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# Diversity and Antibiotic Susceptibility of Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* from the Urine of Human Population Using Bacteria-Contaminated Water in Butembo Urban Area (DR Congo, Central Africa)

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## Authors' contributions

This work was carried out in collaboration among all authors. Authors MN, MW, EBMT, ISK, MAN and PAN designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MW, MN and RN managed the analyses of the study. Authors MN, GB and TSN managed the literature searches. All authors read and approved the final manuscript.

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**ABSTRACT**

**Aims:** This study aims to investigate the extended-spectrum beta-lactamases (ESBLs)-producing *Enterobacteriaceae* in the human urine samples and to understand the potential influence of the contaminated water the urine donors use.

**Study Design and Methodology:** One hundred urine samples from females and the same number from males were collected from donors of different age groups and analysed. One hundred urine samples from females and the same number from males were collected from donors of different age groups. The enterobacterial isolation was done on CLED agar medium. Their identification was carried out first by the enzymatic technic, then using MALDI-TOF MS method. ESBLs-producing enterobacteria were identified by searching the synergy between Clavulanic acid and 3<sup>rd</sup> generation Cephalosporins. The antimicrobial susceptibility tests were carried out using the disk diffusion method.

**Results:** In male urine samples, the identified ESBLs-producing strains belong to *E. coli*, *E. agglomerans* and *K. pneumoniae*. They were in 11 samples: 3 from donors aged 20 years or less, 3 also from those aged 21 to 40 years as well as in those aged 41 to 60 years, and 2 in samples from donors aged over than 60 years. In female urine samples, the identified ESBLs-producing strains belong to *Escherichia coli*, *Enterobacter agglomerans*, *Yersinia frederiksenii*, *Salmonella typhi* and *Klebsiella pneumoniae*. They were in 27 samples: 3 from donors aged 20 years or less, 9 from those aged 21 to 40 years, 12 from those aged 41 to 60 years, and 3 from donors aged over than 60 years. All the strains were sensitive to Imipenem and resistant to Cefotaxim, Ceftazidim, Amoxicillin+Clavulanic acid and Nitrofurantoin. The Multi-Antibiotic Resistance (MAR) index varied from 0.416 (*E. coli* strains, youngest male donors) to 0.916 (*E. coli* strains, old female donors). Most of strains react differently from one antibiotic to another ( $P < 0.05$ ), with the exception of *E. coli* strains from most samples. Most of the identified ESBLs-producing species have been reported in surface and groundwater the population use. These water resources could play a role in the human urinary tract infections.

**Conclusion:** There is a serious health problem with great MAR indices for all bacterial species identified. Treating water before use could reduce the viability and spreading of ESBLs-producing bacteria and genes into the population.

**Keywords:** Human urine; ESBLs-producing enterobacteria; diversity; antibiotic susceptibility profile; bacteria-contaminated water used.

**1. INTRODUCTION**

"Beta-lactamases are a class of diverse enzymes produced by bacteria that degrade the beta-lactam ring, inactivating the beta-lactam antibiotic. Beta-lactamase production in Gram-negative pathogenic bacteria is a public health concern due to the possibility of therapeutic

failure, serious consequences for infection control and increased risk of morbidity and mortality in animals and humans" [1,2]. "Some beta-lactamases are encoded by mobile genetic elements (e.g., plasmids); others are coded on by chromosomes. Both mechanisms give the affected bacteria the ability to hydrolyze a wide variety of Penicillins and Cephalosporins" [3,4].

"There is a diversity of beta-lactamases, sometimes classified by the Ambler classification system on the basis of molecular structure into 4 classes A, B, C and D. Classes A, C and D have a serine residue on the active site, while class B enzymes also called metallo-beta-lactamases, have zinc in the active site. Extended-spectrum beta-lactamases (ESBLs) belong to class A" [4,5].

"Beta-lactamases classifications can also be based on the proteins structure, on their activities or according to their clinical role. The criteria of the classification based on the function are among others, the nature of the substrates and the relative affinity for these substrates" [6-8]. "Members of ESBLs family commonly express beta-lactamases which confer resistance to expanded-spectrum (extended-spectrum) Cephalosporins. The prevalence of ESBL-producing bacteria varies from one geographical area to another" [9,10]. They cause the inactivation of broad-spectrum oxyimino-Cephalosporin (third- and fourth-generation) and Monobactam (Aztreonam) but not Cephamycin (Cefoxitin) or Carbapenems (Meropenem, Imipenem, Ertapenem, and Doripenem) [5,11,12]. Generally, "these enzymes are neutralized by beta-lactamase inhibitors such as Clavulanic acid, Sulbactam, and Tazobactam" [5,11].

"Genes that encode ESBLs are mostly found on transposons or insertion sequences of plasmids in association with other resistance genes. As a result, they can spread rapidly, causing resistance to multiple antimicrobials such as Aminoglycosides, Trimethoprim, Sulphonamides, Tetracyclines Chloramphenicol, and Fluoroquinolone" [5,6]. Most ESBL-producing bacteria are of *Enterobacteriaceae* family [13,14]. Several of them are commonly involved in human infections. This can involve the digestive tract, the respiratory system, blood circulation, the skin or the urinary tract [15].

