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# COEXISTENCE OF *CTX-M*, *GES* AND *IMP* GENES IN GRAM-NEGATIVE BACILLI AND THEIR MULTI RESISTANCE TO BETA-LACTAMS AT SAINT CAMILLE HOSPITAL IN OUAGADOUGOU (HOSCO)

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## ABSTRACT

Extended-spectrum ß-lactamases (ESBLs) are bacterial plasmid or chromosomal enzymes capable of inactivating Penicillins, Cephalosporins, Aztreonam and inactive on Carbapenems. They are encoded by the beta-lactamase genes (bla genes) of the *IMP* family (Imipenemase), *GES* (Guyana Extended Spectrum), *CTX-M* (Cefotaximase – Munich). Bacterial resistance by ESBL production has become a global public health problem because it seriously compromises antimicrobial therapies. The objective of this study was to characterize the *CTX-M* and *GES* type ESBL genes as well as the *IMP* type carbapenemase gene carried by Gram-negative bacilli at Saint Camille Hospital in Ouagadougou (HOSCO). Ninety-five (95) strains of *Enterobacteriaceae* were collected at HOSCO and tested for antibiotics (AMC, ATM, IMP, CTX, CRO and CAZ). Resistant strains, the detection of resistance genes coding for extended spectrum beta-lactamases (ESBLs) were carried out at LABIOGENE by conventional PCR. The strains were mainly isolated in the urine (54.74%), and showed strong resistance to AMC penicillins 90.52%, to

Aztreonam 75.78% to C3Gs: 70.52% CTX, 68.42% CAZ and 64.21% CRO; and imipenem (54.73%). *Escherichia coli* was the most frequent species (51) followed by *Klebsiella spp* (24). The ESBL phenotype was found in 31.57% of the strains, which were positive by conventional PCR. The *IMP* gene (17.89%) dominated the resistance gene profile followed by *CTX-M* (8.42%) and *GES* (5.26%). ESBL *CTX-M* was more produced by *Escherichia coli* (6.31%) and *GES* by *Pseudomonas spp* (3.15%). *Escherichia coli* was the majority species that harbored these genes. Analysis of PCR products after agarose gel electrophoresis confirms the presence of resistance genes coding for the *GES*, *CTX-M* and *IMP* genes. These genes were mainly detected in *E. coli* and then in *Pseudomonas spp*. This study reveals the coexistence of resistance genes of bacterial strains encountered in the clinical environment, hence the need for the establishment and support of an antibiotic resistance monitoring unit.

**KEYWORDS**: BGN, ESBL, bla$_{IMP}$, bla$_{GES}$, bla$_{CTX-M}$, HOSCO.

## INTRODUCTION

Antibiotic resistance occurs when the bacteria that cause an infection survive after being exposed to a drug that, under normal conditions, would kill them or inhibit their growth. As a result, these surviving strains multiply and spread due to the lack of competition from other strains susceptible to the same drug. Due to self-medication, resistant bacteria have become a threat to public health worldwide.[1] The acquisition of several genes by a bacterium leads to resistance to several classes of antibiotics usually called multiresistance. There are four main mechanisms of resistance: bacterial impermeability, target modification, antibiotic inactivation, active efflux.[2] The different classes of antibiotics are: carbapenems, 3rd generation cephalosporins (C3G) that are cefotaxime, ceftriaxone, ceftazidime, monobactams, aztreonam, the only monobactam used in clinic, is active against many Gram-bacteria. Penicillins (Penam) aminopenicillins: amoxicillin + clavulanic acid.

In fact, the problem of antibiotic-resistant bacteria is such that, according to World Health Organization (WHO) predictions, if antibiotic resistance continues to increase at this rate, infections caused by resistant bacteria will become the leading cause of death worldwide, ahead of cancer, diabetes and cardiovascular disease. In 2017, the WHO published a list of antibiotic-resistant bacteria against which there is an urgent need to develop new antibiotics.[3] This list is divided into three categories based on the urgency with which new antibiotics are needed: critical, high and medium. In the critical priority group are *Enterobacteriaceae* resistant to carbapenems and third-generation cephalosporins.

