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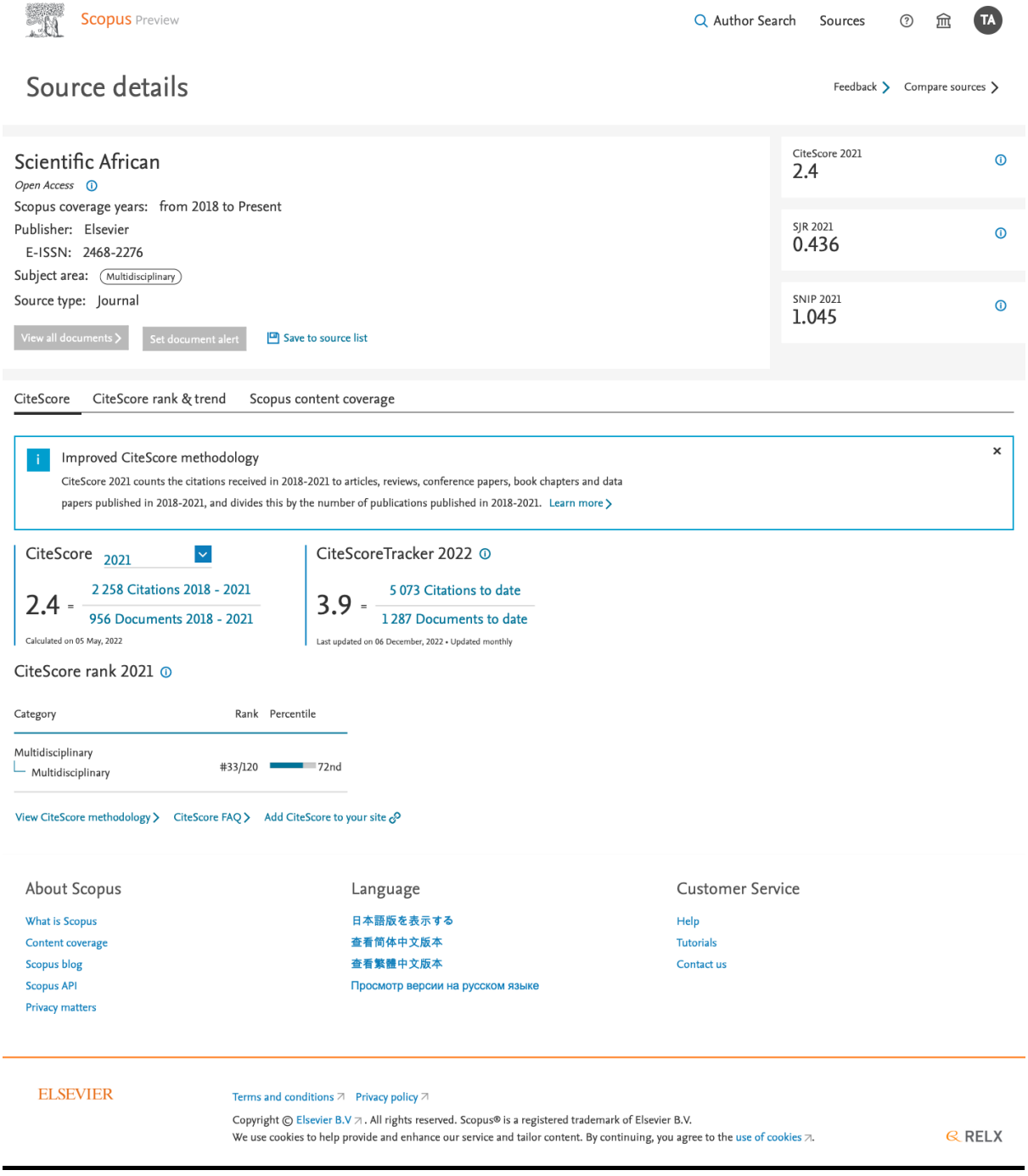

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Genetic diversity and virulence factors of Gram-negative bacilli isolated at the CHU-Z in Abomey-Calavi/So-Ava (Benin)