"Concerning urinary tract, it is known that urine is formed by nephrons present inside the kidneys. In normal healthy individuals, there are several mechanisms that attempt to prevent bacteria from invading the bladder or progressing up through the upper urinary tracts" [16,17]. "The ability of the pathogen to produce infection is influenced by the virulence of the specific pathogen and individual's specific immune response" [16,18]. The urinary tract infection (UTI) can be caused by bacterium that invade

the urinary epithelium cells causing irritation and inflammation of these cells [19]. "The symptoms include a burning feeling when urinating, urine that looks cloudy, urine that appears red, bright pink or cola-colored, or strong-smelling urine" [18]. "Most of the bacterial organisms concerned in the UTIs are sometimes from the gastrointestinal tract and can be linked to the poor microbiological quality of food or water consumed" [20].

"In most African cities in general and in Butembo (DR Congo, Central Africa) in particular, the majority of wastewater is discharged into nature without any treatment, due to the non-existence of wastewater treatment plants. Fecal and non-fecal contents of this wastewater can be found in seepage and then contaminate groundwater. Rainwater, which often feeds groundwater following infiltration, can sometimes be polluted" [21,22]. "The bacteria-contaminants contained in seeping water are rarely all retained before reaching the water table because this process depends on several factors" [23-25].

In Butembo as in most African cities, a large part of the population uses stream and groundwater to meet their water needs, due to the insufficiency of public water supply or channeling difficulties linked to the relief [26,27]. In the natural environment, ESBLs-producing bacteria can be found in various aquatic biotopes including surface water and ground water [14]. In urbanized area of Butembo, various surface and ground water points are contaminated by ESBLs-producing bacteria. Their abundances and diversity in these aquatic environments undergoes significant spatio-temporal variation [14]. The strains antibiotics susceptibility varied depending on the antibiotics, bacterial species and type of aquatic environment hosting the microorganism [28]. Several urinary infections cases among others are often reported in hospitals and populations of Butembo. Little is known about the potential relationships between the reported urinary infections in populations and the poor water microbiological quality they use. It is sometimes indicated that several pathogenic enterobacteria can migrate from the gastrointestinal tract after oral ingestion to other functional systems of the human body [13,15,18]. This study aims to investigate the diversity and antibiotic susceptibility of ESBLs-producing *Enterobacteriaceae* in the human UTI in Butembo and understand the potential role of the contaminated water that the population uses in the transmission of this infection.

## 2. MATERIALS AND METHODS

### 2.1 Urine Samples Collection

The study was carried out in the city of Butembo (DR Congo) from May to July 2023. Urine samples were taken from people who came to the UCG Butembo Central Research Laboratory with a suspected urinary tract infection. They were collected in sterile bottles. Morning urine collection was recommended, or at least 3 hours after the last micturition. The study considered a total of 200 urine samples collected from donors of both sexes, namely 100 samples from females and 100 others from males. Due to the abundance of complaints of relatively high infections for certain age groups compared to others within the populations, the study considered for each sex, 6 samples for donors whose age was younger or equal to 20 years old, 54 samples for donors aged from 21 to 40 years, 34 samples for those aged from 41 to 60 years, and 6 for donors aged over than 60 years.

### 2.2 Isolation and Identification of *Enterobacteriaceae*

The *Enterobacteriaceae* isolation was done after cytological examination of the urine samples. This cytological examination looks for elements such as inflammatory cells (white blood cells, bacteria, among others) and crystals, allowing us to have an idea respectively of a potential infection and of renal functioning.

After homogenization of each of the urine samples, the isolation of the *Enterobacteriaceae* was done by spreading on the surface on Cystine Lactose Electrolyte Deficient (CLED) agar medium (W/ANDRADE INDICATOR) poured into petri dishes and then incubated at 37°C for 18 to 24 hours [29,30]. The macroscopic observation consisted of examining the cultural characters of the bacteria expressed in colonies. These include the color, size, contours and surface configuration of the colony [29,30].

"After sub-culture of each strains in monospecific conditions on agar medium of the strains, their identification was carried out first by the enzymatic method, at Central Research Laboratory, Faculty of Pharmaceutical Sciences, Catholic University of Graben (DR Congo), then using the Matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS), at the Pasteur Center of Yaounde (Cameroon)" [31-33]. The mass-to-

charge ( $m/z$ ) ratios are electrodynamic measurements of how quickly charged ions from the clinical sample material move through the TOF tube and reach a detector.

"From 18 to 24 hours culture, selected colony was applied onto MALDI test plate. Samples were then overlaid with matrix and dried. The plate was subsequently loaded into the MALDI-TOF MS instrument : the sample was bombarded by the laser. This bombardment resulted in the sublimation and ionization of both the sample and matrix. These generated ions were separated based on their mass-to-charge ratio via a TOF tube, and a spectral representation of these ions was generated and analyzed by the MS software, generating an MS profile. This profile was subsequently compared to a database of reference MS spectra and matched to either identical or the most related spectra contained in the database, generating an identification for bacteria and analyzed by software associated with the respective system, allowing rapid identification of the microorganism" [32-34].