*Enterobacteriaceae* pose a threat to public health due to their ability to become resistant to antibiotics by producing extended-spectrum ß-lactamases (ESBLs).

To combat this threat, the medical community has turned to drugs such as carbapenems as a first-line treatment. This new treatment of resistant bacteria had an unexpected result, as it led to a more serious problem, the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE).[4] In particular, ERCs are bacteria belonging to the *Enterobacteriaceae* family that have the ability to survive and grow in the presence of clinically relevant concentrations of carbapenems. Antibiotics are chemicals developed by microorganisms that can inhibit multiplication or kill bacteria.

*Enterobacteriaceae* are rod-shaped gram-negative bacteria that are normal inhabitants of the gut flora and are among the most common human pathogens, causing infections.[5] β-lactamases are enzymes produced by certain bacteria and are responsible for their resistance to antibiotics belonging to the β-lactam family. Antibiotic resistance may be inherent in a particular bacterial species or acquired as a result of mutations or the acquisition of antibiotic resistance genes obtained from other microorganisms. The different mechanisms of resistance are encoded by these genes: decreased cell wall permeability, enzymatic inactivation and production of beta-lactamases that inactivate penicillins in penicillin-resistant *Escherichia coli*. Increased activity of antibiotic efflux pumpIncreased efflux of tetracycline, macrolides, clindamycin or fluoroquinolones. And target changes Decreased affinity of the modified cell wall precursor for vancomycin.[2]

This study is based on the coexistence of *CTX-M*, *GES* and *IMP* resistance genes in gram-negative pathogenic bacilli and their multi-resistance to beta-lactam at the Saint Camille Hospital in Ouagadougou (HOSCO).

## 1. MATERIAL AND METHODS

### 1.1 Mmicrobiological analyses

Ethical approval and consent to participate

The HOSCO/LABIOGENE Institutional Ethics Committee reviewed and approved the study protocol.

The isolation and identification of the bacteria were done at the Saint Camille hospital in Ouagadougou. The biological material consisted of 95 bacterial strains of origin humans responsible for infections in samples such as urine, stool and pus.

Cytobacteriological examination of urine (ECBU) The macroscopic examination consisted of noting the appearance of the urine (cloudy, hematic, clear, straw yellow) As far as the microscopic aspect is concerned, centrifugation at 1500 rpm for 15 min, then the pellet was spread between slide and coverslip and observed under objective 10 of the microscope optical. The presence or absence of epithelial cells, leukocytes, red blood cells, crystals, of some parasites and yeasts was noted. Regarding the culture, the urine was inoculated on the Uri select medium, then incubated at 37 for 24 hours. After incubation, the appearance and color of suspicious colonies were observed then identified on the basis of staining. Pink colonies are suspected as *Escherichia coli* and large, light blue colonies in honey cast suspected as *Klebsiella sp*.

Coproculture or cytobacteriological examination of stools : coproculture consists of isolating and identifying within a complex flora of bacteria pathogens. It is done in three stages like the ECBU and are : The macroscopic examination of the stools was made by noting the aspect of the stools (mucous, mucous, normal or rice water), stool consistency (liquid, semi-liquid, hard or molded) and color (brown, yellowish). The microscopic examination of the stools, meanwhile, consisted in spreading on a slide a small amount of stool diluted in physiological water that has been covered with a coverslip, then we observed elements such as leukocytes, red blood cells, bacteria and a Gram stain was performed. The culture was made by diluting the stool with physiological water. On a petri dish containing the Hektoen medium, the inoculation was done using a loop by making a streak vertical and tight horizontal streaks. Then, the Petri dish was incubated in an oven at 37 degrees for 3 hours and suspicious colonies were identified. There after, a re-isolation on the ordinary medium for the antibiogram and the gallery and a reading of the galleries identifications have been made.

Cytobacteriological examination of pus was done as follows: the macroscopic examination consisted of noting the appearance of the pus (purulent or hematic disorder), the consistency and color (lemon yellow). The microscopic examination consisted of spreading the sample on a slide and a fixation and Gram staining was done. Culture was done on blood agar under CO2, EMB agar, BCP agar or thioglycolate broth. Incubation was done at 37 degrees for 48 hours at least. However, a reading and observation of the colonies were carried out on the 24th hour and an antibiogram of the germ(s) according to CASFM/EUCAST was done.

