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Detection of antibiotic-resistant *Escherichia coli* and *Salmonella* spp. in wastewater from a former slaughterhouse in Ivory Coast

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ABSTRACT: The research presented in this memory was carried out at the Institut Pasteur in Côte d'Ivoire and carried out on E. coli and Salmonella spp. The objective of this study was to isolate E. coli and Salmonella spp. strains resistant to antibiotics from wastewater from the Port-Bouët slaughterhouse. From September 2017 to March 2018, 17 strains of E. coli and 7 Salmonella spp. were isolated in 63 samples of wastewater collected at the slaughterhouse of Port-Bouët. Their identification was performed according to conventional bacteriological tests. An antibiogram according to the disk diffusion method was carried out for 21 antibiotics commonly used in human and veterinary medicine. The prevalence observed was 26.98% for E coli strains. and 11.11% for Salmonella spp. All strains of E. coli showed complete (100%) resistance to 3rd generation cephalosporins, aztreonam, amocixillin + clavulanic acid, and ampicillin. However, family resistance rates of quinolones, aminoglycosides, sulfonamides and cyclins remain very high. The sensitivity of E. coli to imipenem and amikacin was 100%. Salmonella spp. strains, on the other hand, were resistant only to beta-lactam at lower levels compared to E. coli strains. coli. In-depth studies are needed to determine the resistance mechanisms of these bacteria.

KEYWORDS: Escherichia coli, Salmonella spp., beta-lactam, quinolones, sulfonamides, aminoglycosides, cyclins, antibiogram.

1 INTRODUCTION

Contamination by bacterial strains from animals can cause serious pathologies in humans. This contamination occurs through the consumption of soiled foodstuffs of animal origin. It can also occur through contact with the environment, contaminated by live animals via faeces, manure, slurry or slaughterhouse effluents [1]. Slaughterhouse effluents correspond to all liquid discharges produced on the slaughterhouse site, i.e. water resulting from the slaughtering activity (process, washing) and black water (sanitary). By their nature, these effluents are highly loaded with bacteria [2]. Studies indicate that antibiotic-resistant bacteria and/or pathogens of human or animal origin such as Escherichia coli, Listeria monocytogenes, Salmonella spp., Campylobacter spp. are excreted into the environment via slaughterhouse water [3–5]. Ruminants, the main reservoir, are thought to participate in the maintenance of the epidemiological cycle of pathogens [6,7]. The intensive use of antibiotics for therapeutic, preventive or growth-promoting purposes makes livestock farms,

particularly cattle farms, privileged places for resistant pathogens to develop, survive and spread [8]. Indeed, Escherichia coli and Salmonella spp. are the most frequently isolated bacteria from these farms. Studies conducted in several countries show that these bacteria develop resistance to certain antibiotics. Faced with this situation, these countries have developed surveillance networks on antibiotic resistance in these strains. According to the WHO, 80% of the diseases that affect the world's population are linked to water pollution [9]. Indeed, most of the microorganisms that are at the origin of the great historical epidemics of waterborne origin have as their normal habitat the intestines of humans and certain warm-blooded animals. This is why the control and monitoring of water quality, especially wastewater, seems increasingly essential. Located in the south of Côte d'Ivoire, the city of Abidjan is the country's largest city in terms of inhabitants with an estimated population of more than six million. It has only one modern slaughterhouse, that of the municipality of Port-Bouët, which represents the largest slaughterhouse in the country on an area of 6 hectares. Unfortunately, the Port-Bouët slaughterhouse is not equipped with a pre-treatment or wastewater treatment system. The effluents are discharged without treatment into the Ebrié lagoon, used by the surrounding populations for fishing and vegetable crops. Thus, liquid discharges from the slaughterhouse would represent one of the main environmental problems that the municipality of Port-Bouët in particular, but also the city of Abidjan in general, would be confronted with, given the extent of the pollution generated by these effluents, which could have impacts on surface water resources and the health of the population. Faced with this situation and as part of the control and surveillance of diseases due to resistant pathogens, it was considered interesting to carry out a study on the wastewater of the Port-Bouët slaughterhouse. The primary objective of this study is to isolate antibiotic-resistant strains of Escherichia coli and *Salmonella* spp. from the wastewater of the Port-Bouët slaughterhouse. In addition to this main objective, there are two specific objectives: (i) to isolate strains of Escherichia coli and Salmonella spp. from the wastewater of the Port-Bouët slaughterhouse, and (ii) to evaluate the antibiotic resistance of these microorganisms.

