

## Characterization of the blaCTX-M gene in Multidrug-Resistant Enterobacteria Isolated from Wastewater of the Municipal Slaughterhouse of Port-Bouët, Côte d'Ivoire

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DOI: <https://doi.org/10.36347/sajb.2024.v12i10.006>

| Received: 27.09.2024 | Accepted: 02.11.2024 | Published: 26.11.2024

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### Abstract

### Original Research Article

The objective of this study was to characterize the blaCTX-M gene in enterobacteria in wastewater from the Port-Bouët municipal slaughterhouse. For this purpose, 72 wastewater samples were collected. Bacteriological counts were carried out on VRBG medium supplemented with 2 mg/L ceftazidime. Germs were isolated and identified on UTI agar. Sensitivity tests were carried out with beta-lactam antibiotics (AMC, CAZ and IPM), fluoroquinolones (NA and CIP) and aminoglycosides (GM and AN). Enumerations showed high loads of resistant Enterobacteriaceae, ranging from 5.2 to 5.9 Log UFC/100mL. A total of 60 resistant strains of enterobacteria were isolated, including *Escherichia coli* (60%), *Proteus vulgaris* (21.67%), *Klebsiella pneumoniae* (8.33%), *Enterobacter spp* (6.67%) and *Proteus mirabilis* (3.33%). Sensitivity tests revealed 100% resistance in enterobacteria species to amoxicillin + clavulanic acid and ceftazidime. *Proteus vulgaris* species showed high resistance to nalidixic acid (69.2%) and amikacin (46.1%). A search for the blaCTX-M resistance gene revealed that 46.1%, 55.6% and 50% of *E. coli*, *Proteus vulgaris* and *Enterobacter spp* species respectively carried this gene in their genomic DNA. The presence of resistant enterobacteria containing the blaCTX-M resistance gene isolated from wastewater at the Port-Bouët municipal slaughterhouse represents a threat to public health.

**Keywords:** Enterobacteria, wastewater, municipal slaughterhouse, Port-Bouët.

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## INTRODUCTION

Bacterial resistance to antibiotics is a serious public health problem. For several decades now, it has been increasing, causing difficulties in treating patients. It slows down the effectiveness of medicines and increases morbidity worldwide (Opatowski, 2020). According to an estimate by the World Health Organization (WHO), antimicrobial resistance (AMR) was responsible for 1.27 million deaths worldwide in 2019, and contributed to 4.95 million deaths (WHO, 2023). Furthermore, the problem of antibiotic resistance is due to the dissemination of multi-resistant bacteria (MRB) in effluents. This multi-resistance is due to the abusive use of drugs in human and veterinary medicine,

or in animal feed (Boutaiba, 2017), allowing bacteria to adapt to them.

In Côte d'Ivoire, a study carried out on enterobacteria showed the distribution of beta-lactam resistance genes between different bacterial species (Gadou, 2019). These multi-resistant Enterobacteriaceae have developed resistance to beta-lactam antibiotics by producing extended-spectrum beta-lactamases (ESBLs) of the TEM, SHV and CTX-M types. Expanded-spectrum beta-lactamases (ESBLs) are the most common enzyme families, discovered in the 1980s in France, then in Germany, and widely propagated throughout the world (Yassine, 2021). Their increasing frequency is directly linked to selection through the use of different  $\beta$ -

**Citation:** WOGNIN Affou Séraphin, TOULE Aubin Cyrille, TRAORÉ Sylvain Gnamien, ZADI Zohonon Marie Colombe, KOFFI-NEVRY Rose. Characterization of the blaCTX-M gene in Multidrug-Resistant Enterobacteria Isolated from Wastewater of the Municipal Slaughterhouse of Port-Bouët, Côte d'Ivoire. Sch Acad J Biosci, 2024 Nov 12(10): 366-372.

