL isolates carried more than one type of β lactamase genes (Goyal et al., 2009; Bali et al., 2010). The three types of *bla* gene were found within group of children of less than 5 years old. This fact is relevant for using antibiotics for viral infections, that are resistant (Bergus et al., 1996). The limitation of our study is the bacterial ecology which was not identified by any process. We were not able to know for some patients, if resistant bacteria were acquired in the community or hospital.

Conclusion

Our study has shown high prevalence of β -lactamases-isolates producers among clinical strains detected through analysis of biological samples collected in different units of University Hospital Center, Yalgado OUEDRAOGO. CTX-M type (ESBL) was mostly represented, followed by TEM and SHV β -lactamases. *Escherichia coli*, like in

other several studies, was the strain that expressed ESBL frequently and can associate with more than one type. *P. aeruginosa* nosocomial, fastidious strain, known for producing PER currently harbored more and more CTX-M, TEM and SHV. This fact increases risk of ESBL dissemination between plasmid receptive strains or/and between clinical units. The present study has established that β -lactamin antibiotic except cephamycin and carbapenem (imipenem) was sensitive to ESBL. Diameters of inhibition area of oxy-iminocphalosporin (cefotaxim, ceftriazon, ceftazidim) for our isolates were seriously reduced, currently less than 20 mm. ESBL strains are usually multi-drug resistant. So, the practice of routine ESBL testing along with conventional antibiogram would be useful for all cases; it will help in the proper treatment of patients and also prevent further development of bacterial drug resistance. Molecular detection and identification of β -lactamases would be essential for a reliable epidemiological investigation of antimicrobial resistance. It is necessary to control hygiene and antibiotics consumption in hospital centers.

Conflict of interests

The authors did not declare any conflict of interest.

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