2 Materials And Methods

2.1 Study Site

The study was carried out at the slaughterhouse located in the municipality of Port-Bouët. It should be noted that the Port-Bouët slaughterhouse was built a year before the independence of Côte d'Ivoire and covers an area of 6 ha. It represents the largest slaughterhouse in the city of Abidjan and Côte d'Ivoire. In its management, the Port-Bouët slaughterhouse is made up of an administration; a cattle yard; a breeding pen, a slaughterhouse and various points on the site where the animals' heads, feet, tails and skins are buckled. In addition, the slaughterhouse is not equipped with a pre-treatment or wastewater treatment system. The effluent is discharged directly through a pipe system leading to the Ebrié lagoon.

2.2 Sampling

Wastewater samples were taken at eight (8) points on the Port-Bouët slaughterhouse site. The points were selected according to a pipe system that runs from the slaughterhouse to the discharge of the water into the Ebrié Lagoon. These points have been named from P1 to P8 as follows: P1 (slaughterhouse), P2 (exit from the slaughterhouse), P3 (in front of the 16 new administration), P4 (at the level of the dwellings), P5 (point of buckling of cattle heads and dwellings), P6 (outlet of water from the pipe), P7 (livestock farm) and P8 (contact with the Ebrié Lagoon). The sampling sessions were carried out once a week for 7 weeks between 9 and 10 a.m.; hours when the atmosphere in the slaughterhouse is more fluid. The samples were taken in sterile one-litre (1L) glass vials and then transported using a cooler containing cold packs for sample storage. A total of 56 wastewater samples (7 per point) were collected from the Port-Bouët slaughterhouse. The samples were immediately sent to the laboratory for analysis.

2.3 Assessments

2.3.1 Preparation Of The Bacterial Suspension

A volume of 15 mL of wastewater from each sample was introduced into 15 mL vials and centrifuged at 3000 rpm for 5 minutes. After centrifugation, 1 mL of the pellet from each centrifuged sample was removed by a sterile pipette and added to a volume of 9 mL of buffered pepttoned water (BPW) contained in a sterile test tube. The whole thing was homogenized (manually) for two (2) minutes and constituted our bacterial suspension.

- **E. coli testing**

The search for *E. coli* was carried out according to the method described by **Ouattara *et al.* (2014)** [10]. For this purpose, Rapid *E. coli* medium has been substituted for Drygalski medium. Rapid *E. coli* (ReC2) medium supplemented with 2 mg/L of Ceftazidime was used in this study.

- **Preparation of ceftazidime solution**

An amount of 2 mg of ceftazidime (antibiotic) was weighed and then added to 5 mL of sterile distilled water to give an initial concentration of 0.4 mg/mL. The antibiotic solution was stored in a freezer at -20°C until use.

- **Preparation of ReC2+ ceftazidime medium for the isolation of *E. coli***

Following the preparation of Rapid *E. coli* (ReC2) agar according to the manufacturers' instructions, a volume of 20 mL of the medium was dispensed into sterile glass vials. Subsequently, a Vi volume of the originally prepared ceftazidime solution was added to each vial containing 20 mL of ReC2 agar. This volume was determined according to the following formula:

$$C_i * V_i = C_f * V_f \rightarrow\rightarrow\rightarrow C_f = \frac{C_i}{V_f} * V_i$$

***Ci**: initial concentration of ceftazidime solution; **Vi**: initial volume of ceftazidime solution; **Cf**: final concentration of Rapid E. coli agar; **Vf**: final volume of the ReC2 agar*

Thus, to obtain a Rapid *E. coli* agar at a concentration of 2mg/mL, a volume of 100 µL of the ceftazidime solution with a concentration of 0.4 mg/mL was added to each vial containing 20 mL of Rapid *E. coli*. After homogenization, the contents of each vial were poured into a Petri dish.