lactamins (Peirano and Pitout, 2019). Moreover, antibiotic resistance has spread beyond clinical and veterinary contexts to the environment, which is studied as both a receptor and source of the phenomenon, due to the dispersion of resistance genes in the environment via wastewater, particularly that generated in slaughterhouses. Slaughterhouses produce wastewater laden with organic matter (fats, proteins, blood, antibiotic residues, ...) and micro-organisms (bacteria, viruses, and parasites) from slaughtered animals (Saizonou *et al.*, 2010, Mravili, 2013, El OualiLalami *et al.*, 2014). Indeed, wastewater from slaughterhouses and the faeces of slaughtered animals are rich in pathogenic micro-organisms that are potentially resistant to antibiotics. In addition, the spread of ESBL-producing Enterobacteriaceae or their resistance genes is a problem that needs to be resolved in order to protect the health of the human population. However, previous work has shown high levels of multi-resistant Enterobacteriaceae resistance in abattoir wastewater (Zurfluh *et al.*, 2013).

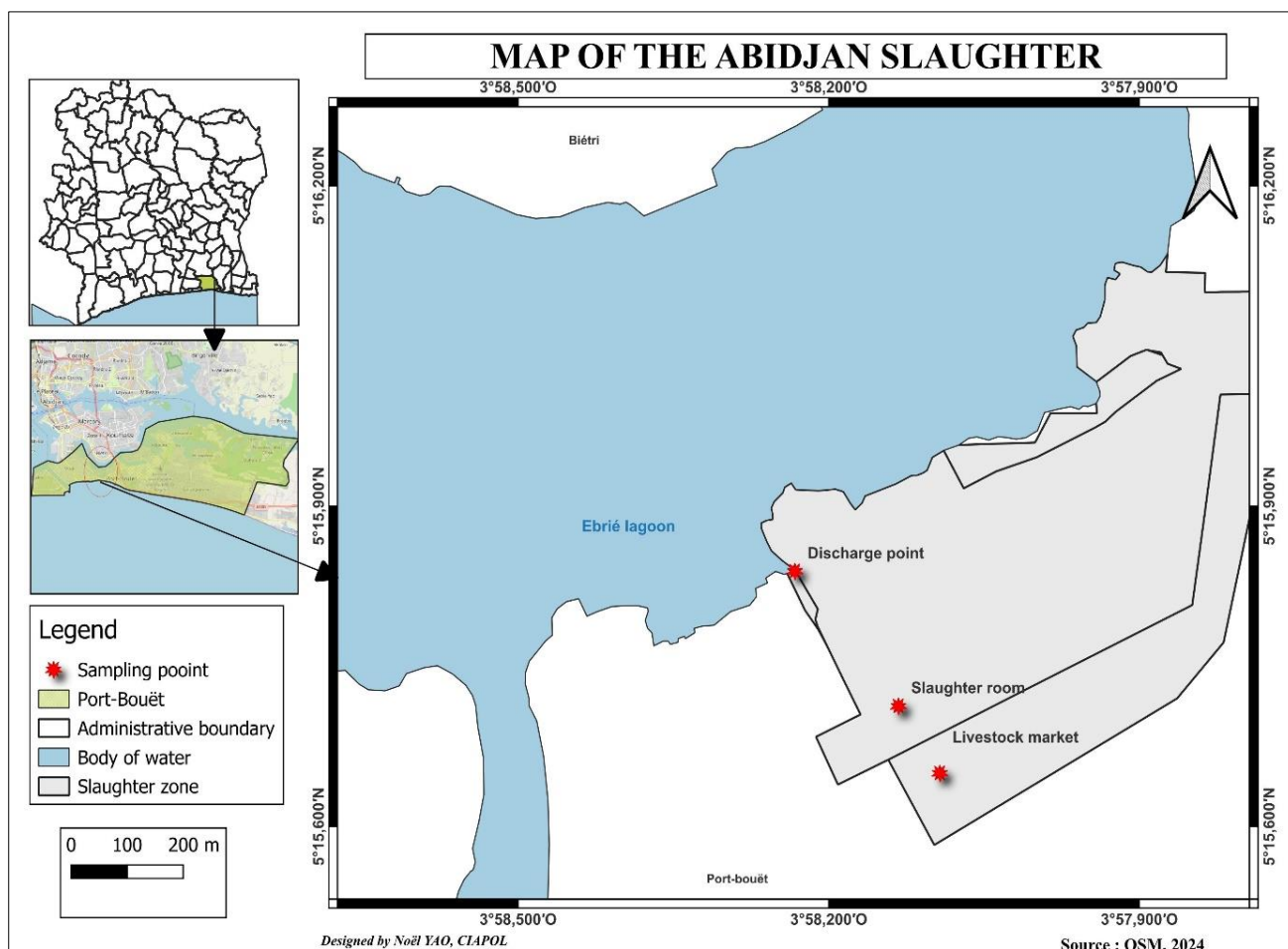
This is the background to the present study, the general aim of which is to characterize the blaCTX-M gene in multi-resistant enterobacteria isolated from wastewater from the Port-Bouët municipal abattoir. Specifically, this consisted in:

- Identify enterobacteria in wastewater samples collected at the Port-Bouët municipal slaughterhouse;
- Determine levels of resistance to antibiotics used in human therapy;
- Characterize the bla CTX-M gene in multi-resistant enterobacteria.

## 1. MATERIALS AND METHODS

### 1.1. Sampling

The study was conducted between March and June 2024. Wastewater samples were collected over 8 sampling campaigns at the Port-Bouët municipal abattoir. During each campaign, 9 samples were collected. These samples were taken at 3 sampling points, namely the slaughter room, the cattle yard and the point of discharge into the environment (Figure 1). Using a pole, they were collected and placed in 500 mL sterile vials. A total of 72 wastewater samples were collected, placed in a cooler containing frozen carboglass and transported to the microbiology laboratory of the Ivorian Anti-Pollution Center (CIAPOL) for microbiological analysis.



**Figure 1: Map of Port Bouet municipal slaughterhouse with sampling sites**

## 1.2. Enumeration of Enterobacteria in Wastewater Samples

Wastewater was used directly as a stock suspension for decimal dilutions. Inoculation was carried out using the surface spreading technique. After dilutions, a 0.1 mL volume of each dilution retained was taken and spread in Petri dishes containing VRBG medium. The same technique was used for inoculation on VRBG medium supplemented with ceftazidime, in order to detect enterobacteria resistant to this antibiotic. Petri dishes were then incubated in an oven for 18 to 24 hours at 37°C. Enumeration was carried out in accordance with ISO 21528-2: 2017.

## 1.3. Enterobacteriaceae Identification

Enterobacteria colonies were isolated on VRBG + Ceftazidime medium and subcultured on UTI medium, a chromogenic medium. Red colonies were presumptive *E. coli* or *Proteus*, and blue colonies were presumptive *Enterobacter* spp or *Klebsiella*. The blue and red colonies were then plated on nutrient agar to perform Ferguson's urea-indole test.

## 1.4. Antimicrobial Sensitivity Testing

Sensitivity testing of isolated Enterobacteriaceae strains to antibiotics was carried out using the diffusion method for the antibiotic contained in the discs on Müeller-Hinton agar. The antibiotics used in this test were nalidixic acid (NA, 30µ), ciprofloxacin

(CIP, 5µ), ceftazidime (CAZ, 10µ), amoxicillin + clavulanic acid (AMC, 20µ), imipenem (IPM, 10µ), gentamicin (GM, 10µ) and amikacin (AN, 30µ). Readings were taken by measuring the diameter of the zones of inhibition obtained around the antibiotic discs using a caliper, and interpretation was made according to the terms Susceptible (S), Intermediate (I) or Resistant (R) based on the criteria defined by CASFM (2023). The *Escherichia coli* ATCC 25922 strain was used as a reference for quality control.

## 1.5. Molecular Analysis

Genomic DNA from Enterobacteriaceae strains was extracted by the phenol-chloroform method (Maron *et al.*, 2005), then used for PCR amplification of the blaCTX-M resistance gene. The sequence of primers used for PCR is recorded in Table I (Al-Mayahie, 2013). The polymerase chain reaction was carried out in a final volume of 20 µL. Amplification was carried out in a thermal cycler (TECHNE 3Prime) with the following conditions: a first initial denaturation step at 94°C for 5 minutes, The second step groups together 35 cycles each of which is composed of a denaturation phase at 94°C for 30 seconds, a hybridization phase at 70°C for 45 seconds and an elongation phase at 72°C for 1 minute.

