Original Research Article

Antibiotic resistance and production of extended spectrum β-lactamases by clinical Gram-negative bacteria in Benin.

ABSTRACT

Aims: The aim of this work was to determine the resistance profile and to investigate the production of extended spectrum β-lactamases (ESBL) ~~of~~ by clinically relevant Gram-negative Bacillus (GNB) strains.

Methodology: About 191 strains were isolated from 1823 samples collected at the HKM National Hospital and University Center of Cotonou (Benin). Species identification was done with the Api 20th gallery. Two methods were used to search for β-lactamase production: the liquid acidimetric test for penicillinases and double halo method for ESBL. The susceptibility to conventional antibiotic molecules was investigated by the disk diffusion method. Polymerase Chain Reaction (PCR) was used to identify~~for the research of~~ $_{bla}$TEM and $_{bla}$SHV genes in ~~coding for~~ the ~~production of~~ β-lactamases.

Results: A prevalence of 10.48% of GNB was recorded. Among the isolated strains, 51.31% came from samples collected from in~~side hospital~~-patients and 48.69% from out-patients' ~~of hospital~~ samples. The most contaminated samples were urin~~eating~~ (43.98%), pus (34.58%) and blood (9.42%). Majority ~~Five~~ of the isolated species included~~were in the majority~~: *Klebsiella pneumoniae* (28.27%), *Acinetobacter spp*. (18.32%), *Pseudomonas aeruginosa* (15.72%), *Escherichia coli* (14.15%) and *Enterobacter cloacae* (12.04%). More than the half (57.07%) of the strains ~~were found to~~ produced penicillinases; whereas 16.76% ~~of the isolated strains~~ were ESBL-produc~~ers~~~~ing~~ and these occurred only among ~~. Only four species, namely~~ *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli* and *Enterobacter agglomerans* ~~were ESBL-producers~~. The ESBL-producing strains were~~are~~ cross-resistant to beta-lactams. Imipenem is the most effective antibiotic on all isolated strains. ESBL-producing GNB strains possessed both the $_{bla}$*TEM* gene and the $_{bla}$*SHV* gene in a proportion of 25%; 37.5% of the strains had only the $_{bla}$TEM gene and 12.5% of the strains had only the $_{bla}$SHV gene.

Conclusion: ESBL-producing strains of *K. pneumonia* in the hospital environment were the major carriers of $_{bla}$*TEM* and $_{bla}$*SHV.* Given this situation, it is necessary to continue research to identify resistance genes.

Key words: Gram-negative bacilli, ß-lactamases, antibiotics, clinic, Benin.

1. INTRODUCTION

Bacterial diseases are the second leading cause of death and los~~t years~~ of productive life years worldwide [1]. These bacteria are ~~being~~ involved in 70% of mortality in ~~due to~~ human~~s~~ infectious diseases [2]. Gram-negative bacilli represent a very important place among the

responsible microorganisms for infections [3]. These pathogens are part of different bacterial families such as *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Proteus mirabilis*, *Serratia marcescens* etc.), *Pseudomonas*, *Stenotrophomonas* and *Acinetobacter* species. Thought displaying various virulence level, these bacteria have inherent ~~natural~~ (natural resistance), or develop (acquired resistance) resistance to antibiotics [4-7].

The discovery of antibiotics has been one of the most important therapeutic advances of the twentieth century; their use has reduced significantly the rate of morbidity and mortality associated with infectious diseases [8]. However, it has been at the origin of a strong increasing antibiotic resistance affecting more species with the use of a number of more and more antibiotics [9]. Since the beginning of the 1960s, there is an increase in the number of bacteria resistant to antibiotics, and the emergence of new resistance [10]. Thus, the emergence and dissemination of resistance to antibiotics represent a real threat to global public health [11-13]. The antibiotic resistance continues to increase~~grow~~ in both the industrialized and the developing country [14]. The situation is alarming in countries with limited resources where infectious diseases, poverty and malnutrition are endemic [15].