2.3.2 ISOLATION OF DIFFERENT BACTERIAL STRAINS

- **Isolation of *E. coli***

Isolation consisted of inoculating the bacterial suspension on Rapid *E. coli* agar supplemented with ceftazidime (ReC2+Ceftazidime) by the streak depletion technique. The seeded Petri dishes were then incubated at 44 °C for 24 hours. Presumptive purple *E. coli* colonies on agar were subsequently identified from oxidase, catalase and Leminor's reduced rack tests.

- **Isolation of *Salmonella* spp.**

The search for strains of *Salmonella* spp. was carried out in accordance with the **NF ISO 6579** standard [11]. on the search for salmonella in foodstuffs and environmental samples. It was carried out in three main stages, namely first, the pre-enrichment stage, which consisted of incubating the bacterial suspension previously prepared for 24 hours at 37°C. Then in the enrichment step, a volume of 0.1 ml of the pre-enriched solution was inoculated in 10 ml of Rappaport Vassiliadis. The homogenized mixture was incubated at 44 °C for 24 hours to form the enriched solution. Enrichment in a selective medium (Rappaport Vassiliadis) allowed the development of salmonella while retarding or inhibiting the growth of other microorganisms. Finally, the isolation stage, which consisted of seeding the enriched solution on the selective Hecktoen medium by the streak depletion technique. The seeded boxes were then incubated at 37 °C for 24 hours. After incubation, presumptive colonies of *Salmonella* spp. of translucent green color with or without black center were identified from oxidase, catalase and Leminor's reduced rack assays.

2.3.3 DETERMINATION OF THE ANTIBIOTIC RESISTANCE PROFILE OF E. COLI AND SALMONELLA SPP. STRAINS

The determination of the antibiotic resistance profile of the E. coli and Salmonella spp. strains consisted of testing 21 antibiotic discs belonging to the beta-lactam, aminoglycoside, cyclin, phenicolate, sulfonamide and quinolone families. The strains confirmed E.coli and Salmonella spp. following the reduced Leminor rack were purified on ordinary agar and subjected to the determination of the resistance profile. For this purpose, a colony fragment isolated from ordinary agar

was collected using a dropper pipette and suspended in a physiological water solution (10 ml). The inoculum, well homogenized, constituted the bacterial suspension. Turbidity measurement was performed using the BioMerous Densimat at 0.5 McFarland.

The inoculum obtained was used to inoculate the Müller-Hilton agar (HD) previously poured into the petri dishes. To do this, a sterile cotton swab was dipped into the inoculum. Excess liquid was removed by rotating the swab over the tube walls to prevent over-flooding of the agar. The swab was performed on the entire surface of the agar in three directions according to EUCAST/ CA-SFM 2017.

Antibiotics (**Table 1**) were deposited on agar plates of HD inoculated using the disc dispenser (7 per dish). The dishes were then incubated at 37 °C for 24 hours. After incubation, the reading of the inhibition zones was carried out using a software, ADAGIO, in order to define the sensitive, intermediate or resistant categories of the isolated strains.

Table 1. Table of antibiotic discs used

Group	Antibiotics tested	Abbreviations	Charge	Critical Concentration (mg/L)		Critical Diameters (mm))	
				S ≤	R >	S ≥	R<
β-lactamines	Amoxicilline+ Acide clavulanique	AMC	30 µg	8	8	19	19
	Ceftazidime	CAZ	30 µg	1	4	22	19
	Aztreonam	ATM	30 µg	1	4	26	21
	Cefoxitine	FOX	30 µg	8	16	19	15
	Cefotaxime	CTX	30 µg	1	2	20	17
	Imipenème	IMP	30 µg	2	8	22	16
	Ampicilline	AMP	30 µg	8	8	14	14
	Ceftriaxone	CRO	30 µg	1	2	25	22
	Céfépime	FEP	30 µg	1	4	27	21
	Céfixime	CFM	5 µg	1	1	17	17
Quinolons	Ciprofloxacine	CIP	5 µg	0,25	0,5	26	24
	Acide nalidixique	NAL	30 µg	16	16	19	14
	Norfloxacine	NOR	5 µg	0,5	1	22	19
Aminosides	Gentamicine	GMI	15 µg	2	4	17	14
	Amikacine	AKN	30 µg	8	16	16	13
	Tobramycine	TMN	10µg	2	4	17	14
Cyclins	Tétracycline	TET	30 µg	4	8	19	17
	Minocycline	MNO	30 µg	4	8	19	17
Phenicoles	Chloramphénicol	CHL	30 µg	8	8	17	17
Sulfamides	Triméthoprime/ Sulfamethoxazole	SXT	25 µg	2	4	14	11
Other	Colistin	CST	50 µg	2	2	15	15