Finally, the third step is final elongation at 72°C for 10 minutes (Yassine, 2021). PCR products were revealed by agarose gel electrophoresis (0.8%).

**Table 1: Primers used to search for resistance genes**

	Primers	Sequences (5'→ 3')	Hybridization temperature	PCR product size (pb)
Gene <i>bla</i> <sub>CTX-M</sub>	CTX-M F	ATGTGCAGTACCAGTAAGGTGATGGC	70°C	608
	CTX-M R	TGGGTGAAGTAGGTGACCAGAATGAGCGG		

Source: Al-Mayahie (2013)

## 1.6. Statistical Analysis

The results of the enumeration of enterobacteria in wastewater from the Port-Bouët municipal slaughterhouse were translated into base-10 decimal logarithm along with the means ± standard deviations. Graphs were produced using Excel 2019 software.

## 2. RESULTS

### 2.1. Enterobacteria Loads in Wastewater Samples from the Port-Bouët Municipal Slaughterhouse

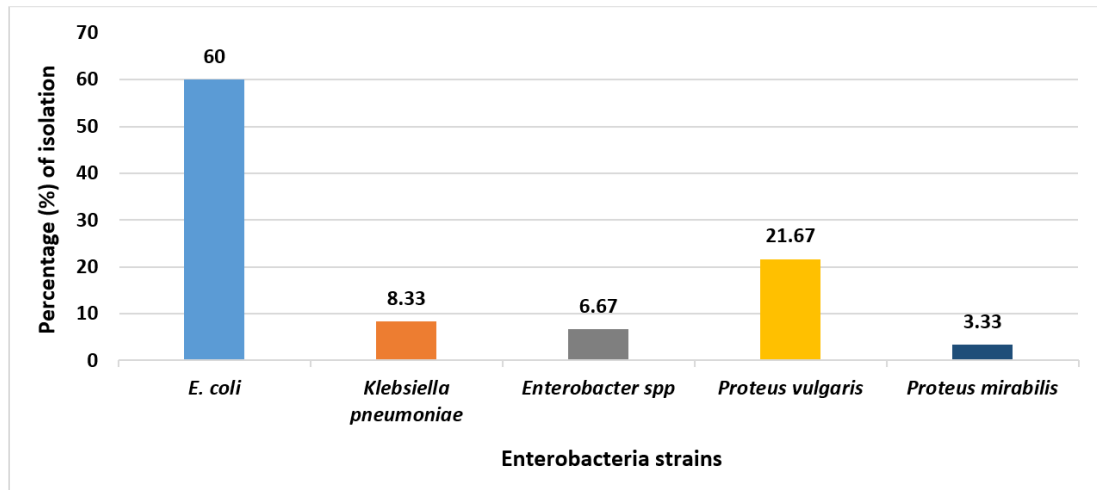
Enterobacteria strains counted on VRBG medium ranged in mean load from  $7.3 \pm 0.17$  to  $7.8 \pm 0.06$  Log UFC/100mL. On VRBG + CAZ medium, the average load of enterobacteria enumerated ranged from  $5.2 \pm 0.27$  to  $5.9 \pm 0.47$  Log UFC/100mL in wastewater (Table 2).

**Table 2: Enterobacteria loads in wastewater samples from the Port-Bouët municipal slaughterhouse**

Waste water	Enterobacteria load (Log CFU/100mL)	Loads of resistant Enterobacteriaceae (Log CFU/100mL)
Slaughterhouse	$7.8 \pm 0.06$	$5.5 \pm 0.15$
Livestock yard	$7.6 \pm 0.15$	$5.9 \pm 0.47$
Discharge point	$7.3 \pm 0.17$	$5.2 \pm 0.27$

### 2.2. Frequency of Isolation of Resistant Enterobacteriaceae in Wastewater from the Port-Bouët Municipal Slaughterhouse

The number of resistant Enterobacteriaceae species isolated from wastewater samples was 60. *E. coli* had a frequency of isolation of 60%, followed by *Proteus vulgaris* with a frequency of 21.67% (Figure 2).