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ABSTRACT

Nosocomial infections are increasingly recurrent in health facilities and represent a serious public health concern. Apart from patients, health workers are also at high risk of infection. The risk factors associated with this type of infection are still not fully characterized. The present study aimed at characterizing Gram-negative bacillus strains isolated from surfaces and medico-technical equipment at the CHU-Z in Abomey-Calavi/Sô-Ava. 128 samples were collected by dry swabbing in five departments of the Abomey-Calavi University Hospital Center. Identification of the strains and antibiograms were done using the API 20 E Gallery and CASFM recommendations. The pathogenic potential of the isolates was evaluated by i) analyzing the biofilm formation ability and ii) examining the presence of the beta-lactam resistance gene (blaSHV). In addition, *E. coli* strains were analyzed for their enterohemorrhagic potential through the screening for the gene encoding for shigatoxin (stx). The proportion of contaminated samples by enterobacteria strains was 23.43%. Twelve species of Gram-negative bacillus were identified with a high predominance of *Klebsiella oxytoca* (20%), followed by *Acinetobacter baumanni* (16.66%), *Chryseomonas luteola* (13.33%). Most strains were resistant to tetracycline (87%) and ceftriaxone (80%). However, most of them were sensitive to norfloxacin (17%), ciprofloxacin (17%), and imipenem (13%). All strains of *Escherichia coli, Enterobacter sakazakii, Klebsiella terrigena, Serratia rubidaea, Citrobacter youngae* and 50% of *Chryseomonas luteola* formed biofilm. The results of PCR amplification showed that 6.66% of the strains carry the blaSHV gene and none of *E. coli* strains have the gene coding for Shigatoxin. These data suggest a significant risk of severe infections

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for patients and health workers at the CHU-Z in Abomey-Calavi/Sô-Ava. Additional investigations are required to better characterize the presence of pathogenic strains in hospital environment.

Introduction

Nosocomial infections, also called healthcare-associated infections, are infections acquired by a patient during a hospital stay that were neither present nor incubating at the time of admission [1]. Nosocomial infections are gradually becoming a major public health problem due to their frequency and social and economic impact [2–3]. Indeed, nosocomial infections are often polymicrobial [4] and can be caused by the contamination of staff hands during epidemics [5]. Among the commonly isolated bacteria in hospital diseases, Gram-negative bacilli are the leading cause of death [6–8].

Although it is difficult to accurately assess surfaces and hospital environment in the origin of nosocomial infections, recent work mentioned the presence of Gram-negative bacillus strains in the hospital environment of the CNHU in Cotonou [9] and the CHU-Z Abomey-Calavi/Sô-Ava [10]. This confirms that hospital surfaces are microbial reservoirs that can contaminate the hands of caregivers or patients directly [11]. In addition, bacterial resistance to antibiotics is a longstanding phenomenon and remains a major public health problem nowadays [8]. The situation appears particularly worrying in hospitals, where staphylococci and some Gram-negative bacilli, including *Enterobacteriaceae*, are often responsible for multi-resistant strains infections [8]. Infection and carriage rates increase with the use of antimicrobial agents, which generally leads to the development of broad-spectrum beta-lactamases, which also induce resistance to antibiotics [12,13].

Taking into account recent work in Benin, and particularly at the CHU-Z of Abomey-Calavi/Sô-Ava, which does not address the pathogenicity and molecular aspect of Gram-negative bacilli, the present study aims to investigate the virulence factors and the molecular characterization of Gram-negative bacillus strains isolated from surfaces and technical equipment at the CHU-Z of Abomey-Calavi/Sô-Ava.

Material and methods

Sampling and samples collection

Samples were collected from five medical units of CHU-Z Abomey-Calavi/Sô-Ava, taking into account their classification according to the infectious risk [14]: i) moderate risk zone [central sterilization room (7 samples)]( ); ii) high-risk area [maternity (8 samples) and pediatrics (10 samples)]( ); and iii) very high-risk area [neonatology (10 samples) and operating room (7 samples)]( ). Per sampling, the number of points where samples are taken varies from five to eight. Thus, five points were sampled from the central sterilization room and operating room, six from the neonatology, and eight from maternity and pediatrics. The targeted sample collection points include beds, soils, trolleys, baby toilet tables, weighs baby, benchtops, cupboards, cesarian boxes, etc.