3 RESULTS

3.1 ISOLATION FREQUENCY

The results of the microbiological analyses were used to determine the biochemical characteristics of the E. coli and Salmonella spp. strains summarized in the following table:

Table 2. Biochemistry of E. coli and Salmonella spp strains

Tests	*Gram*	*Ox*	*Cat*	*Urée*	*Ind*	*Glu*	*Lac*	*H_2S*	*Gaz*	*LDC*	*LDA*	*Man*	*Cit*
E. coli	-	-	+	-	+	+	+	+/-	+/-	+	-	+	-
Salmonella spp.	-	-	+	-	-	+	-	+/-	+/-	+	-	+	+

Of the 56 samples collected and analyzed, 17 strains of E. coli were isolated, representing a prevalence of 30.36 %, and 5 strains of Salmonella spp., representing a prevalence of 8.93%, met these characteristics.

3.2 Difference In Contamination According To Points

The diagrams below have shown the differences in contamination according to the sampling points. Points 1 and 7 corresponding respectively to the point of the slaughterhouse and the point near the breeding farm were the most contaminated, (**Figure 1., E. coli**) and (**Figure 2.,** Samonella spp.).

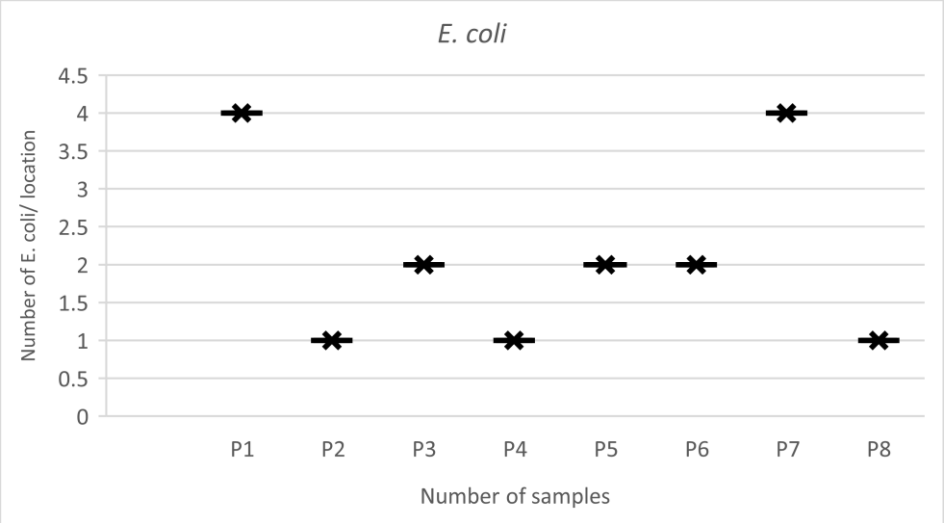

Fig. 1. Difference in E. coli contamination depending on the sampling points

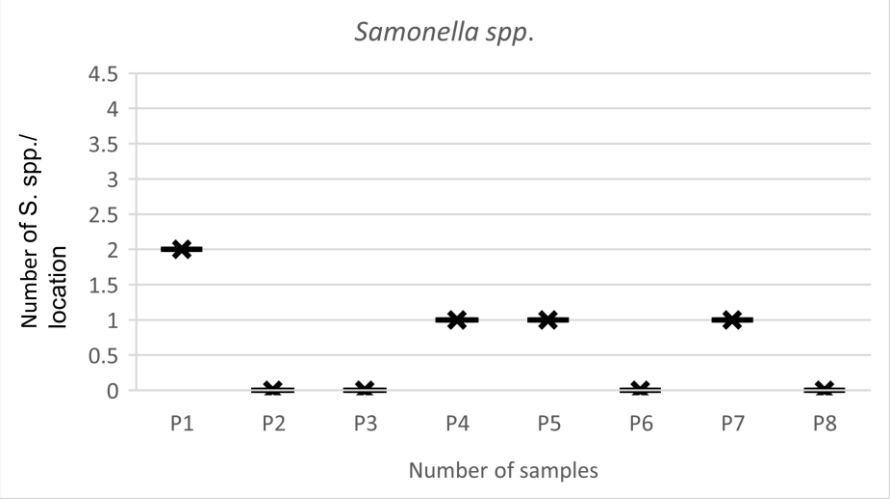

Fig. 2. Difference in Salmonella spp. contamination by sampling location

3.3 Antibiotic Resistance Pattern Of E. Coli And Samonella Spp. Strains

The results showed that the *E. coli* strains isolated from the wastewater analyzed were all susceptible to imipenem (17/17 or 100 %). Regarding the other antibiotics of the beta-lactam family, the strains showed complete resistance (17/17