**Figure 2: Distribution of enterobacteria strains isolated from wastewater at the Port-Bouët municipal slaughterhouse**

### 2.3. Antibiotic Resistance Levels of Enterobacteriaceae Strains Isolated from Wastewater at the Port-Bouët Municipal Slaughterhouse

All species of enterobacteria identified showed very high resistance (100%) to AMC and CAZ. On the other hand, *E. coli*, *Proteus vulgaris* and *Enterobacter spp.* showed relatively high resistance rates to MPI of 22.2%, 23.1% and 25% respectively. However,

*Klebsiella pneumoniae* and *Proteus mirabilis* showed no resistance to either MPI or aminoglycosides. *E. coli* and *Proteus vulgaris* showed varying degrees of resistance to the other families. On the other hand, *Enterobacter spp* was resistant to nalidixic acid (75%) and GM (50%), while showing no resistance to CIP and AN. However, *Klebsiella pneumoniae* showed less resistance to fluoroquinolones, while *Proteus vulgaris* species was only resistant to nalidixic acid at 50% (Table 3).

**Table 3: Antibiotic resistance levels of enterobacteria identified in wastewater samples from the Port-Bouët municipal slaughterhouse**

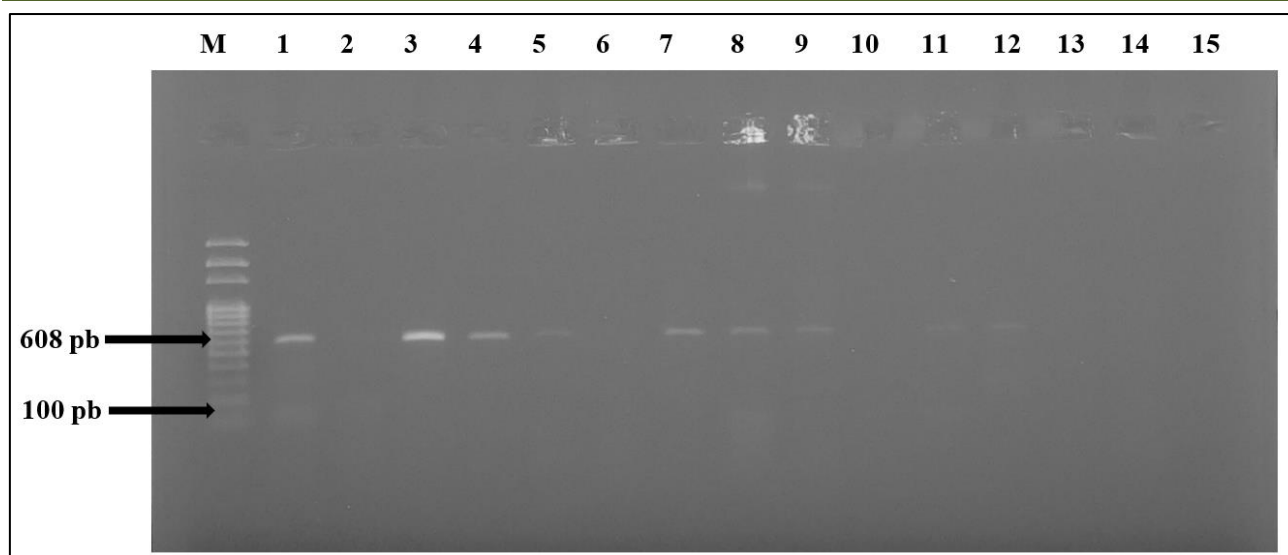
Antibiotic families	<i>E. coli</i> (N = 36) n (%)		<i>K. pneumoniae</i> (N = 05) n (%)		<i>Enterobacter spp</i> (N = 04) n (%)		<i>P. vulgaris</i> (N = 13) n (%)		<i>P. mirabilis</i> (N = 02) n (%)	
	R	S	R	S	R	S	R	S	R	S
<b>Beta-lactams</b>										
Amoxicillin + clavulanic acid (AMC)	36 (100)	0 (0)	5 (100)	0 (0)	4 (100)	0 (0)	13 (100)	0 (0)	2 (100)	0 (0)
Ceftazidime (CAZ)	36 (100)	0 (0)	5 (100)	0 (0)	4 (100)	0 (0)	13 (100)	0 (0)	2 (100)	0 (0)
Imipenem (IPM)	8 (22,2)	28 (77,8)	0 (0)	5 (100)	1 (25)	3 (75)	3 (23,1)	10 (76,9)	0 (0)	2 (100)
<b>Fluoroquinolones</b>										
Nalidixic acid (NA)	9 (52,8)	17 (47,2)	2 (40)	3 (60)	3 (75)	1 (25)	9 (69,2)	4 (30,8)	1 (50)	1 (50)
Ciprofloxacin (CIP)	13 (36,1)	23 (63,9)	1 (20)	4 (80)	0 (0)	4 (100)	7 (53,8)	6 (46,1)	0 (0)	2 (100)
<b>Aminosides</b>										
Gentamicin (GM)	4 (11,1)	32 (88,9)	0 (100)	5 (100)	2 (50)	2 (50)	5 (38,4)	8 (61,5)	0 (0)	2 (100)
Amikacin (AN)	3 (8,3)	33 (91,7)	0 (0)	5 (100)	0 (0)	4 (100)	6 (46,1)	7 (53,8)	0 (0)	2 (100)