A sampling of surfaces and medico-technical equipment was performed using the dry swab method according to ISO/DIS14698–1 standards. In brief, swabs were passed over the targeted point in parallel streaks close together by rotating them slightly. The swabs were then returned to their protective cases. Collected samples were carried on ice (4 °C) to the laboratory for microbial analysis. A total of 128 samples were collected for this study.

Microbiological analyses

Identification of gram-negative bacillus

Once in the laboratory, 5 ml of Mueller Hinton broth was added to each case. The cases were then incubated for 24 h at 37 °C. After this incubation period, cloudy cases indicating bacterial growth were chosen for germ tests. Isolation of Enterobacteriaceae was performed on Eosin Methylene Blue (EMB) agar. Isolation was performed by subculturing the isolated colony to obtain a pure culture. Colonies from the pure culture were used to identify Enterobacteriaceae with API 20 E commercial kit (Biomerieux, Marcy l'Etoile, France). Identification of isolated colonies was performed by plating with Enterobacteriaceae API 20 E, commercial kit (Biomerieux, Marcy l'Etoile, France).

Antibiotic susceptibility of isolates

The susceptibility of isolates to 10 conventional antibiotic molecules was analyzed using the diffusion method [15]. The antibiotics tested are amoxicillin-clavulanic acid (AC 30 µg), ceftriaxone (CI 30 µg), cefoxitin (FOX 30 µg), gentamicin (G 10 µg), imipenem (IMP 5 µg), fosfomycin (FOS 50 µg), erythromycin (E 15 µg), tetracycline (TE 30 µg), norfloxacin (NOR 30 µg), ciprofloxacin (CF 5 µg).

Biofilm formation

The capacity to produce biofilm of the isolated microorganisms was determined *in vitro* according to a previously described method [16]. Thus, 24-well microplate was used to qualitatively assess the formation of biofilms due to the appearance of a visible film.Briefly, 10 µl of 18 h old Gram-negative bacillus bacteria suspension was diluted with 150 µl of Brain Health Infusion (BHI) before incubating for 24 h at 37 °C. After incubation, wells were washed three times with about 0.2 ml sterile physiological. Biofilms formed by the adhesion of sessile organisms to the microplate in each well were stained with crystal violet (0.1%) for 10 min. The excess of dye was removed by thorough washing, and the plates were left at room temperature to dry [17]. Violet color indicates a positive well.

Molecular characterization

DNA extraction

DNA extraction was done as previously described by Rasmussen and Morrissey [18]. Briefly, 1.5 ml tubes containing bacterial strains were centrifuged for 5 min at 12,000 rpm. After discarding the supernatant 500 µl of sterile distilled water was mixed with the bacterial pellet. The mixture was then heated for 15 min at 95 °C and centrifuged for 5 min at 12,000 rpm. The supernatants were recovered in new tubes, and 500 µl of absolute ethanol was added before centrifugation for 5 min at 12,000 rpm. The DNA pellets were suspended in 50 µl sterile distilled water and maintained at 4 °C for immediate use or at −20 °C for long-term storage.

The 16S rRNA gene primers [19] were used for the molecular confirmation of the *Acinetobacter baumanii* isolates. The used primer sequences are F (5′- CGGGATCCCAGGCCCGGGAAC-3′) and R (5′- GTGCCAGCAGCCGCGGTAAT-3′). The PCR reaction was performed on 20 µl containing 14 µl 10x GoTaq mix (PROMEGA, USA); 0.5 µl primer F (10 µM); 0.5 µl primer R (10 µM) and 5 µl extracted DNA. The amplification program is composed of an initial denaturation (95 °C for 5 min); 35 cycles of denaturation cycles (95 °C, 30 s); hybridization (50 °C, 30 s), and elongation (72 °C, 30 s) and a final elongation (72 °C, 10 min).