or 100 %) to ceftazidime, ceftriaxoma, amoxicillin + clavulanic acid, aztreonam, cefotaxime and ampicillin. The resistance rate remains high for cefepime and cefixime (16/17 or 94.12 %) and lower for cefoxitin with 5/17 or 20.41%.

The rates of resistance of the strains to aminoglycosides were lower with 3/17 or 17.65 % for gentamicin and 6/17 or 35.29 % for tobramycin. All E. *coli* strains were sensitive to amykacin (0/17 or 0 %). For quinolones, the resistance rates were 10/17 or 58.82 % for norfloxacin, 9/17 or 52.94 % for nalidixic acid and 8/17 or 47.06 % for ciprofloxanin. Cyclin resistance rates were high for tetracycline with 16/17 or 94.12 % and slightly lower for minocycline with 9/17 or 52.94 %. For the other antibiotics, a high level was obtained with trimethoprim/sulfamethoxazole (15/17 or 88.24 %), a lower level with chloramphenicol (3/17 or 17.65 %) and a low level with colistin (1/17 or 5.88 %) (**Figure 3**).

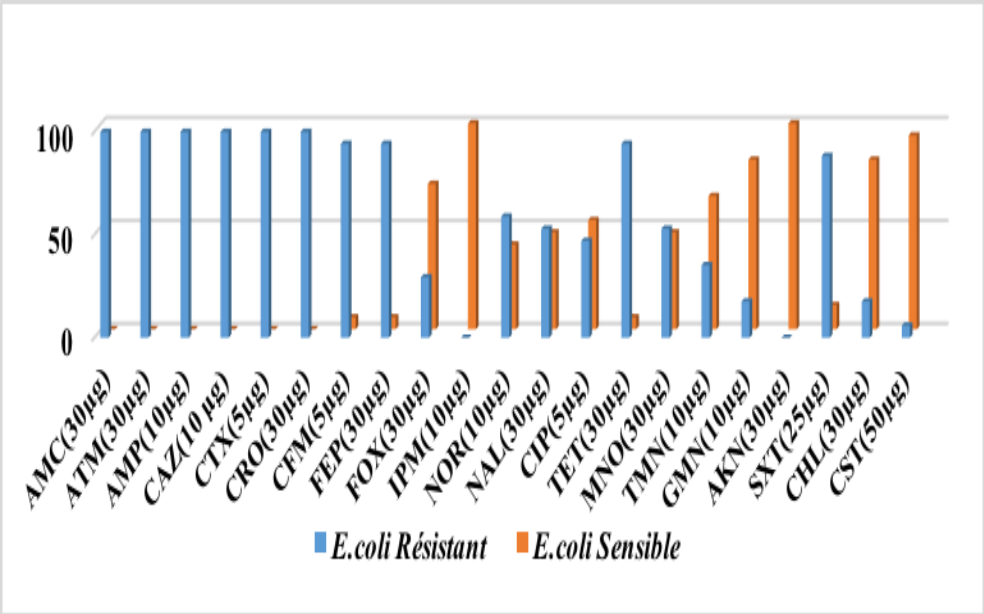

Fig. 3. Resistance rates of isolated E. coli by antibiotic

The results show that only 4 strains of *Salmonella* spp. were found to be resistant to an antibiotic from the beta-lactam family. Resistance rates were 1/5 or 20 % for the combination of amoxicillin + clavulanic acid, cefoxitin, ampicillin and ceftazidime. No strains of *Salmonella* spp. showed resistance to imipenem, ceftriaxone, aztreonam, cefepime, cefixime and cefotaxime.