During the last ten years, bacterial resistance to antibiotics, particularly by AmpC enzymes and β-lactamases with extended spectrum (ESBL) production, has become a major problem of public health [16]; because of the multidrug resistance and the choice of limited antibiotics molecules [17]. ESBL-producing microorganisms were associated with the expansion of hospital-acquired infections leading to prolonged hospitalization, increased morbidity and mortality [18-19]. Despite the importance of the problem and economic consequences, West Africa is having national program of surveillance to fight against resistance as recommended by the World Health Organization [14]. Furthermore, there is a lack of data assessing the impact of antimicrobial resistance in developing ~~ment~~ countries~~y such~~ as in West Africa [20]. The emergence and dissemination of ~~the~~ ESBL in West Africa is therefore reported to be related to the expansion of CTX-M-15 allelic type [21]. Other ESBL allelic type of CTX-M type were reported by Duval et al. [22] in Mali (CTX-M-14), in Nigeria by Ogbolu et al. [23] and in Senegal by Breurec et al. [24] the identified form was CTX-M-3. Besides~~Apart of~~ the CTX-M enzymes, SHV enzymes (3 and 12) were recently characterized from various isolates in Mali [25] and Nigeria [26]. Plasmids carrying ESBL gene can also host other resistance genes conferring resistance to other families of antibiotics, including cotrimoxazole, fluoroquinolone and ~~to~~ aminoglycosides [27].

In Benin, there is a scarce or paucity of information on the prevalence of ESBL enzymes. Thus, this study is a contribution on the epidemiology of clinical ~~isolated~~ Gram Negative Bacilli. The aim of our study was to isolate GNB from various clinical samples, to determine their prevalence, to make their biochemical characterization, to evaluate their antimicrobial resistance and to check the presence of some resistance genes.

2. MATERIAL AND METHODS

2.1. Study design and sampling

The study focused on clinical ~~the biological samples carried to~~processed at the National Hospital and University center of Cotonou (Benin) from July–September 2012. Thus during 3 months, 1823 specimen were collected from both hospitalized ~~patient~~ and community patients. ~~The specimen coming from hospitalize patient are considered as inside hospital patient specimen. If not, they are out-of-hospital specimen.~~ All ~~The entire~~ specimens randomly carried to the reference center during the period were taken as the ~~our~~ sample size for the study and were ~~. These specimens~~ collected following the conventional protocols [28-29]. These include urethral, vaginal, urinary, semen, blood, catheters, Intra Uterine Devices (IUD) and cerebrospinal fluid specimen. For the study, only specimen with majority or pure culture (10^5 cfu/ml) of GNB were taken into account.

2.2. Isolation and identification of Gram negative bacilli

Specimen were cultured~~seeded~~ on Eosin Methylene Blue Agar (Oxoid, England) and incubated overnight~~for 24 h~~ at 37°C. From the obtained colonies, the Gram stain and the identification by the Gallery Api 20E was carried out.

2.3. Susceptibility of Bacilli Gram negative bacilli isolates to antibiotics

The susceptibility of the identified bacteria to 21 conventional antibiotics, provide by Oxoid (England) was performed using EUCAST recommended methods and interpretation [30]. The 21 antibiotics tested (Bio Mérieux, France) were: cefotaxime (CTX 5 µg), amoxicillin (AMX 30 µg), amoxicillin + clavulanic acid (AMC 20/10 µg), ceftriaxone (CRO 30 µg), cefuroxime (CXM), cefoxitin (FOX 30 µg), imipenem (IPM 10 µg), nalidixic acid (NA 30 µg), ciprofloxacin (CIP 5 µg), ofloxacin (OFX 5 µg), norfloxacin (NOR 10 µg), gentamicin (GM 10 µg), netilmicin (NET 10 µg), chloramphenicol (C 30 µg), trimethroprime sulfonamide (SXT 1.25/23.75 µg), nitofurantoin (FT 300 µg), ticarcillin (ICT 75 µg), cefixim (CFM 30 µg), aztreonam (ATM 30 µg), doxycycline (DO 30 µg) and tetracycline (TE 30 µg).