R: Resistant; S: Sensible; N: Number of bacteria

### 2.4. PCR Results for Multi-Resistant Enterobacteriaceae Species in Wastewater from Port-Bouët Municipal Slaughterhouse

The polymerase chain reaction was tested on 29 species of multi-resistant Enterobacteriaceae. The results

showed that 12 species contained the bla<sub>CTX-M</sub> resistance gene. Amplification revealed a bla gene size of 608 bp. Figure 2 shows the electrophoretic profile of some species.



**Figure 3: Electrophoretic profile of the bla<sub>CTX-M</sub> gene amplification product of some enterobacterial species isolated from wastewater at the Port-Bouët municipal slaughterhouse**

Column M: Molecular weight marker (1 kb DNA Ladder plus)

Tracks 1 to 15: Bacterial strains

### 2.5. Genotypic Characterization of Enterobacteriaceae Species in Wastewater from the Port-Bouët Municipal Slaughterhouse

Detection of ESBL-encoding genes in wastewater revealed that 46.1% (n = 6/13), 55.6% (n =

5/9) and 50% (n = 1/2) of multidrug-resistant *E. coli*, *Proteus vulgaris* and *Enterobacter spp* species contained the bla<sub>CTX-M</sub> gene, respectively (Table 4).

**Table 4: Genotypic profile of Enterobacteriaceae species in wastewater from the Port-Bouët municipal slaughterhouse**

	Gene bla <sub>CTX-M</sub> , n (%)
<i>E. coli</i> , N = 13	6 (46,1)
<i>Proteus vulgaris</i> , N = 9	5 (55,6)
<i>Enterobacter spp</i> , N = 2	1 (50)
<i>Proteus mirabilis</i> , N = 2	0 (0)
<i>Klebsiella pneumoniae</i> , N = 3	0 (0)
Total, N = 29	12 (41,4)

## 3. DISCUSSION

This study on the characterization of the bla<sub>CTX-M</sub> gene in multi-resistant enterobacteria isolated from wastewater samples revealed high loads of enterobacteria ranging from 7.3 to 7.8 Log CFU/100 mL. These results could be due to the slaughterhouse's lack of hygiene, particularly in the cattle yard and slaughter room. Slaughterhouse wastewater is full of germs from slaughtered animals (Sai. zonou *et al.*, 2010 ; Mravili, 2013 ; El OualiLalami *et al.*, 2014). The lack of hygiene confirms the comments of Coulibaly (2023), who described the insalubrity of this establishment. The loads of resistant enterobacteria observed in slaughterhouse wastewater reflect the high quantities of antibiotics used in the livestock sector.