For the families of aminoglycosides (tobramycin, gentamicin, and amikacin), cyclins (tetracycline and minocycline), quinolones (norfloxacin, nalidixic acid, and ciprofloxacin), and other antibiotics used in this study (trimethoprim/sulfamethoxazole, chloramphenicol, and colistin), no strain of *Salmonella* spp. showed resistance (0 %) (Figure 4).

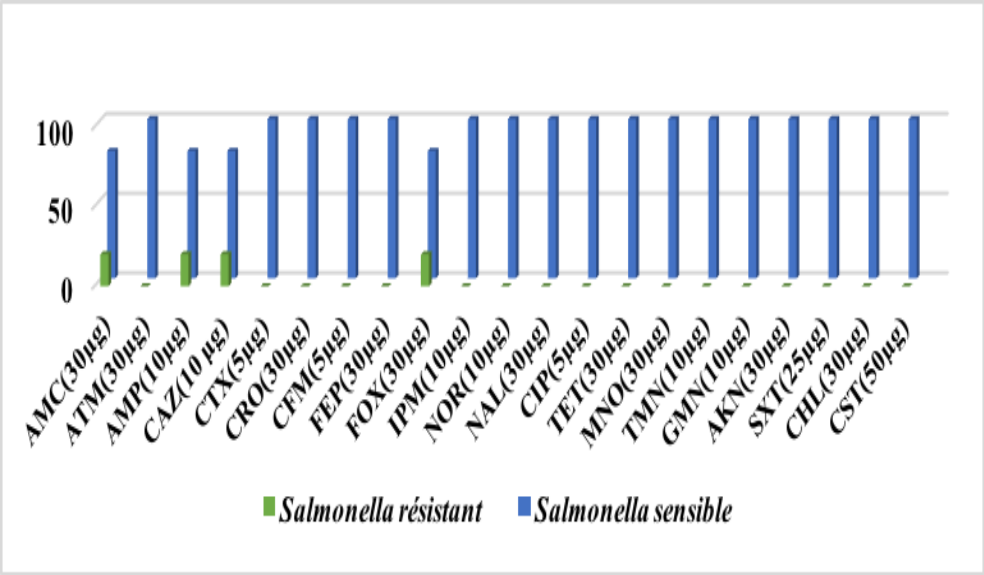

Fig. 4. Resistance rates of isolated Salmonella spp. by antibiotic

4 DISCUSSION

This study shows a high prevalence rate of *E. coli* strains (17/56 or 30.36 %) in the effluents of the Port-Bouët slaughterhouse. The results of [4]. show a higher prevalence rate in slaughterhouse effluents (1.18%) compared to those obtained in urban effluents. These studies also demonstrated that 25 % of samples isolated from slaughterhouse effluents carried pathogenicity. The prevalence of Salmonella has also been highlighted with a rate of 8.93 %, results close to those of [5] and [11]. which obtained *Salmonella* levels of 5 % and 6.66 % respectively. These results confirm the postulate that animals are reservoirs of *E. coli* and *Salmonella* spp. strains.

The antibiotic susceptibility profile of the 17 E. *coli* strains isolated in our study shows high resistance to the beta-lactam family, including 3rd generation cephalosporins, aztreonam and the penicillin group (100 %). These results do not agree with those obtained by [15], which revealed the absence of resistance to cefotaxime (0 %), ceftazidime (0 %) in 33 strains of *E. coli* isolated from purified raw water and cultures in Morocco. These results can be explained by the high use of antibiotics belonging to the beta-lactam family in the treatment of diseases in the Port-Bouët slaughterhouse. However, these strains remained susceptible to amykacin and imipenem (0 %). These results are in agreement with those obtained by [8]. (36.4 % for amykacin and 100 % for imipenem). One might think that these antibiotics are not commonly used in the treatment of diseases at the Port-Bouët slaughterhouse and therefore occupy a prominent place in the therapeutic treatment of severe infections with multi-resistant bacteria. Moreover, this sensitivity of the isolated *E. coli* strains remains quite close to the ESBLs isolated by [8]. This suggests the chances of finding extended-spectrum beta-lactam (ESBL) - producing strains. For quinolones, the resistance rates of *E. coli* were 47.06 % for ciprofloxacin, 52.94 % for nalidixic acid and 58.82 % for norfloxacin. These results are close to those of [7], (54.44 % with nalidixic acid). Quinolones are currently the largest group of antibiotics. Their interest is linked to their low toxicity and especially to the absence of plasmid resistance [3].Studies conducted by INRA researchers [1-2], showed that highly resistant quinolone strains of *Escherichia coli* of animal origin appeared by a significant modification of the target. For aminoglycosides, the most active molecule was tobramycin with 35.29 % resistance. This result is in contrast to that of Guessennd *et al.* (2008) [16]. which achieved a resistance rate of 36.4 % for amikacin. This rate is still lower than that obtained in Turkey, which is 64 % [6]. Regarding the other antibiotics, the study revealed a high rate of resistance to trimethopine/sulfamethoxazole (88.24 %) and the cyclin family; tetracycline (94.12 %) minocycline (52.94 %). Only colistin had good activity with 5.88 % resistance.