Detection of genes encoding for toxins

The primers used to target the *blaSHV* gene were those designed by Pagani et *al.* [20]. The primer sequences are: *blaSHV* F: 5′- ATGCGTTATATTCGCCTGTG −3′ and *blaSHV* R: 5′- TTAGCGTTGCCAGTGCTC-3′. To target the Sxt gene, the primer used were F: 5′- GAAGAGTCCGTGGGATTACG-3′ and R: 5′- AGCGATGCAGCTATTAATAA −3′ [21]. The PCR reaction was performed on 20 µl containing 14 µl 10x GoTaq mix (PROMEGA, USA); 0.5 µl primer F (10 µM); 0.5 µl primer R (10 µM) and 5 µl extracted DNA. The amplification program was composed of an initial denaturation (95 °C for 5 min); 35 cycles of denaturation cycles (95 °C, 30 s); hybridization (50 °C, 30 s), and elongation (72 °C, 30 s) and a final elongation (72 °C, 10 min). The expected fragment size of the PCR products is 400 pb (for *blaSHV*) and 150 bp (for Stx1).

Data processing and analysis

The data collected was coded and processed using Microsoft Excel 2016 spreadsheet software. Graph Pad Prism 8 software was used for statistical analyses. Student T and Fischer tests were used for number series. Significance was accepted when $p < 0.05$.

Results

Gram-negative bacillus contamination rate

Out of the 128 samples, 23.43% were contaminated by the Gram-negative bacillus strains. However, considering the targeted units, it appears that pediatrics was the most contaminated (42%) by Gram-negative bacilli, followed by neonatology (33%). The central sterilization unit is the least contaminated by Gram-negative bacilli, with a rate of 20% (Fig. 1).

Identification of gram-negative bacillus strains

Using the API 20E gallery, 12 Gram-negative bacillus species have been identified. Thus, it should be noted that the major species is *Klebsiella oxytoca* (20%), followed by *Acinetobacter baumanni* (16.66%). In contrast, the weakly represented species were *Enterobacter amnigenus, Klebsiella terrigena, Serratia rubidaea, Pantaea* spp, *Citrobacter youngae* (3.33%) (Fig. 2).

Susceptibility of isolates to antibiograms

Antibiotic resistance has enabled us to assess the level of sensitivity of our strains of Gram-negative bacilli species to antibiotics.

Variable resistance levels were observed, ranging from 13% to 87% (Fig. 3). Indeed, the highest resistance rates were observed with tetracycline (87%) and ceftriaxone (80%). Lower rates of resistance were observed with norfloxacin (17%), ciprofloxacin (17%), and imipenem (13%).

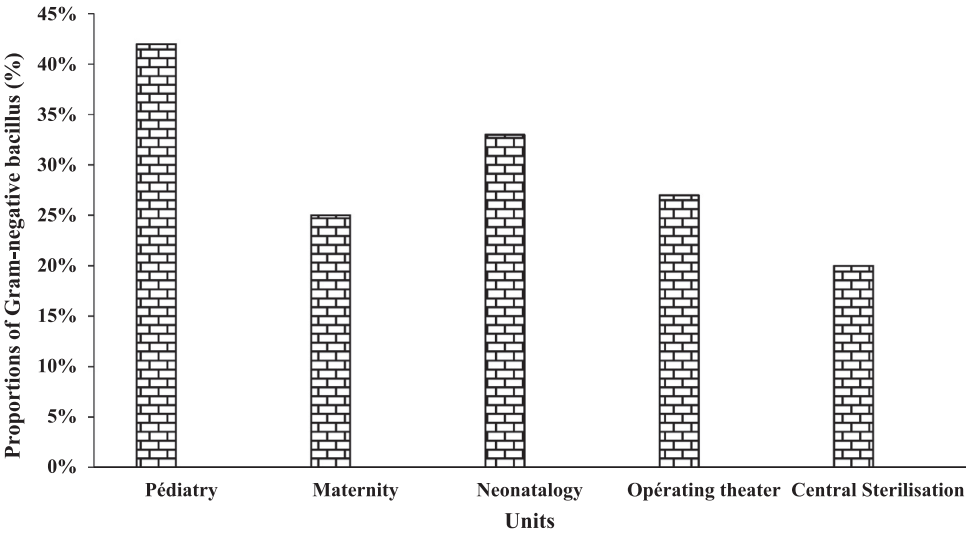

Fig. 1. Distribution of Gram-negative bacillus contamination in investigated units.