The collection was made after the isolation and identification of the bacteria. To do this, we transplanted the pure colonies of the bacteria concerning our study into petri dishes containing MH. Then, we quickly sent the boxes to LABIOGENE where we put in the oven for incubation for 24 hours. We collected a total of 95 strains bacteria.

All isolates were also tested for susceptibility to 6 different antimicrobial agents using the disk diffusion method on Mueller Hinton II agar (Oxoid, England) following the recommendations of the Antibiogram Committee of the French Society of Microbiology (CASFM). The following antimicrobial disks (Oxoid) used were ceftriaxone ( 30 µg ), ceftazidime ( 30 µg), cefotaxime ( 30 µg ), imipenem (10µg), amoxicillin +clavulanic acid (30 µg), and aztreonam (30 µg).

### 1.2. DNA extraction and polymerase chain reaction (PCR) for resistances genes detection

The extraction of DNA was done by the boiling method. An isolated colony was taken from MH Petri dishes and suspended in 200 µl of distilled water in labeled Eppendorf tubes. Then, the tube was soaked in a water bath at 100°C for 15 minutes to release the genetic material of the bacteria. After, a centrifugation of 10 min at 12000 rpm; the supernatant containing the released DNA was transferred to a new Eppendorf tube.

After quantification and verification of DNA purity with the nanodrop, part of the supernatant was used for amplification and the rest was stored at -80 °C.

PCR (Polymerase Chain Reaction), the polymerase chain reaction was performed in a reaction mixture of 20 µL. This reaction mixture was prepared using 4 µL of 5X Firepol® Master Mix + 0.5 µL of primer sense + 0.5 µL of antisense primer + 14 µL of water PCR + 1 µL of bacterial DNA of each strain.

The PCR programme used is recorded in Table I. Primer sequences for the different carbapenemase and beta-lactamase genes are shown in Table II.

**Table 1: PCR program by gene type.**

| Parameters / Genes | Condition / duration | | |
|---|---|---|---|
| | ***bla*$_{IMP}$** | ***bla*$_{GES}$** | ***bla*$_{CTX-M}$** |
| Initial denaturation | 96 °C / 5 mn | 96 °C / 7 mn | 96 °C / 5 mn |
| Denaturation | 96 °C / 30s | 96 °C / 1mn | 96 °C / 1mn |
| Hybridization | 54 °C / 30s | 60 °C / 1mn | 50 °C / 1mns |

| | | | |
|---|---|---|---|
| Elongation | 72 °C / 30s | 72 °C / 1mn | 72 °C / 1mn |
| Final elongation | 72 °C / 7 mn | 72 °C /10 mn | 72 °C /10 mn |
| Number of cycles | 30 | 35 | 35 |

**Table 2: Nucleotide sequences of different primers.**

| Genes sought | Sequences (5'-3') | Waist (pb) | References |
|---|---|---|---|
| $bla_{CTX-M}$ | For : 5'GTTACAATGTGTGAGAAGCAG 3' | 1000 | **(Pagani et al., 2003)** |
| | Rev : 5' CCGTTTCCGCTATTACAAA 3' | | |
| $bla_{GES}$ | For : 5' ATGCGCTTCATTCACGCAC 3' | 863 | **(Moubareck et al., 2009)** |
| | Rev : 5' CTATTTGTCCGTGCTCAGG 3' | | |
| $bla_{IMP}$ | For :5' CATGGTTTGGTGCTTGT 3' | 500 | **(Huang et al., 2012)** |
| | Rev :5' ATAATTTGGCGGACTTTGGC 3' | | |

### 1.3. Agarose gel electrophoresis

The PCR-amplified DNA fragments were separated by agarose gel electrophoresis (1.5%) prepared in a 1X tris base - borate - EDTA solution and containing 8 µL of ethidium bromide. The 1000 bp molecular weight marker was used to assess the expected band sizes. The migration was carried out for 30 minutes at a voltage of 110 V with an intensity of 65 mA. The resulting migration products were visualized under UV light with the transilluminator (Vilber E-Box) and the photos were recorded.