### 2.3 Detection of ESBLs-Producing *Enterobacteria*

ESBLs-producing enterobacteria have been highlighted by the search for synergy between Clavulanic acid and 3<sup>rd</sup> generation Cephalosporins. Enzymes are highlighted by the disc method which consists of looking for an image of champagne cork synergy between an antibiotic disc which contains a  $\beta$ -lactamase inhibitor and the 3<sup>rd</sup> generation Cephalosporin discs. The antibiotic discs used were Ceftazidim (30  $\mu$ g), Cefotaxim (30 $\mu$ g), Ceftriaxon (30 $\mu$ g), Amoxicillin/Clavulanic Acid (20/10 $\mu$ g) and Aztreonam (30 $\mu$ g). The production of ESBLs results in the appearance of an image of "Champagne cork" synergy between the discs of Amoxicillin/Clavulanic Acid and 3<sup>rd</sup> generation Cephalosporins [35].

### 2.4 Antibiotic Susceptibility Tests

The antimicrobial susceptibility tests were carried out using the disk diffusion method according to the recommendations of the FMS-EUCAST [36]. The medium used was Müller Hinton agar (Biorad) poured into Petri dishes. The thickness of the agar was approximately 4 mm. The surface of the agar medium was dried before use. From an 18-24 h culture on non-selective agar medium (Plate Count Agar), a bacterial suspension in saline solution (0.9% NaCl) with a

turbidity equivalent to that of the standard 0.5 of the range of McFarland was carried out, which corresponds to a bacterial density of approximately  $1 \times 10^8$  CFU/100 mL. The inoculum was then diluted 1/10 ( $1 \times 10^7$  CFU/100 mL) before inoculation.

The agar was inoculated with the bacterial inoculum using swab method. The entire surface of the agar medium was swabbed in three directions. The antibiotic discs were placed on the surface of the inoculated and dried agar. The gap between the discs was 3 cm in order to avoid overlapping of the inhibition diameters. The Petri dishes were then incubated within 15 min following the depositing of the discs, at 37 °C aerobically for 24 h.

"The antibiotic molecules considered depend on the uses of the population and also on their availability in the laboratory. Those considered were Ceftriaxon, Cefotaxim, Aztreonam, Cefotaxim, and Amoxicillin+Clavulanic acid, Gentamicin, Ciprofloxacin, Chloramphenicol, Doxycyclin, Imipenem, Nalidixic acid and Nitrofurantoin. The inhibition diameters (ID) were measured using the caliper and the results were scored as resistant, sensitive or intermediate according to CA-SFM recommendations" [36,37].

## 2.5 Data Analysis

"The ESBLs-producing strains were grouped per species. After the antibiotic susceptibility tests, the percentages of sensitive, resistant or intermediate strains were calculated for each species. The comparison using the Kruskal-Wallis test has been carried out amongst the inhibition diameters values for all ESBLs-producing strains for the same antibiotic, then from one antibiotic to another for the same strains per sex and age of urine donor. The average values of the inhibition diameters (and standard deviations) have been calculated for each ESBLs-producing species, considering the sex and age of urine donor patients, then illustrated by histograms. The isolates which displayed resistance to three or more than three classes of antibiotics were designated as multidrug resistant bacteria. The Multi-Antibiotic Resistance (MAR) Index was calculated by using the following formula" [38]:

MAR Index =

$$\frac{\text{Number of antibiotic to which an isolate showed resistance}}{\text{Total number of antibiotics used}}$$

## 3. RESULTS

### 3.1 ESBLs-Producing Enterobacteria Isolates

Amongst the 100 urine samples given by male donors, 19 samples were infected with bacterial strains belonging to the *Enterobacteriaceae* family. They included 3 samples from donors aged 20 years or less, 7 from those aged 21 to 40 years, 7 from those aged 41 to 60 years and 2 from those aged over 60 years. Amongst the 19 *Enterobacteriaceae* strains, 11 produced ESBLs and were distributed as follows: 3 strains in the urine samples of donors aged 20 years or less, 3 also in those of donors aged 21 to 40 years old as well in those of donors aged 41 to 60 years, and 2 in urine samples of donors aged over 60 years.

Amongst the 100 urine samples given by female donors, 48 samples were infected with bacterial strains belonging to the *Enterobacteriaceae* family. They included 3 samples from donors aged 20 years or less, 18 from those aged 21 to 40 years, 24 from those aged 41 to 60 years, and 3 from those aged over 60 years. Amongst the 48 *Enterobacteriaceae* strains, 27 produced ESBLs and were distributed as follows: 3 strains in the urine samples of donors aged 20 years or less, 9 in those of donors aged 21 to 40 years, 12 in those of donors aged 41 to 60 years, and 3 in urine samples of donors aged over 60 years. Abundance of ESBLs-producing strains as well as the number of strains with respect to bacterial species is presented in Table 1. It can be noted that in the urine from male donors, the majority of ESBLs-producing strains are *Escherichia coli*, followed by *Klebsiella pneumoniae*. In the urine of female donors, most of ESBLs-producing strains are also *E. coli*, followed by *Enterobacter agglomerans*, *Yersinia frederiksenii*, *Salmonella typhi* and *K. pneumoniae*. The number of ESBLs-producing strains seems to vary first with the sex of the patients, then with their ages (Table 1).