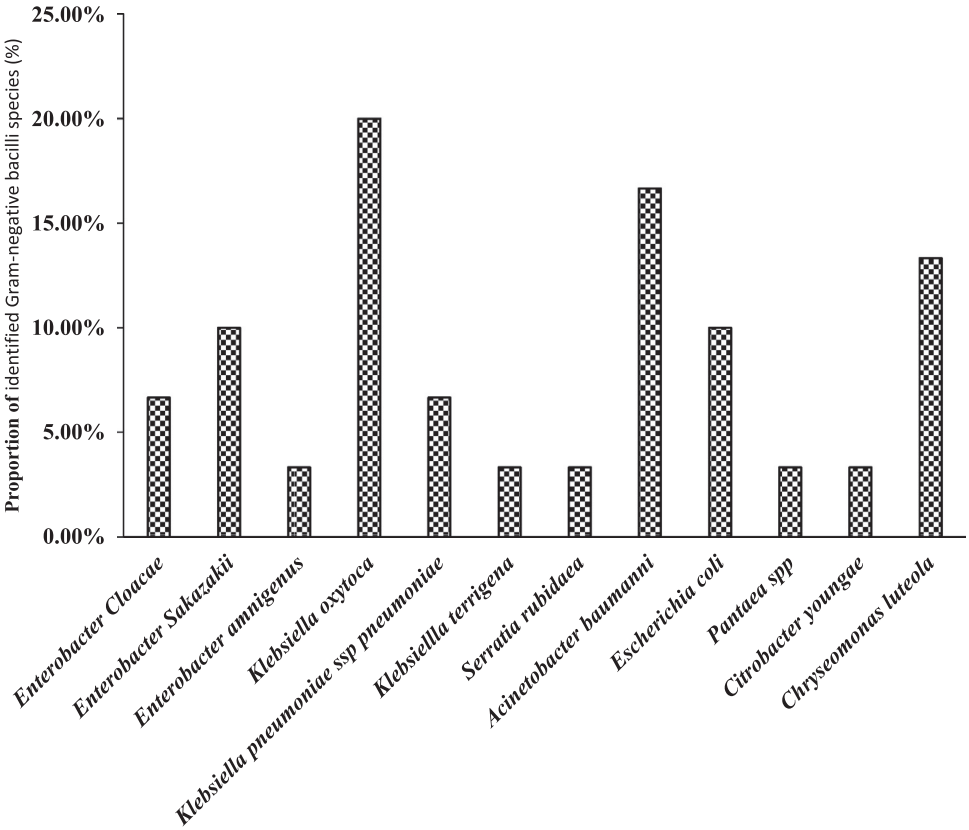

Fig. 2. Distribution of identified Gram-negative bacilli species.

TE30: tetracycline, NOR: norfloxacin, AC30: amoxicillin-clavulanic acid, IMP: imipenem, CF5: ciprofloxacin, E15: erythromycin, FOS50: Fosfomycin, FOX 30: cefoxitin, G10: gentamicin, CI30: ceftriaxone.

Biofilm formation

All strains of *E. coli, Enterobacter sakazakii, Klebsiella terrigena, Serratia rubidaea, Citrobacter youngae* were biofilm formers, whereas only half of the *Chryseomonas luteola* species were biofilm-forming. In contrast, no biofilm formation was observed in *E. cloacae, E. amnigenus, K. oxytoca, K. pneumoniae,* and *A. baumanni* (Fig. 4).