2.4. Penicillinase production assay by test acidimetrique in tubes

The production of the penicillinase was performed on all ~~to~~ GNB isolates by the broth medium method as previously describe~~d~~ [31]. The reagent used is a solution prepared extemporaneously with 600 mg of benzylpenicillin powder dissolved in 400 µl of sterile distilled water and 300 µl of 1N NaOH has been added to the mixture to obtain a pH of 8. Finally, to get a purple red color, 300 µl of 1% phenol red was added in distilled water. Two fresh ~~young (24 h)~~ colonies were ~~have been~~ emulsified in 500 µl of physiological water. Then, 150 µl of reagent to the benzylpenicillin previously prepared have been added. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 35657 were used as control. After an hour~~1 h~~ of incubation at 37°C, a shift in the color of the original red purple to orange or yellow in the tubes indicates that the tested strain is penicillinase producing.

2.5. Analysis of the spectrum β-lactamase expanded (ESBL) by the method of double halo

Determination ~~Research~~ of ~~the~~ ESBL production on all the GNB isolates were~~as~~ performed using the method of disk synergy between third-generation cephalosporin and clavulanic acid [28]. On the Mueller Hinton agar previously inoculated ~~seeded~~ with the test strain, a disc of 3rd generation cephalosporin: (Cefotaxime (CTX) and Ceftriaxone (CRO) with clavulanic acid (amoxicillin + clavulanic acid), was applied and incubated for 18 h at 37°C [26]. The disc of amoxicillin + acid clavulanic (AMC) was ~~has been~~ placed in the center of the two other cephalosporins at a distance of 30 mm. The positive result reveals~~is~~ a potentiation of the inhibition zone around the disks of CTX and CRO, in the presence of the antibiotic AMC, because of the synergy of beta lactams with clavulanic acid [32-33].

2.6. Detection of resistance genes

Polymerase chain reactions was performed on all extracted ~~total~~ DNA of all confirmed ESBL producer GNB to detect genes encoding multidrug resistance (TEM and SHV) The DNA template was extracted by suspending a loop of GNB colony in 500-µL sterile, pure water and boiling for 10 min at 95°C. The suspension was then centrifuged for 5 min at 12000 rpm, and 10 µL of the supernatant was used as target DNA. DNA extracts were stored at -20°C until used.

The primers for $_{bla}$*TEM*, and $_{bla}$*SHV* were used for multidrug resistance gene investigation by PCR amplification in 30 µL containing for each: 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 PCR program used for amplification consisted, for $_{bla}$*TEM* (initial denaturation 94°C for 5 min followed by 30 cycles 94°C at 30 s, 52°C for 30 s, 72°C for 1 min and a final elongation step 10 min at 72°C). For $_{bla}$*SHV* (initial denaturation was performed at 96°C for 5 min, 30 cycles at 96°C for 15 s, 50°C for 15 s, 72°C for 1 min and a final elongation step 10 min at 72°C). The primers sequences and the expected fragments are presented in the Table 1.

Table 1. Primers used to search genes in this study.

Targuets genes	Primers	Primers sequences (5' ⟶ 3')	Amplicon size (bp)	Reference
bla_{TEM}	OT-F	5'-TTGGGTGCACGAGTGGGTTA-3'	465	[34]
	OT-R	5'-TAATTGTTGCCGGGAAGCTA-3'		
bla_{SHV}	SHV-F	5'-CGCCGGGTTATTCTTATTTGTCGC-3'	620	[35]
	SHV-R	5'-TCTTTCCGATGCCGCCGCCAGTCA-3'		

PCR products (10 µL) were visualized after electrophoresis at 150 V for 30 min on a 1.5 % agarose gel containing ethidium bromide and visualized with an UV trans-illumination. A 100 bp ladder standard was used as molecular weight ladder.