### 3.2 Antibiotics Inhibition Diameters Against ESBLs-Producing Enterobacteria Isolates

The identified ESBLs-producing bacterial strains belong to *E. coli*, *K. pneumoniae*, *E. agglomerans*, *S. typhi* and *Y. frederiksenii*. For each bacterial species, the average value of the inhibition diameter for all the strains identified in

the presence of each antibiotic was calculated with respect to donor sex (male or female) and age group. These inhibition diameters are presented in Fig. 1. It is observed for all the strains of each of the 5 species that the highest inhibition diameters were recorded in the presence of Imipenem.

With *Y. frederiksenii* strains isolated from the urine of only female donors aged from 41 to 60 years, the Smallest Inhibition Diameter (SID) was 8mm recorded in the presence of Ceftazidim. In the presence of *S. typhi* strains isolated only from urine samples of female donors aged from 41 to 60 years too, the SID was 6mm, recorded in the presence of Aztreonam and Nalidixic acid. With *K. pneumoniae* strains isolated from the urine samples from females aged from 20 years or less, it was 6mm, recorded in the presence of Ceftazidim, Ciprofloxacin and Chloramphenicol. For other strains of the same species isolated from male aged over 60 years, it was also 6mm, recorded in the presence of Ceftazidim and Amoxicillin+Clavulanic acid. These SID with *E. agglomerans* strains isolated in the urine of only females aged from 21 to 40 years was 8mm, recorded in the presence of Ceftriaxon.

Several ESBLs-producing *E. coli* strains have been identified in the urine of patients of both sexes and of various age groups. In the urine of males aged from 20 years or less, the SID was 6.33 mm recorded in the presence of Doxycyclin. Still in the urine of males aged from 21 to 40 as well as those aged from 41 to 60, it was 7mm all recorded in the presence of Nitrofurantoin. For strains isolated from the urine of females aged from 20 years or less, it was 6.5 mm, recorded in the presence of Cefotaxim and Ceftazidim. In the urine of females aged from 21 to 40 and from 41 to 60 years, it was 9.12 mm and 6.66 mm, respectively, recorded in the presence of Doxycyclin and Ceftazidim. For strains isolated from the urine of females aged over 60 years, the SID was 8.33 mm recorded in the presence of Chloramphenicol (Fig. 1).

### 3.3 Antibiotics Susceptibility of ESBLs-Producing Enterobacteria Isolates

Antibiotic susceptibility tests showed that the percentages of sensitive, resistant or intermediate strains vary relatively from one bacterial species to another for all the antibiotics tested, and from one antibiotic to another for the same bacterial species. The percentage of

sensitive strains for all antibiotics varied from 0 to 100 (Fig. 2).

All strains of the 5 bacterial species were sensitive to Imipenem. Additionally, all strains of *K. pneumoniae* were sensitive to Ciprofloxacin and Gentamicin. All strains of *Y. frederiksenii* were also sensitive to Ceftriaxon. The same observation was noted for *E. agglomerans* against Doxycyclin. Amongst all the antibiotics used, all strains of *E. coli* were resistant to 3 (Ceftazidim, Nitrofurantoin, Doxycyclin), those of *K. pneumoniae* resistant to 6 (Ceftriaxon, Ceftazidim, Cefotaxim, Nitrofurantoin, Chloramphenicol, Amoxicillin+Clavulanic acid), those of *E. agglomerans* resistant to 5 (Ceftriaxon, Cefotaxim, Aztreonam, Amoxicillin+Clavulanic acid, Nalidixic acid), those of *S. typhi* resistant to 7 (Ceftriaxon, Cefotaxim, Aztreonam, Ceftazidim, Amoxicillin+Clavulanic acid, Nalidixic acid, Chloramphenicol), and those of *Y. frederiksenii* resistant to 8 (Cefotaxim, Ceftazidim, Amoxicillin+Clavulanic acid, Ciprofloxacin, Nalidixic acid, Nitrofurantoin, Gentamicin and Doxycyclin) (Fig. 2).

The resistant, sensitive or intermediate character of the groups of ESBLs-producing bacterial strains identified with respect to sex and age group of urine donors is presented in Table 2. It can be noted that all the bacterial strains identified are sensitive to Imipenem. In addition, *E. coli* strains isolated from the urine of male and female donors aged 20 years or less, *E. agglomerans* isolated from the urine of female donors aged from 21 to 40 years, as well as *S. typhi* strains isolated from the urine of female donors aged from 41 to 60 years are sensitive to Gentamicin (Table 2). Several other *E. coli* strains from the urine of male and female donors aged 20 years or less, as well as from male donors aged from 41 to 60 years, and *K. pneumoniae* isolated from the urine of male donors aged over 60 years, have an intermediate status for more than one antibiotic tested (Table 2).