Five species of enterobacteria were isolated from wastewater at the Port-Bouët municipal slaughterhouse, including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Proteus mirabilis* and *Enterobacter spp*. Among the species isolated, *E. coli*

accounted for 60%. This result is higher than those of Mehran *et al.*, (2024), who also isolated these species, with *E. coli* present at 24.3% in slaughterhouse wastewater in Iran. However, it is still lower than that of Hemen *et al.*, (2019), who showed the predominance of *E. coli* at 75% in their study carried out in slaughterhouse wastewater in Addis Ababa, Ethiopia. Antibiotic sensitivity tests carried out on these strains showed high resistance (100%) to beta-lactam antibiotics (AMC and CAZ) in wastewater. These results concur with those of Mehran *et al.*, (2024), who also observed a high rate of resistance (100%) to Amoxicillin + clavulanic acid and Ceftazidime in  $\beta$ -lactamase-producing Enterobacteriaceae isolated from the wastewater of cattle and poultry slaughterhouses in Iran.

Homeier-Bachmann *et al.*, (2021) showed a 95% resistance rate to ceftazidime in their study of antibiotic-resistant Enterobacteriaceae in slaughterhouse wastewater in Germany. *Klebsiella pneumoniae* strains showed no resistance to imipenem or amikacin. This

result is similar to that of Ejikeugwu *et al.*, (2018), who conducted a study of *Klebsiella* species in a slaughterhouse in Nigeria. Indeed, these authors observed no resistance from this species during their research. Similarly, Homeier-Bachmann *et al.*, (2021) presented *Klebsiella pneumoniae* strains with no resistance to imipenem. The Enterobacteriaceae species present in the wastewater were resistant to at least three classes of antibiotics. The multi-resistance of these species in different matrices is thought to be due to the exchange of their genetic material with other bacteria in the receiving environment. Enterobacteriaceae have a great capacity to acquire a number of virulence genes and to transmit them horizontally and vertically (Dognon *et al.*, 2018).

The study also looked for the blaCTX-M resistance gene in multi-resistant enterobacteria species in this matrix. PCR results revealed that the enterobacter species carrying the resistance gene in the wastewater were *E. coli* (46.1%), *Proteus vulgaris* (55.6%) and *Enterobacter spp* (50%). The *E. coli* rate is higher than that of Mykhailo *et al.*, (2020), who showed 33.3% of *E. coli* species carrying blaCTX-M. However, it is lower than those of Mehran *et al.*, (2024) with 100% *E. coli*. The significant increase in ESBL incidence in Enterobacteriaceae has been attributed to the spread of CTX-M family members according to studies carried out by Sudarwanto *et al.*, (2016) in Indonesia. The global spread of CTX-M-type ESBLs in *E. coli* are a matter of concern in human and veterinary medicine (Tamang *et al.*, 2014).

#### 4. CONCLUSION

The aim of this study was to identify the blaCTX-M gene in multidrug-resistant enterobacteria isolated in wastewater from the municipal slaughterhouse in Port-Bouët. It showed more loads of ceftazidime-resistant enterobacteria. Several resistant species were identified, including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter spp*, *Proteus vulgaris* and *Proteus mirabilis*. Sensitivity testing showed multi-drug resistance to beta-lactams, fluoroquinolones and aminoglycosides in species isolated from wastewater samples. A search for the blaCTX-M resistance gene in resistant Enterobacteriaceae revealed that *Escherichia coli*, *Enterobacter spp* and *Proteus vulgaris* were carriers of this gene. This gene was therefore characterized at genomic DNA level in this study.

The presence of resistant enterobacteria harboring the blaCTX-M resistance gene isolated in wastewater at the Port-Bouët municipal abattoir represents a health threat for consumers and workers.

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