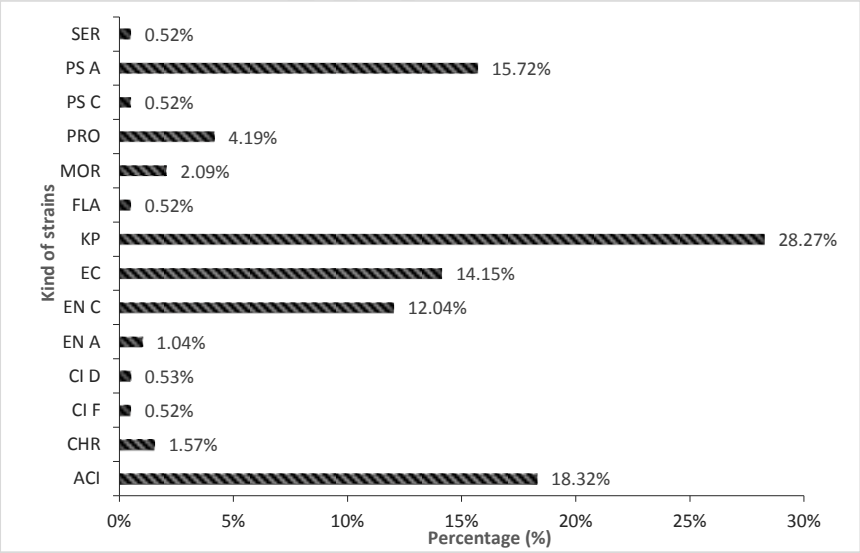
2.7. Data analysis

The Microsoft office Excel 2010 spreadsheet was used for processing data. GraphPad Prism 5 was used to perform the Chi-square test and make graphs. The test was considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Prevalence of GNB isolates

From the 1823 collected specimens, 191 strains (10.47%) of GNB were identified. The 191 strains were identified from both male (23.56%) and female (76.44%) patients. Thus, the females samples displays significant infectioncontamination rate compared to the males (p= 0.0001). Fifteen different species of GNB were isolated. The species five mostly represented werespecies are *K. pneumoniae* (28.27%), *Acinetobacter* spp. (18.32%), *P. aeruginosa* (15.72%), *E. coli* (14.15%) and *E. cloacae* (12.04%) (Figure 1).



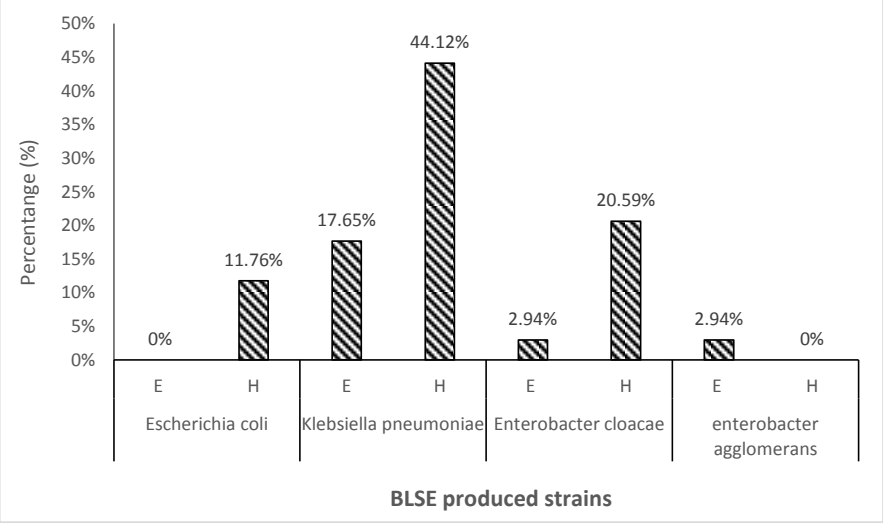
ACI : *Acinetobacter* spp, PS A: *Pseudomonas aeruginosa,* PS C : *Pseudomonas cepacia,* EC : *Escherichia coli,* KP : *Klebsiella pneumoniae*, EN A : *Enterobacter agglomerans,* EN C : *Enterobacter cloaceae,* PRO : *Proteus mirabilis,* MOR : *Morganella morgnii,* CHR : *Chryseomonas luteola,* CI D : *Citrobacter diversus*, CI F : *Citrobacter freundii*, FLAV : *Flavobacterium indologenes* et SER : *Serrati ficania.*

Figure 1. Distribution of different species of Gram-negative bacilli isolated from specimen.