It is noted that all ESBLs-producing isolates identified are multidrug resistant bacteria. The Multi-Antibiotic Resistance (MAR) Index has been calculated. Its values varied from 0.416 to 0.916. The highest was recorded with *E. coli* strains isolated from female urine donors aged from 41 to 60 years old and over, and the lowest was registered from *E. coli* strains isolated from male urine donors aged of 20 years old or less (Table 2).

Table 1. ESBL-producing *Enterobacteriaceae* species identified in urine infected with respect to the urine donors ages and sex

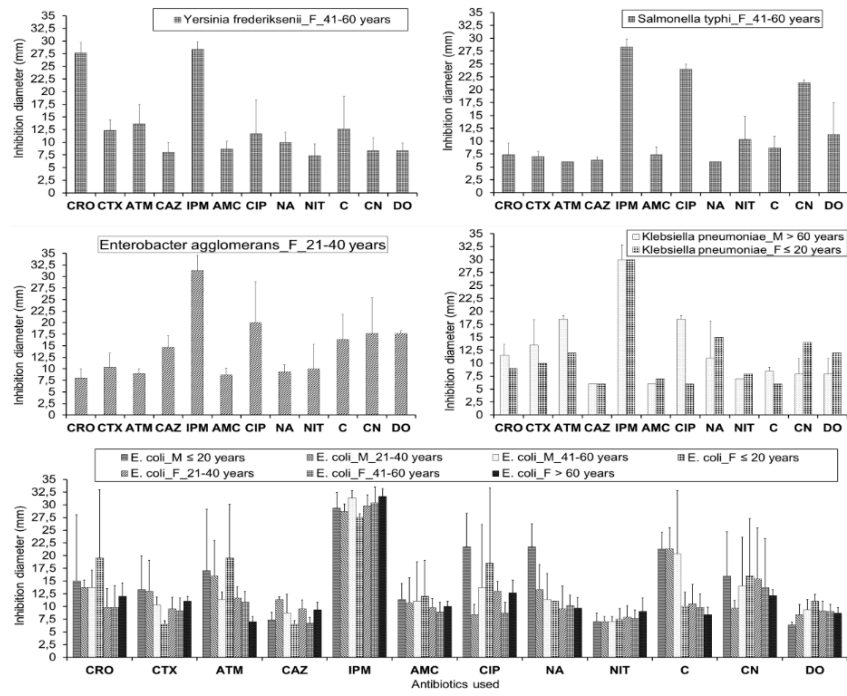
Urine donors ages	Male			Female		
	Urine infected	Urine infected and <i>Enterobacteriaceae</i> strains identified	Urine infected and ESBL-producing strains identified	Urine infected	Urine infected and <i>Enterobacteriaceae</i> strains identified	Urine infected and ESBL-producing strains identified
≤ 20 years old (6 samples)	(6)	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3	(4)	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>K. pneumoniae</i>	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>K. pneumoniae</i>
21 to 40 years old (54 samples)	(17)	(7) <i>E. agglomerans</i> -1 <i>E. agglomerans</i> -2 <i>E. agglomerans</i> -3 <i>K. pneumoniae</i> <i>E. coli</i> -1 <i>E. coli</i> -2	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3	(37)	(18) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3 <i>E. coli</i> -4 <i>E. coli</i> -5 <i>E. coli</i> -6 <i>E. coli</i> -7 <i>E. coli</i> -8 <i>E. agglomerans</i> -1 <i>K. pneumoniae</i>	(9) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3 <i>E. coli</i> -4 <i>E. coli</i> -5 <i>E. coli</i> -6 <i>E. agglomerans</i> -1 <i>E. agglomerans</i> -2 <i>E. agglomerans</i> -3 <i>E. agglomerans</i> -4 <i>E. agglomerans</i> -5 <i>E. agglomerans</i> -6 <i>Y. frederiksenii</i> -1 <i>Y. frederiksenii</i> -2 <i>Y. frederiksenii</i> -3 <i>K. pneumoniae</i>
41 to 60 years old (34 samples)	(15)	(7) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3 <i>E. coli</i> -4 <i>E. coli</i> -5 <i>E. coli</i> -6 <i>E. agglomerans</i>	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3	(31)	(24) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3 <i>E. coli</i> -4 <i>E. coli</i> -5 <i>E. coli</i> -6 <i>E. coli</i> -7 <i>E. agglomerans</i> -1 <i>E. agglomerans</i> -2 <i>E. agglomerans</i> -3 <i>E. agglomerans</i> -4 <i>E. agglomerans</i> -5 <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3 <i>Y. pseudotuberculosis</i> -1 <i>Y. pseudotuberculosis</i> -2 <i>Y. pseudotuberculosis</i> -3 <i>S. typhi</i> -1 <i>S. typhi</i> -2 <i>S. typhi</i> -3	(12) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3 <i>E. coli</i> -4 <i>E. coli</i> -5 <i>E. coli</i> -6 <i>Y. frederiksenii</i> -1 <i>Y. frederiksenii</i> -2 <i>Y. frederiksenii</i> -3 <i>S. typhi</i> -1 <i>S. typhi</i> -2 <i>S. typhi</i> -3
> 60 years old (6 samples)	(3)	(2) <i>K. pneumoniae</i> -1 <i>K. pneumoniae</i> -2	(2) <i>K. pneumoniae</i> -1 <i>K. pneumoniae</i> -2	(3)	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3	(3) <i>E. coli</i> -1 <i>E. coli</i> -2 <i>E. coli</i> -3
Total	41	19	11	75	48	27