## 2. RESULTS AND DISCUSSION

### 2.1. Bacterial strains

Among the 95 resistant gram-negative bacterial strains, we had resistance to at least one of the antibiotics that are: 3rd generation cephalosporins C3G (ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX)), carbapenem imipenem (IMP), amoxicillin + clavulanic acid (AMC), and aztreonam (TMA), the different species that have been studied are *E. coli* (n=51), *Klebsiella spp*, (n=24), *Pseudomonas spp*, (n=16), *Proteus spp* (n=4). The frequency of bacterial strains is shown in the following table (Table III)

**Table 3: Frequency of bacterial strains.**

| Bacteria | Number (%) |
|---|---|
| *E.coli* | 51 (53.68) |
| *Klebsiella* | 24 (25.26) |
| *Pseudomonas* | 16 (16.84) |
| *Proteus* | 4 (4.21) |
| **Total** | **95 (100)** |

These strains were isolated from various biological samples such as urine (n = 52), stool (n = 32) and pus (n = 11). The following table (Table IV) shows the distribution of bacterial strains by pathological products.

**Table 4: Distribution of bacterial strains by pathological products.**

| Pathological products / Strains | Urine (%) | Stool (%) | Pus (%) | Total (%) |
|---|---|---|---|---|
| *E.coli* | 31 (32.63) | 17 (17.89) | 3 (3.10) | **51 (53.68)** |
| *Klebsiella spp* | 13 (13.68) | 9 (9.47) | 2 (2.10) | **24 (25.26)** |
| *Pseudomonas spp* | 8 (8.42) | 2 (2.10) | 6 (6.31) | **16 (16.84)** |
| *Proteus spp* | 0 (0) | 4 (4.21) | 0 (0) | **4 (4.21)** |
| **Total** | **52(54.74)** | **33 (33.64)** | **11(11.57)** | **95 (100)** |

### 2.2. Resistance profile

The isolated bacterial strains show high resistance, to cefotaxime (70.52%), ceftazidime (68.42%) and ceftriaxone (64.21%). The most active antibiotics on these strains were amoxicillin + clavulanic acid (90.52%), aztreonam (75.78%). Imopenem (IMI) is the only carbapenem used in this study. Of the 95 strains tested, 54.73% strains were resistant to this antibiotic.

The frequency of resistance of strains to β-lactam is illustrated in Figure 1

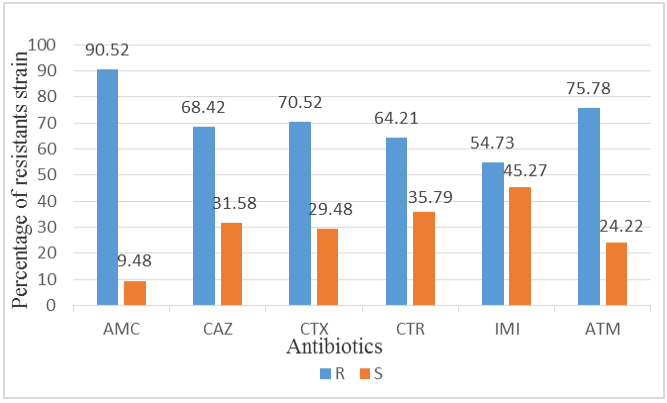


**Fig. 1: Beta-lactam resistance rate.**

Legend:

**AMC**: Amoxicillin + clavulanic acid; **ATM** : Aztreonam; **IMI**: Imipenem; **CAZ**: Ceftazidime; **CTX**: Cefotaxim; **CTR**: Ceftriaxone; **R**: Resistant; **S**: Sensible

### 2.3. Molecular characterization of genes encoding the production of ESBLs

The DNA of the 95 strains was subjected to the PCR technique for the search for the ESBL gene type using the specific primers for *blaGES*, *blaCTX-M*, *blaIMP*. Examination of PCR products after agarose gel electrophoresis noted that 30 isolates were found to carry at least

one of the genes investigated and one (1) isolate harbored both CTX-M and GES genes. Out of the 30 strains isolated, 5 strains had produced amplicons of 863 bp (figure 2) with *blaGES primers* indicating the production of ESBLs of the GES type, 8 strains were positive for the *blaCTX-M* gene found at 1000 bp (figure 3) and 17 bla*IMP* positive strains found at 500 bp (figure 4).