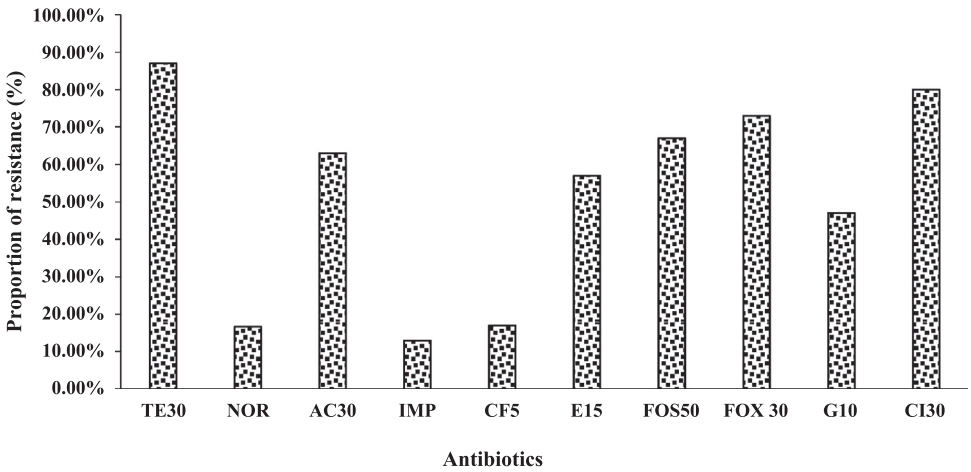

Fig. 3. Variability of the resistance rates of the strains isolated to tested antibiotics.

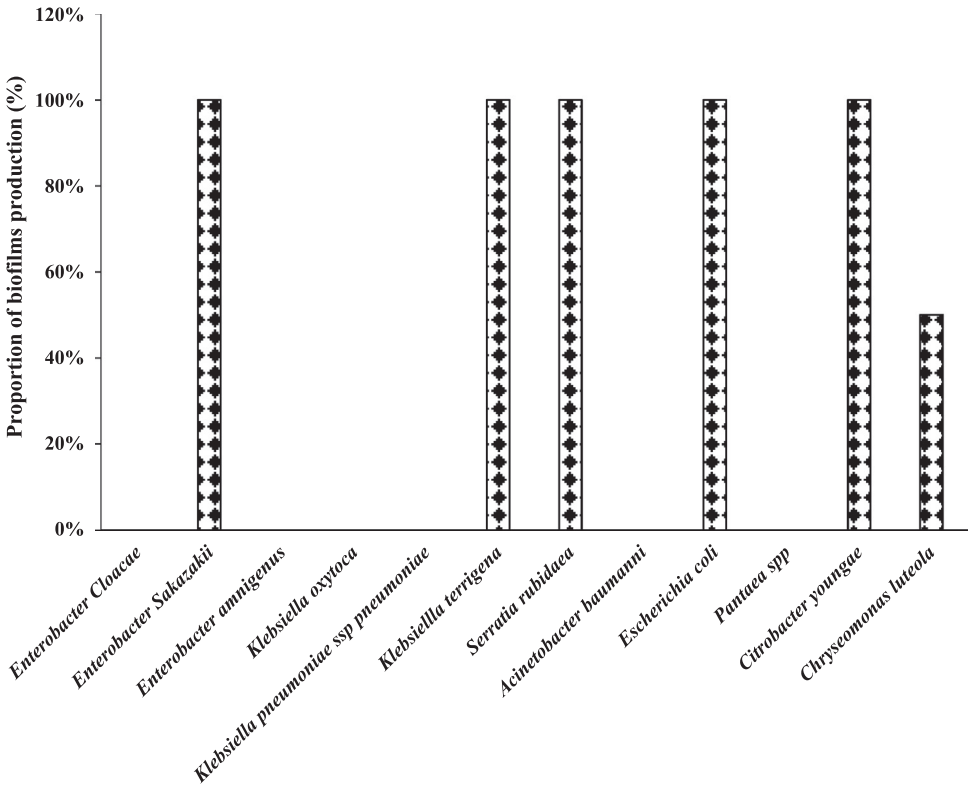

Fig. 4. Production of biofilms by the isolated strains.

Search for the genes of shiga toxins and blaSHV

No *E. coli* strain expressed this virulence gene. However, from the 30 strains of Gram-negative bacilli obtained, 6.66% host blaSHV gene, which codes for resistance to beta-lactams.

Discussion

This study assesses the level of pathogenicity of strains of Gram-negative bacilli isolated from the hospital environment of CHU-Z Abomey-Calavi/Sô-Ava. Overall, 23.43% of our samples were contaminated with Gram-negative bacilli. Our results are similar to the 21% obtained in Morocco on samples taken from the hospital environment [22]. The presence of Gram-

negative bacilli strains in hospital environment suggests insufficient hygiene measures. In addition, it was observed that the pediatric unit was the most contaminated by Gram-negative bacilli. Recent work has also reported this high contamination of the pediatric department by other germs [9–10]. The highest contamination level of the pediatric unit could be explained by the fact that this unit receives more patients, especially children, and therefore more visitors since children are usually accompanied by their parents.