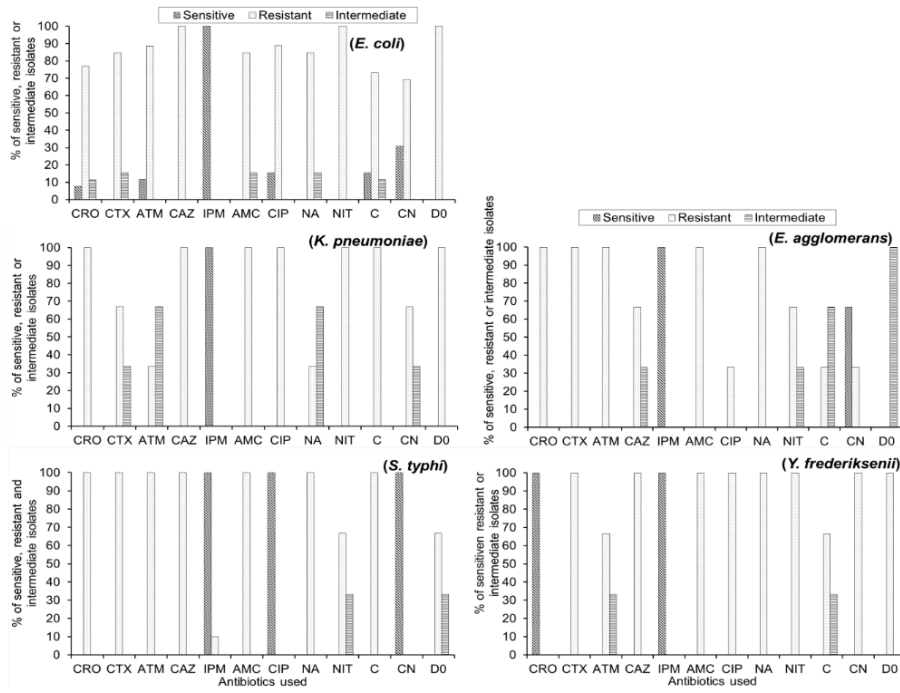
**Table 2. Profile of antibiotics activity (R : resistant ; S : sensitive ; I : intermediate) against ESBL-producing *Enterobacteriaceae* identified in urine samples with respect to the urine donor sex and age and Multi-Antibiotic Resistance (MAR) Index**

Groups of cells strains based on the urine donor age and sex	Antibiotics used and MAR Index												MAR Index
	CRO	CTX	ATM	CAZ	IPM	AMC	CIP	NA	NIT	C	CN	DO	
<i>E. coli</i> _M ≤ 20 years old	I*	R	I*	R	S*	R	I*	S*	R	I*	S*	R	0.416
<i>E. coli</i> _M_21-40 years old	R	R	R	R	S*	R	R	R	R	I*	R	R	0.833
<i>E. coli</i> _M_41-60 years old	R	R	R	R	S*	R	R	R	R	I*	I*	R	0.75
<i>E. coli</i> _F ≤ 20 years old	I*	R	I*	R	S*	R	I*	R	R	R	S*	R	0.583
<i>E. coli</i> _F_21-40 years old	R	R	R	R	S*	R	R	R	R	R	I*	R	0.833
<i>E. coli</i> _F_41-60 years old	R	R	R	R	S*	R	R	R	R	R	R	R	0.916
<i>E. coli</i> _F > 60 years old	R	R	R	R	S*	R	R	R	R	R	R	R	0.916
<i>E. agglomerans</i> _F_21-40 years old	R	R	R	R	S*	R	I*	R	R	R	S*	I*	0.666
<i>K. pneumoniae</i> _M > 60 years old	R	R	I*	R	S*	R	I*	R	R	R	R	R	0.75
<i>K. pneumoniae</i> _F ≤ 20 years old	R	R	R	R	S*	R	R	I*	R	R	R	R	0.833
<i>S. typhi</i> _F_41-60 years old	R	R	R	R	S*	R	S*	R	R	R	S*	R	0.75
<i>Y. frederiksenii</i> _F_41-60 years old	S*	R	R	R	S*	R	R	R	R	R	R	R	0.833

M : male urine donor; F : female urine donor; CRO : Ceftriaxon; CTX : Cefotaxim; ATM : Aztreonam; CAZ : Ceftazidin; IPM : Imipenem; AMC : Amoxicillin+Clavulanic acid; CIP : Ciprofloxacin; NA : Nalidixic acid; NIT : Nitrofurantoin; C : Chloramphenicol; CN : Gentamicin; DO : Doxycyclin.