Strains of *E. coli* isolated from this study, showed fairly high percentages of resistance to several families of antibiotics. Beta-lactams are currently the largest group of these antibiotics. On the contrary, these results showed that Salmonella strains were still largely sensitive (0 %) to several families of antibiotics. However, the salmonella isolated in this study was resistant to only one family of antibiotics, beta-lactams, with a prevalence rate of 4/5, or 80 %. These results are in contrast to those obtained by [7]. which show a prevalence of 81.1 % of *Salmonella spp*. strains. antibiotic and more. These strains showed multiple resistance (68.49 %) to five antibiotics (ampicillin, trimethoprim, trimethoprim-sulfametoxazole, tetracyclines, sulfonamides). These percentages are comparable to those reported in other studies in France [17] and Ethiopia [19]. *E. coli* strains showed higher levels of resistance than those of Salmonella, as reported in the literature [12,

18]. It is likely that the isolated *E. coli* strains were under pressure from previous antibiotic therapies. The more a bacterial species or a serotype is encountered in pathology, the higher the frequencies of resistance, [13] Cross-resistance of ceftazidime-resistant *E. coli* strains from the strain isolation stage was observed for all beta-lactams. Salmonella also showed cross-resistance to this same family of antibiotics. Cross-resistance has a chromosomal origin and it only concerns antibiotics of the same family, therefore having a common site of action. Thus, resistance to an antibiotic can be accompanied by resistance to other antibiotics in the same family. It was noted from this study that antibiotic-resistant strains of *E. coli* and *Salmonella* spp. respectively 7/17 (41.17 %) and 1/5 (20 %) were found close to contact with the Ebrié Lagoon (breeding park). These strains are released into the environment and therefore represent a risk to public health through contamination of surface wastewater. It should be noted that the water from the Ebrié lagoon in direct contact with the effluents of the slaughterhouse is used by the surrounding populations for fishing and vegetable crops, which constitutes a real threat to the health of the population.

In short, the extent of bacterial resistance observed in this study reflects the previous use of antibiotics, in particular beta-lactams, in cattle farms at the Port-Bouët slaughterhouse as a curative or prophylactic measure. Better control of the evolution of bacterial resistance to antibiotics is only possible by disciplining the use of these products simultaneously in humans and animals.

5 CONCLUSION

The results of this study show that effluent discharges from the Port-Bouët slaughterhouse contribute to the maintenance of the environmental cycle of antibiotic-resistant *E. coli* and *Salmonella* spp. strains. The environment would therefore constitute a reservoir of multi-resistant strains where numerous gene exchanges would take place at the origin of the emergence of new pathogenic and/or resistant clones for humans. Antibiotic resistance is a constantly evolving phenomenon that concerns the entire bacterial world and all families of therapeutic antibiotics. This situation makes it difficult to choose effective measures to limit the erosion of the antibiotic spectrum. To prevent the spread of resistance, the Port-Bouët slaughterhouse should set up a network for monitoring the resistance of bacteria of animal origin. It would also be interesting to select a more representative number of strains of Salmonella and *Escherichia coli,* and to repeat this work in order to assess the impact of the spread of resistant bacteria in the environment on public health. An analysis of strains using molecular biology tools could make it possible to acquire information on resistance phenotypes and to distinguish within bacterial populations, the phenomena of clonal diffusion of resistant strains or the transfer of resistance genes.

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