Varieties of proportions of GNB were observed according to the type of specimen. The urine and pus ~~are the specimen that contain~~ produced more GNB with ~~respectively, the~~ proportions of 43.98% and 34.58% respectively. Blood samples (9.45%), semen (2.61%), cerebrospinal fluid (2.61%), vaginal specimen (2.09%), pleural fluid (2.09%), catheters (1.05%), IUDs (1.05%) and specimen of unknown origin (0.52%) respectively followed them.
The GNB isolates were grouped into hospital isolates (i.e., isolates from the specimen taken from patients hospitalized in different services of the CNHU) and community~~outside~~ isolates (i.e. the isolates obtained from patients outside of the hospital environment~~'s~~ specimen). The hospital GNB's isolates (51.31%) were higher than those from outside the hospital (48.69%), and the difference was not ~~to be~~ statistically significant ($p > 0.05$).

3.2. Production of penicillinase and ESBL by the GNB isolates

A proportion of 57.07% of the isolates ~~were~~ produced ~~ing~~ penicillinases and these ~~. The isolates producing penicillinases~~ occurred both ~~were well found among~~from in-patient ~~hospital~~ and out-patient (community)~~extra-hospital~~ isolates. Among the strains producing penicillinases, 61.47% are from the ~~community~~ out-patients ~~hospital~~ and 38.53% from the in-patients~~hospital environment~~. Thus, we find that in-patient~~hospital~~ isolates produced more penicillinase than the isolates from outpatient~~side~~ specimens ~~the hospital~~; and the ~~proportion~~ difference were~~as~~ statistically significant ($p < 0.05$).
Among the GNB isolates, 32 (16.76%) were extended ~~panded~~ spectrum β-lactamases producers (ESBL). The ESBL producing isolates were observed ~~recorded~~ both in-patient ~~hospital~~ and out-patient clinical samples, ~~extra-hospital environment. H~~however, the in-patient ~~hospital~~ ESBL-producing isolates were substantially higher (75%) than the out-patient isolates~~extra-hospital environment producers~~ (25%) ($p < 0.005$). Among ESBL-producing isolates, only *E. coli*, *K. pneumoniae*, *E. cloacae* and *E. agglomerans* strains~~'~~ produced ESBL; and these include 44.12% ~~. The ESBL producers are~~by *K. pneumoniae* ~~(44.12%)~~, *E. cloacae* (20.58%) and, *E. coli* (11.76%) strains isolated from the in-patients~~hospital environment~~ (Figure 2).



E: Extra-Hospital isolates and H: Hospital isolates

Figure 2. Gram Negative Bacillus strains producing ESBLs depending on the source.

3.3. Susceptibility to antibiotics of isolated strains of GNB

The susceptibility results ~~investigation on the antibiotic resistance~~ of the isolates revealed that imipenem had ~~s experienced~~ the lowest resistance (9.8%) and the ticarcilline ~~has experienced~~ the highest resistance (100%) rate (Figure 3). All the intermediate resistance observed by the GNB were ~~too strong, each being~~ more than 50%. Thus, resistance level located between 50 and 80% was recorded for 12 antibiotics (gentamicin, netilmicin, ciprofloxacin, aztreonam, trimethroprime sulfonamide, cefoxitin, ofloxacin, ceftriaxone, cefotaxim, norfloxacin, nitofurantoin and cefixim). In addition, seven antibiotics (amoxicillin +

clavulanic acid, nalidixic acid, cefuroxime, chloramphenicol, amoxicillin, tetracycline and doxycycline) displayed an inhibition activity on between 80% and less than 100% of the tested GNB.