**Fig. 1.** Mean values of the antibiotics inhibition diameters against ESBLs-producing bacterial strains ((CRO : Ceftriaxon; CTX : Cefotaxim ; ATM : Aztreonam ; CAZ : Ceftazidim ; IPM : Imipenem ; AMC : Amoxicillin+Clavulanic acid ; CIP : Ciprofloxacin ; NA : Nalidixic acid ; NIT : Nitrofurantoin ; C : Chloramphenicol ; CN : Gentamicin ; DO : Doxycyclin)



**Fig. 2. Percentage of sensitive, resistant or intermediate *E. coli*, *K. pneumoniae*, *E. agglomerans*, *S. typhi* and *Y. frederiksenii* isolates with respect to antibiotics used (CRO : Ceftriaxon; CTX : Cefotaxim ; ATM : Aztreonam ; CAZ : Ceftazidin ; IPM : Imipenem ; AMC : Amoxicillin+Clavulanic acid ; CIP : Ciprofloxacin ; NA : Nalidixic acid ; NIT : Nitrofurantoin ; C : chloramphenicol ; CN : Gentamicin ; DO : Doxycyclin)**

The comparison using the Kruskal-Wallis test has been carried out amongst the inhibition diameters values for all ESBLs-producing strains for the same antibiotic, then from one antibiotic to another for the same strains with respect to sex and age of urine donor. It appears that each identified ESBLs-producing *Enterobacteriaceae* strains react differently from one antibiotic to another ( $P < 0.05$ ), except the *E. coli* strains isolated from the urine of male donors aged less than or equal to 20 years and those aged from 41 to 60 years, as well as the *E. coli* strains isolated from the urine of female donors aged from 21 to 60 years. This test also showed that Aztreonam, Ceftazidim, Chloramphenicol and Doxycyclin react differently from one ESBLs-producing *Enterobacteriaceae* strain to another ( $P < 0.05$ ), while the activities of the other antibiotics do not differ significantly from one strain to another ( $P > 0.05$ ).

#### 4. DISCUSSION

##### 4.1 Antibiotics Susceptibility of ESBLs-Producing Enterobacteria Isolates

The identified ESBLs-producing enterobacterial isolates were *E. coli*, *K. pneumoniae*, *E. agglomerans*, *S. typhi* and *Y. frederiksenii* species. Some authors have isolated many strains of the same species or the genus from human and animals, and also from food-producing animals and animal-derived foods, with some studies indicating genetic similarities between these isolates and those found in human infections [39-41].

This study showed that all the identified ESBLs-producing isolates in urine samples are multidrug resistant. This has also been noted by Mwanzo et al [14] about isolates from surface and ground water samples in the urbanized area of Butembo where ESBLs-producing bacterial abundances and diversity undergoes significant spatio-temporal variation. It has also been noted that the antibiotic ESBLs-producing bacteria varied depending on the antibiotics, bacterial species and type of aquatic environment hosting the microorganism [28]. It is sometimes indicated that such resistance develops in bacteria due to genetic mutations and/or acquired genomes [42].

Some mechanisms of ESBLs-producing bacterial resistance have been suggested. Antibiotics spread out in the cell through the occurrence of mutations in the gene which

specifically encodes the outer membrane porin protein [43,44]. Through strong efflux pumping, the number of antimicrobials are launched out of the cell. Their overexpression allows resistance to be formerly effective [44,45]. By changing the arrangement of the targets, the binding affinity of antibiotics can be reduced [44,46].

The MAR index varied from 0.416 to 0.916. It is known that bacteria having MAR index  $\geq 0.2$  originate from a high-risk source of contamination where several antibiotics are used [47]. Development of resistance to certain drugs such as macrolids, lincosamines, and streptogramins can be attained by doing methylation of their binding site and the 16 s rRNA by the action of enzyme called erythromycin ribosomal methylase [44,48]. "The development of the concerned antibiotic resistance takes place due to horizontal distribution of ESBL plasmids through bacteriophages or through horizontal gene transfer which engage the genes for the concerned antibiotic binding proteins" [49]. "Attainment of the van gene cluster is mainly responsible for the development of resistance that ultimately causes less binding affinity to the target [50]. This can lead to enzymes modification like phosphotransferases, nucleotidyltransferases, acetyltransferases, or through mutation and efflux mechanisms" [50]. Resistance could also be due to the mutations occurring in the subunits of the DNA gyrase and topoisomerase [44,50].

It is also indicated that genes that encode ESBL are mostly found on transposons or insertion sequences of plasmids in association with other resistance genes. As a result, they can spread rapidly, causing resistance to multiple antimicrobials such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines, chloramphenicol, and fluoroquinolone [5,6].