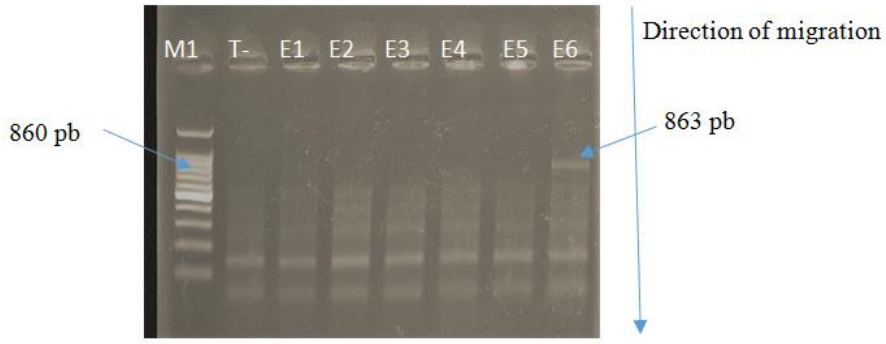


**Fig. 2: Electrophoretic profile of *blaGES* gene amplicons at 863 bp.**

Legend:

M1: Molecular weight marker (100pb DNA Ladder).

T-: Negative control

The numbers E1 - E6 represent the samples: E1 = stool; E2 = stool; E3 = urine; E4 = urine; E5 = pus; E6 = urine. The direction of migration of electrophoresis is from top to bottom.

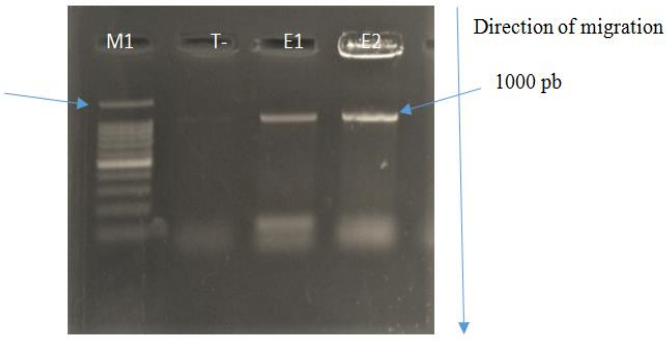


**Fig. 3: Electrophoretic profile of *blaCTX-M* gene amplicons at 1000 bp.**

M1: Molecular weight marker (100pb DNA Ladder), T-: Negative control, E1 – E2 represent the samples: E1 = urine; E2 = urine. The direction of migration of electrophoresis is from top to bottom.

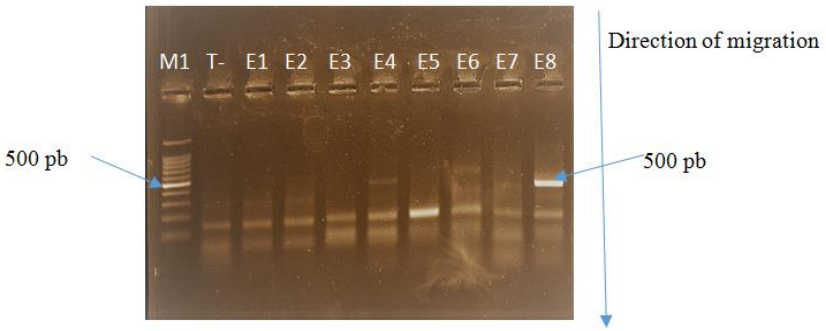


**Fig. 4: Electrophoretic profile of *blaIMP* gene amplicons at 500 bp.**

M1: Molecular weight marker (100pb DNA Ladder), T-: Negative control, E1 – E8 represent the samples: E1 = urine; E2 = urine; E3 = stool; E4= pus; E5 = urine; E6 = stool E7 = urine E8 = stool. The direction of migration of electrophoresis is from top to bottom.