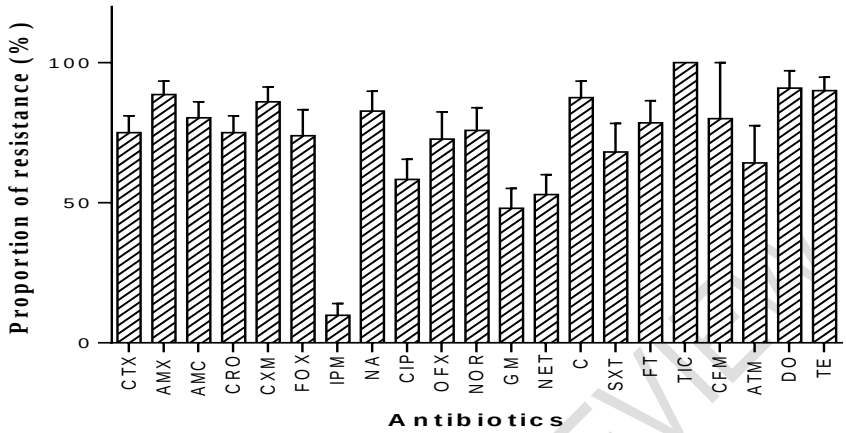


Figure 3. Antibiotic resistance of isolated Gram-negative Bacillus strains. CTX: cefotaxime, AMX: amoxicillin, AMC: amoxicillin + clavulanic acid, CRO: ceftriaxone, CXM: cefuroxime, FOX: cefoxitin, IPM: imipenem, NA: nalidixic acid, CIP: ciprofloxacin, OFX: ofloxacin, NOR: norfloxacin, GM: gentamicin, NET: netilmicin, C: chloramphenicol, SXT: trimethroprime sulfonamide, FT: nitofurantoin, TIC: ticarcillin, CFM: cefixime, ATM: aztreonam, DO: doxycycline and TE: tetracycline.

3.4. Antibiotic resistance of GNB according to the production of ESBL

Globally, for the majority of the tested antibiotics (18), GNB isolates producing ESBL were more resistant than those that do not produce. However, Figure 4 shows a variation of resistance proportion according to the type of antibiotic. Eight antibiotics (aztreonam, nalidixic acid, ticarcillin, cefixim, tetracycline, doxycycline, amoxicillin and chloramphenicol) showed a resistance level between 2% and 15%. On the other hand, resistance of isolated strains varying between 20% and 38% was observed for eight antibiotics (Cefotaxim, amoxicillin + clavulanic acid, norfloxacin, ceftriaxone, ofloxacin, ciprofloxacin, trimethroprime sulfamide and imipenem). Finally~~To end~~, thea~~-~~ highest resistance was obtained for gentamicin (52.46%) and netilmicin (56.67%).

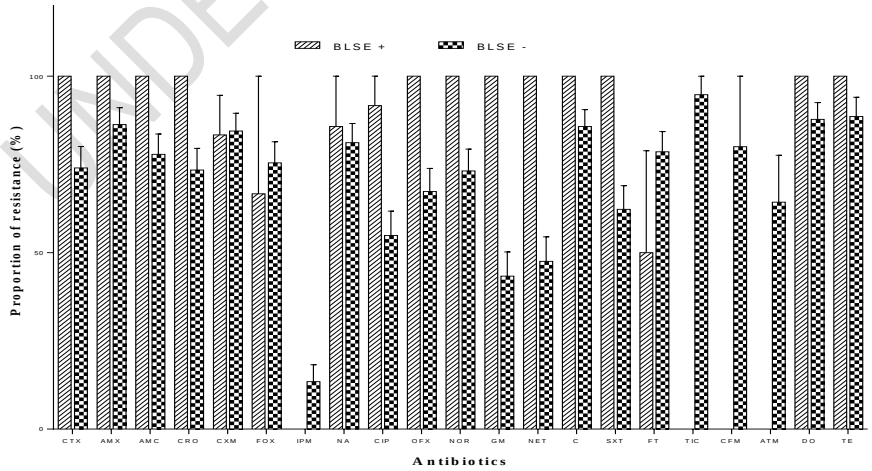


Figure 4. Antibiotic resistance of Gram Negative Bacilli strains depending on whether or not they produce ESBL. CTX: cefotaxime, AMX: amoxicillin, AMC: amoxicillin + clavulanic acid, CRO:

ceftriaxone, CXM: cefuroxime, FOX: cefoxitin, IPM: imipenem, NA: nalidixic acid, CIP: ciprofloxacin, OFX: ofloxacin, NOR: norfloxacin, GM: gentamicin, NET: netilmicin, C: chloramphenicol, SXT: trimethroprime sulfonamide, FT: nitofurantoin, TIC: ticarcillin, CFM: cefixime, ATM: aztreonam, DO: doxycycline and TE: tetracycline.