Considering the identification of the isolated strains, it should be remembered that a total of 12 Gram-negative bacilli species were isolated. The predominant species was *Klebsiella oxytoca* followed by *Acinetobacter baumanni*. Different results have been reported at Suru-Léré University Hospital in Cotonou, where the *Enterococcus faecalis* species was more represented [23]. The strong presence of Enterobacteriaceae could be explained by the fact that these bacteria adhere easily to surfaces and have a high survival capacity. Indeed, they preferentially attack intubated patients who have weaker immunity, like patients with catheters and who are immunocompromised [24]. These observations justify that the hospital environment is an ecological niche for opportunistic microorganisms, which mainly cause nosocomial infections [25].

For years, clinicians have been facing multidrug-resistant infections linked to the strong selective pressure undergone by microorganisms in hospital settings through the excessive use of antibiotics and disinfectants. Regarding the resistance of our strains to various antibiotics, a strong resistance to tetracycline and ceftriaxone was noticed. However, imipenem was shown to be the most effective against Gram-negative bacilli. Similar observations were made in Algeria and Morocco where, except for imipenem, sensitivity to the antibiotics tested shows total resistance to the majority of 3rd generation cephalosporins, as well as very frequent resistance to aminoglycosides; fluoroquinolones and colistin [22]. Imipenem is the most effective against isolated Gram-negative bacilli as previously reported [26].

Bacteria use biofilm formation to survive unfavorable environmental conditions. Biofilm formation results from a massive production of alginate (polymer of D-mannuronic acid and L-guluronic acid) used as a barrier against the host defenses and antibacterial. This promotes bacterial persistence and virulence [27]. In our study, *E. coli, Enterobacter sakazakii, Klebsiella terrigena, Serratia rubidaea, Citrobacter youngae* displayed the capacity to form biofilm. However, no biofilm formation was observed with *E. cloacae, E. amnigenus, K. oxytoca, K. pneumoniae,* and *Acinetobacter baumannii*. Few data exist on biofilm formation by Enterobacteriaceae, which makes it difficult to compare our results with the current literature in the field. However, new effective control strategies need to be investigated. Indeed, it was reported that minimum effective antibiotics concentrations on sessile bacteria are 10 to 1000 times higher than those effective in their planktonic form [28].

The blaSHV gene was found in two strains (*Enterobacter amnigenus* and *Serratia rubidaea*) which are resistant to Fosfomycin and ceftriaxone. It should be remembered that these two antibiotics belong respectively to the family of glycopeptides and cephalosporins, which are included in the large group of beta-lactams. Also, those two strains are isolated in the neonatology unit. This highlighted the high risk incurred by newborns, their parents, and health personnel. Similar results on the blaSHV gene were obtained by Degbey et al. [23] on the *Enterobacteriaceae* strains isolated at CHU Suru-Léré in Cotonou.

In several studies, the majority of VHS-type ESBLs have been described in strains of K. pneumoniae. However, these enzymes can be found in other species, such as Citrobacter *freundii, Citrobacter diversus*, *Escherichia coli, Enterobacter cloacae* [29]. Although *Enterobacteriaceae* possessing genes encoding beta-lactamases are observed in our hospital strains, the possibility of disseminating these multidrug-resistant germs in the community is increasingly worrying. Since the transmission, mainly plasmid, of the genes encoding ESBLs is responsible for their rapid dissemination and, thus, for the increase in the prevalence of ESBL-producing bacteria worldwide [30]. The other phenotypic resistance to the beta-lactam families observed in our study may be linked to the involvement of other genes, such as blaTEM and blaCTX-M.

Conclusion

The present study suggests that the CHU-Z of Abomey-Calavi/Sô-Ava is highly exposed to nosocomial infections mainly due to Gram-negative bacilli. Since these germs are often transmitted by contact with fomites, hygiene measures in and around this hospital should be improved in order to reduce infection risk. The present study also raises the thorny issue of the multi-resistance of bacterial strains mainly due to self-medication. We recommend public awareness campaigns on the risks associated with self-medication and its implications for immune reactivity.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data availability

The data used to support the findings of this work are available from the corresponding author upon request.

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