At the biological sample level, urine harbours more strains producing ESBL type *CTX-M* followed by pus and stool. Isolates that produce ESBLs of the IMP type are much more common in feces and in equal numbers in urine and pus. The number of ESBL-producing strains of GES type is found in urine and pus followed by stool, and Table V shows the distribution of ESBL genes according to biological samples.

**Table 5: Distribution of ESBL genes according to biological samples.**

| Pathological products / Genes | Pus N (%) | Stool N (%) | Urine N (%) | Total N (%) |
|---|---|---|---|---|
| GES | 2 (2.10) | 1 (1.05) | 2 (2.10) | **5 (5.26)** |
| CTX-M | 2 (2.10) | 1 (1.05) | 5 (5.26) | **8 (8.42)** |
| IMP | 5 (5.26) | 7 (7.36) | 5 (5.26) | **17 (17.89)** |
| **Total** | **9 (9.47)** | **9 (9.47)** | **12 (12.63)** | **30 (31.57)** |

The *IMP* gene (CARBAPENEMASE-like ESBL) dominated the profile of ESBL resistance genes followed by *CTX-M* and *GESs*. *The CTX-M* ESBL was much more produced by *E. coli* than by *Klebsiella spp* and *Pseudomonas spp*. On the other hand, it was the *GES* that dominated at *Pseudomonas spp*. The frequency of *IMP* was 9.47% in *E. coli* and 4.21% in *Klebsiella spp*. Table VI shows the distribution of genes according to bacterial species. We observed that only one strain of *E. coli* produced more than one resistance gene (*GES/CTX-M*).

**Table 6: Distribution of genes according to bacterial species.**

| Genes Species | $bla_{GES}$ | $bla_{CTX-M}$ | $bla_{IMP}$ | Total |
|---|---|---|---|---|
| | N (%) | N (%) | N (%) | |
| *E. coli* | 1 (1.05) | 6 (6.31) | 9 (9.47) | **16 (16.84)** |
| *Klebsiella spp.* | 0 (0) | 1 (1.05) | 4 (4.21) | **5 (5.26)** |
| *Proteus spp.* | 1 (1.05) | 0 (0) | 1 (1.05) | **2 (2.10)** |
| *Pseudomonas spp* | 3 (3.15) | 1 (1.05) | 3 (3.15) | **7 (7.36)** |
| **Total** | **5 (5.26)** | **8 (8.42)** | **17 (17.89)** | **30 (31.57)** |

## 2.4. DISCUSSION

Since their first detection in 1983, *Enterobacteriaceae* producing extended-spectrum beta-lactamases have spread widely throughout the world with varying isolation frequencies even from one department to another within the same hospital institution. ESBLs today are the majority of multidrug-resistant BMR bacteria that cause potentially severe infections and prescriptions for broad-spectrum antibiotics, which threaten the future activity of late-line molecules. Their involvement in nosocomial infections is a real public health problem.[6]

These bacterial strains consisted of 4 species with a prevalence of 53.68% for *E. coli*, 25.26% of *Klebsiella spp*., 16.84% of *Pseudomonas spp*, and 4.21% of *Proteus spp* from biological samples such as urine (n=52), stool (n=32) and pus (n=11), the rate is lower than that recorded by Kalambry et al., in Mali (*E.coli* 63%; *Klebsiella spp* 32 .8%)[7], this could be explained by the fact that our samples did not contain too many microorganisms.