3.5. Genotypic detection of gene resisting to *bla*TEM and *bla*SHV

~~The Table 1 shows~~ The genotypic analysis of ~~that among~~ the GNB isolates revealed that ~~producing ESBL,~~ 25% did not have *bla*TEM and *bla*SHV genes, but 25% had at same time the two genes. Among the GNB producing ESBL, 3.5% of the strains had only *bla*TEM gene and 12.5% of the strains had only *bla*SHV gene. *K. pneumoniae* isolated from hospital specimen were the most carriers of the genes *bla*TEM and *bla*SHV then followed by ~~come the~~ hospital isolated *Enterobacter cloacae* strains. *Enterobacter agglomerans* and *E. coli* strains isolated from hospital and ESBL producers did not carry the *bla*TEM and *bla*SHV resistance genes either.

Comment [PA1]: Table 1 had nothing to do with the percentage of genes identified but the primers used.

4. DISCUSSION

The five most important isolated species in this study were *Klebsiella pneumonia*, *Acinetobacter* spp., *P. aeruginosa*, *E. coli* and *E. cloac~~e~~ae*. The 4.40% ~~was~~ recorded for *E. coli* appear lower than the 11% previously obtained by Ahoyo AT et al ~~–~~[36] in Benin. This observation suggest an improvement in the management of *E. coli* infections in the country~~ies~~. ~~This observation can also be due to the emergence of other species.~~

The proportion of in-patient~~nosocomial~~ isolates are~~is~~ high because the hospitals are clusters of patients with infections, increasing the probability of infecting~~to infected~~ “safe” people in the environment. The occurrence of hospital-associated infections is also favored by many factors including patient-related factors, exposures to invasive medical devices and deficiencies in quality of care [37]. The same is true for cleaning, disinfection and sterilization defects, which are also potential factors for infections associated with care [38-39].

The specimen containing the majority of these bacteria in this current study were ~~our work are~~ urine, pus and blood . These results corroborate those obtained in a study conducted in Saudi Arabia where urine specimen were more contaminated than those of pus [40]. Toudji et al. [41] in Togo noted ~~in there~~ that the pus specimen (47.90%) were the most contaminated than the urine (40.78%) and blood specimen (7.77%). Despite this result, the percentage of contaminated urine obtained in our study is higher than that obtained by Toudji et al. [41]. These results show that colonization of urine is becoming a major public health problem [42].

The proportion of GNB producing penicillinase is 57.07%, while non-producing *Enterobacteriaceae* is 43.93%. Studying *A. baumannii* for example, the second major strain in our study, Endimiani et al. [43] found in Italy that the proportion of penicillinase-producing strains was 42%, a result not far from ours.

In Camero~~o~~n, Gangoué-Piéboji et al. [34] reported 31% of GNB producing extended spectrum β-lactamases. This difference could be explained by the greater use of antibiotics, which increase the acquisition of resistance in this country. However, lowest (7.5%) rate of GNB producing extended spectrum β-lactamases have been ~~were~~ recorded in Barcelona [44]. A proportion of 75% of GNB producing extended spectrum β-lactamases strains was from hospital environment. During their investigations in Italy, Endimiani et al. [43] revealed that most of the strains isolated outside of the ~~from extra~~ hospital patients had previously been hospitalized at least once during the last 12 months before the study. Thus, there is probable likelihood between strains isolated from outside ~~extra~~ hospital specimen strains and hospitals.~~,~~ About 4.5% of the GNB producing extended spectrum β-lactamases were from newborns; indicating that healthy individuals could be infected as non-carriers [45]. The acquisition of resistance can be a result of horizontal transfer of resistance genes [46].

Imipenem shows the lowest resistance (9.8%) and is in agreement with ~~corroborates~~ that obtained by Ahoyo et al [36] and Toudji et al. [41]. Imipenem exerts a total inhibition on GNB that are resistant to many antibiotics such as cefotaxim, ceftriaxone, aztreonam and cefoxitin [47]. The loss of sensitivity to cephamycins (eg. cefoxitin) and carbapenems (e.g. imipenem), that is normally maintained even in ESBL-producing strains [48]. The reason for this high susceptibility in imipenem ~~,~~ is believed to be due ~~to resistance acquired by these bacteria due~~ to overuse~~abuse~~ of certain antibiotics; which calls for greater medical supervision. This

antimicrobial resistance occurs naturally over time, usually because of genetic modification but the excessive ~~or excessive~~ use of antibiotics speeds up the process [49]. The proportion of bacteria resistant to antimicrobials in Benin could be explained by self-medication in patients and the use of antibiotics of dubious quality. Dougnon et al. [50] proposed ~~erceived~~ that the quality of the antibiotic discs used for the performance of antibiograms could be a factor favoring the bacteria resistance.