Other betalactam antibiotics inactivation mechanisms by Gram-negative bacteria have been reported. The periplasm of Gram-negative bacteria releases beta lactamase which has a higher affinity to betalactam antibiotics than that of betalactam antibiotics to their targets. The gene coding betalactamase may be located in the immobile genetic chromosomes or extra-chromosomal mobile genetic elements such as a plasmid, integrin, or a transposon. The resistant genes evolve either by gene-level mutations or acquisition of resistant genes from other bacteria of the same or different species [5]. Bacteria having MAR index  $\geq 0.2$  also confirm the

presence of multidrug-resistant genes originating from the environment where there is an abuse of these drugs and also that the plasmids contain one or more resistance genes, each encoding a single antibiotic resistance phenotype [47,51]. With the exception of Imipenem (Carbapenemes class), all identified strains were resistant to the majority of antibiotics used. Imipenem is currently among the latest molecules in clinics [52]. Excessive use of this molecule could lead to the development of resistance.

#### 4.2 Potential Relationship between Human Urine Infection and Poor Bacterial Water Quality in Butembo

Mwanzo et al [14] noted in urbanized area in Butembo that various aquatic environments including well, river water and hospital wastewater harbored various species of ESBLs-producing enterobacterial strains. They were mainly *Escherichia coli*, *Ewingella americana*, *Erwinia* spp, *Citrobacter freundii*, *Edwardsiella* spp, *Klebsiella ozaenae*, *Shigella* spp, *Enterobacter aerogenes*, *E. agglomerans*, *Citrobacter diversus*, *Proteus mirabilis*, *Serratia fonticola*, *Serratia ficana*, *Moellerella wisconsinensis*, *Klebsiella rhinoscleromatis* and *Providencia rettgeri* species. Debabza et al [53] analysed more than 250 *Enterobacteriaceae* strains isolated from influent wastewater, effluent wastewater, and sludge in a municipal wastewater treatment plant and from river water receiving their effluent in Algeria. They noted that 56.30% were defined as extended-spectrum beta-lactamase producers with 17.50% found in the river water. In the urban area without wastewater treatment plant, the municipal sewage may be a reservoir of the dissemination of ESBLs-producing *Enterobacteriaceae* into the environment, thereby contaminating natural water resources including groundwater sometimes mostly drunk by the population. This can also disseminate mobile genetic elements which are involved in spreading ESBLs-genes among the bacterial population [5].

The presence of multiresistant ESBLs-producing bacteria in soil have been reported by some authors [10,54], and the phylogenetic analysis of the prevalent ESBLs-carrying organisms in soil indicated that the genes can be horizontally transferred across different bacterial orders and classes [55]. It is known that the soil rarely retains all the chemical and biological pollutants contained in the seeping water from the soil surface to the groundwater. This retention depends on several factors including

the mineralogical and petrographic properties of the soil particles, the anatomical and physiological properties of the germs contained in these infiltration waters, as well as the chemical characteristics of this seeping water [23-25]. Following the infiltration of microbial pollutants from the soil to the water table during the infiltration of runoff water, for instance, these genes and enzymes could then be found in groundwater which will be consumed by populations.

In addition, the use of antimicrobial agents has precipitated the proliferation of antimicrobial resistant strains of bacteria within the commensal microbiota, engendering a potential hazard to human health via the consumption of contaminated water and foods [41,56]. The unregulated and excessive use of antimicrobials in aquatic environments has also been identified as a significant driver of antimicrobial resistance ESBLs-producing enterobacteria. For example, it has been indicated that aquatic animals such as fish and shrimp can serve as reservoirs for antimicrobial resistance genes [57,58], which could lead as a threat to human health through the consumption of raw seafood, thus contributing to a public health crisis [59].

A relative variation in the number of ESBL-producing strains and species depending on the sex and age of the urine samples donor has been noted. Some authors believe that the risk factors of ESBLs-producing enterobacteria are numerous and include male sex, higher age, Charlson comorbidity index score (assessment tool designed specifically to predict long-term mortality, with regard to its reliability, concurrent validity, sensitivity, incremental and predictive validity), history of cystitis or hospitalization and prescriptions for certain antibiotics or proton pump inhibitors in the past years, among others [60,61]. The vulnerability to ESBL-producing bacteria would also depend on the personal and collective efforts for good lifestyle practices, as well as the sanitation of the immediate environment.

#### 5. CONCLUSION

The ESBLs-producing bacteria represent a real danger for human health, because of their multi-resistance to antibiotics. In areas without public drinking water for various reasons, people should treat groundwater before consumption. This can be done by simple and inexpensive means such as boiling water or adding chlorine or ozone. This would reduce the viability of the ESBLs-

producing bacteria and genes in drinking water as well as the level of human infections. This could also reduce the spread of these microbial contaminants into the natural environment.

#### DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

#### CONSENT

This study was approved by the health ethics committee, Provincial Directorate of North Kivu (authorization ref.: CNES/DP-NK 002054125001-105/2023).

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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