Resistance to C3G has been reported in ESBL-producing strains studied in Cameroon with 44.6% for CAZ and 45.4% for CTR)[8], in Togo, 97.28% for CAZ, 97.16% for CTR and 100% for CTX) ([9]) and in Burkina-Faso with 87.8% for CTX and 68.9% for CRO).[10] The resistance rate to C3Gs in our study is (64.21% for CTR, 68.42% for CAZ, 70.52% for CTX) and the high resistance of these strains to C3Gs could be due to the intense prescription of these C3Gs in BGN infections which would lead to a follow-up of the spread of bacterial resistance to these antibiotics, in particular by production of enzymes that inactivate Beta-lactams, namely ESBLs.[11]

The characterization of these strains has highlighted the production of ESBLs of *GES* types, *CTX-M* and carbapenemase type *IMP* by the isolated species of bacteria. Analysis of PCR products after agarose gel electrophoresis revealed that: 5 strains had produced amplicons indicating the production of ESBLs of the GES type, 8 strains were positive for the bla*CTX-M* gene 17 strains positive for the bla*IMP* gene. The *IMP* gene (CARBAPENEMASE-like

ESBL) dominated the profile of ESBL resistance genes followed by *CTX-M* and *GESs*. This dominance is due to the fact that *Enterobacteriaceae* become resistant to carbapenems through three main mechanisms: enzyme production, efflux pumps and porin mutations, as well as the misuse of antibiotics, mobile genetic elements, travel between countries and ineffective infection control measures are the main predisposing factors for the emergence of resistance. Travel within and outside the country is a major risk factor for the transmission of resistant strains of *Enterobacteriaceae.* Carbapenem resistance in *Enterobacteriaceae* is linked to the association of decreased outer membrane permeability with overexpression of beta-lactamases with limited carbapenemase activity (cephalosporinase [AmpC] or extended-spectrum beta-lactamase (ESBL, mainly CTX-M, inhibited by clavulanic acid).[12]

The *CTX-M ESBL* was much more produced by *E. coli* than by *Klebsiella* spp and *Pseudomonas spp*. On the other hand, it was the *GES* that dominated at *Pseudomonas spp*. The frequency of *IMP* was 9.47% in *E. coli* and 4.21% in *Klebsiella spp*. We observed that only one *E. coli* strains produced more than one resistance gene (*GES/CTX-M*). In this survey, PCR results showed that the *blaIMP* gene (17.88%) was the most common followed by bla*CTX-M* (8.41%) and bla*GES* (5.25%). Although the prevalences are relatively different, *CTX-M* has been found to be the majority in other studies, such as Burkina Faso (65.49%) and Morocco (70%) (Amama et al., 2014[13] and a minority in India (7.6%).[14] The positivity of the strains assumes the presence of ESBL type CTX-M, confers resistance to Ceftazidime which is the most widespread. For the IMP gene, these results are slightly lower than ours, 16% in China according to the studies of[15] and lower than those of Tanzania (12%)[16] and higher than the studies of which are 43% in Uganda.[17] This high level of resistance through ESBL production is due to the acquisition of antibiotic resistance factors, following the excessive and unregulated use of these different classes of antibiotics, especially in poor hygiene conditions in poor countries where access to care and infection control are poor.

## CONCLUSION

The misuse or inappropriate use of antibiotics is mainly responsible for the emergence of antibiotic resistance. The spread of resistant strains producing extended-spectrum β-lactamase poses a public health threat by reducing treatment for severe infections. This public health problem requires careful monitoring and implementation of antibiotic use policy both in the community and in the hospital. Public education, banning the sale of antibiotics without a

prescription and minimizing probabilistic antibiotic therapy in inpatient wards could be solutions to limit antibiotic resistance. The control of this phenomenon does not necessarily require the discovery of new antibacterial agents but rather strict compliance with simple hospital hygiene measures and the more rational use of antibiotics.

Analysis of PCR products after agarose gel electrophoresis confirms the presence of resistance genes encoding the *GES, CTX-M* and *IMP* genes. These genes were mainly detected in *E. coli* and then in *Pseudomonas spp*. We also found co-existing genes, including *blaGES* & *blaCTX-M.*

**Author contributions**

All authors have made substantial contributions to data acquisition or data analysis and interpretation; participated in the drafting of the article or its revision critical for significant intellectual content; gave the final approval of the version to be published; and agree to be responsible for all aspects of the job.

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**Competing interests**

The authors declare that they have no competing interests.

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**Availability of data and hardware**

All datasets used to support the conclusions of this study are available upon request from the corresponding author.

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