GNB producing extended spectrum β-lactamases resisted more than those that do not produce. Aibinu et al. [51], in Nigeria, reported ~~founded~~ that most of ESBL producing ~~ESBL~~ *Enterobacter spp* were more resistant ~~resisted better~~ than non-producing ones. Indeed, the ability to synthesize such enzymes allows bacteria to face a greater number of antibiotics as shown by our results and those of Aibinu et al. [51]. Our results revealed the existence of three antibiotics (Cefuroxime, Cefoxitin and Nitofurantoin) to which non-ESBL-producing GNB isolates resisted better than those ESBL-producing did. This could be explained by the absence of these antibiotics in the spectrum of action of these enzymes whose ability of synthesis would result from a mutation of the β-lactamase gene, which acted against these antibiotics.

Certainly, extended spectrum β-lactam resistance genes strains have the *blaCTX*-M that is the third gene that codes for the production of ESBLs. No *E. agglomerans* strains carried $_{bla}$*SHV* gene and 12.50% of *E. cloacae* strains had both the gene $_{bla}$*TEM* and $_{bla}$*SHV* gene. This result can be explained by the fact that ESBL-type are very rarely found in certain genera of *Enterobacteriacea* such as *Enterobacter*, *Serratia*, *Citrobacter*, in which the chromosomal cephalosporinase is predominant [52]. It was observed in our study, ~~a~~ the ~~simultaneous~~ presence of both $_{bla}$*TEM* and $_{bla}$*SHV* (12.50% for *E. cloacae* and 12.50% for *K. pneumoniae*). These associations are frequently reported in several studies. Thus, it was reported in Algeria the association of TEM -CTX-M1 and SHV-TEM genes in a group of strains [53]. The associations TEM-SHV, SHV-CTX-M-1 and TEM-CTX-M-1 were also reported [54]. The coexistence of the different β-lactamases in the same series may pose a diagnostic and therapeutic problem [55].

The ESBL-producing strains of *K. pneumonia* in the hospital environment were the major carriers of $_{bla}$*TEM* (15.62%) and $_{bla}$*SHV* (12.50%). A previous study proves that the enzymes initially derive plasmid-like narrow-spectrum penicillinases-temoneira (TEM 1/2) and variable sulphhydryl (SHV-1) by the modification of their active site were observed mainly in hospital strains of *K. pneumonia* [56]. It should also be noted that the different types of ESBL (TEM and SHV) found in our study are part of so-called transferable ESBLs, which explains the ease of transfer between *Enterobacteriaceae* [57]. The presence of ESBL-producing hospital strains of different types can act as a tank of resistance genes, which can be transmitted horizontally to out of ~~extra~~ hospital or environmental strains. In addition, it is a risk factor for nosocomial epidemics [58]. Therefore, the inanimate environment of patients (surfaces and objects), contaminated by multi-resistant bacteria can serve as a secondary reservoir for cross-transmission [59]. We thus recommend to the patient to avoid the auto medication and to the practitioners to be sure of the strains before using antibiotics.

5. CONCLUSION

The study of GNB is an important scientific subject because of the strategic position of the gut, natural surroundings of life in the human body. The originality of our investigation was to explore the whole family by studying together its entire species encountered as pathogens in our environment. In this condition, bacteria living together often share their different characters over time. *K. pneumoniae*, *Acinetobacter spp*., *P aeruginosa*, *E. coli* and *E. cloacae* were founded in majority with the capacity to produce penicilinase. We observed that ESBL-producing strains of *K. pneumonia* in the hospital environment were the major carriers of $_{bla}$*TEM* and $_{bla}$*SHV*. However, this study would be interesting if the patient was followed for a long time. Especially with regard to patients visiting ~~coming~~ from the outside the country~~world~~. It would also be interesting to expand this study to other hospitals across the country. For the depth of this study to explain the observations of our study, it will be interesting to study further the other resistance genes.

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