

## ISOLATION AND PHENOTYPIC CHARACTERIZATION OF MULTIDRUG RESISTANCE NON-*E. COLI* ENTEROBACTERIACEAE FROM POULTRY IN WESTERN ALGERIA: RESISTANCE TO MANY FIRST-LINE ANTIMICROBIAL AGENTS

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### ABSTRACT

This study aimed to evaluate the antimicrobial resistance to the commonly used antibiotics with particular attention to the first-line antimicrobial agents and to investigate the multidrug resistance levels of non-*E. coli* Enterobacteriaceae from poultry in Western Algeria. Enterobacteriaceae culture was done on MacConkey agar and their identification was determined by AP20E kit, *Salmonella*-positive isolates serotyping were performed according to Kauffmann-White-Le Minor's scheme. For susceptibility testing, the Kirby-Bauer disk diffusion method to a panel of 14 antibiotics was used according to Clinical and Laboratory Standards Institute (CLSI) guidelines. A total of 141 non-*E. coli* Enterobacteriaceae isolates (53 *Proteus spp.*, 42 *Enterobacter spp.*, 11 *Citrobacter spp.*, 10 *Klebsiella spp.*, 8 *Serratia spp.*, 6 *Salmonella spp.*, 4 *Yersinia spp.*, 4 *Edwardsiella spp.*, 2 *Hafnia spp.* and one *Providencia spp.*) were isolated in this study. Four different serotypes of *Salmonella* strains were identified in this work. The predominant isolates exhibited high levels of resistance to various first-line antibiotic classes, notably betalactams, quinolones. Only the serotype *Salmonella Kentucky* was resistant to ciprofloxacin. All the strains isolated in this study were resistant to at least one antibiotic. Overall, 130 out of 141 isolates (92,19 %) demonstrated multidrug resistance (MDR). The dramatic increase in the rates of resistance to various first-line antimicrobial agents and the rapid spread of MDR in non-*E.coli* Enterobacteriaceae isolates of poultry origin can have major implications for public and animal health that should be approached urgently and pro-actively.

**Keywords:** multidrug resistance, non-*E. coli* enterobacteriaceae, first-line antimicrobial agents, poultry, Algeria.

## INTRODUCTION

Poultry production can face many constraints, especially microbial contaminations such as Gram-negative bacteria, which have a serious implications for public health and the surrounding environment (Skóra et al., 2016). Gram-negative bacteria of the Enterobacteriaceae family are important causes of urinary tract infections (UTIs), bloodstream infections, hospital- and healthcare-associated pneumonias, and various intra-abdominal infections. Within this family, *E. coli* is a frequent cause of UTIs, *Klebsiella spp.* and *Enterobacter spp.* are important causes of pneumonia, and all of the Enterobacteriaceae have been implicated in bloodstream infections and in peritonitis, cholangitis, and other intra-abdominal infections. Poultry and their products are considered the main vehicle for pathogenic bacteria such as *Salmonella (S.)* serovars, *E. coli* and *Klebsiella spp.* that cause foodborne infections in humans (EFSA, 2008; Sukumaran et al., 2012; Yadav et al., 2015). Enterobacteriaceae infections are of clinical concern, because several of the most important members of this family are becoming progressively more resistant to currently available antimicrobials (Paterson, 2006; Denton, 2007; Kilonzo-Nthenge et al., 2013; Benameur et al., 2014). Members of family Enterobacteriaceae, such as *E. coli*, *Klebsiella*, *Proteus*, and *Salmonella*, have been reported for developing antimicrobial resistance (Cortes et al., 2010; Ansari et al., 2014). Antimicrobial resistance in bacteria from the family Enterobacteriaceae is an essential sign of the occurrence of resistant bacterial strains in the community (Ojo et al., 2012). The abusive use of antibiotics in intensive livestock farming can be an important factor of the selection of antibiotic-resistant bacteria. Farm animals exposed to an antimicrobial agent for prolonged periods develop a microbial flora resistant to the agent (Swartz, 2002), which play a crucial role on the occurrence of MDR. Emergence of multidrug-resistant bacteria is of particular concern because of their potential for widespread dissemination and acquisition of additional resistance genetic elements (Karlowsky, 2003). The importance of infections due to multidrug resistant (MDR) Enterobacteriaceae has been increasingly recognized in recent years (Mercedes et al., 2013). Infections caused by multidrug-resistant Enterobacteriaceae are associated with increased morbidity and mortality than those caused by their susceptible counterparts (Bushen et al., 2021). This is mainly related to delay in providing active therapy, and also the bacterial infections increasingly failing to first-line and “last-resort” antibiotic therapies (Murray et al., 2021).

In different regions of Algeria, many published data reported alarming antimicrobial resistance levels of *E. coli* in poultry (Hammoudi and Aggad, 2008; Aggad et al., 2010; Messai et al., 2013; Benameur et al., 2016). Recent findings described clear evidence of direct transmission of multidrug-resistant *E. coli* isolates from food-producing animals or food to humans (Magiorakos et al., 2012; Adelowo et al., 2014; Bryan et al., 2020; Ramadan et al., 2021). However, few data were found in other Enterobacteriaceae species. The main goal of the present study was to evaluate the levels of antimicrobial resistance, notably to first-line antimicrobial agents, in non-*E. coli* Enterobacteriaceae strains from poultry and to investigate the presence of multidrug resistance.

## MATERIALS AND METHODS

Between January 2017 and December 2019, a total of 290 samples were collected from poultry farms and hatcheries located in six provinces, namely: Mascara, Mostaganem, Relizane, Tiaret, Tissemsilt, and Chlef. The samples consisted of healthy and diseased broiler and layer breeders, one day-old broiler and layer chicks, broilers, laying hens, and farm swabs. The samples were transported to the Laboratoire Vétérinaire Régional de Mostaganem, Algeria, and processed immediately for bacterial isolation.

Birds were necropsied immediately upon arrival in the laboratory using aseptic techniques. Internal organs (liver, spleen, or pericardium), fecal samples and farm swabs were used for bacterial isolation. The samples were examined using a previously described method (Benameur et al., 2018), with slight modifications. Briefly, one mL of sample was mixed with nine mL of buffered peptone water (Pasteur Institute of Algeria) vortexed and incubated overnight at 37 °C. Then, a drop of broth was streaked on MacConkey agar medium (Biochemica, Spain) and incubated overnight at 37 °C. The Enterobacteriaceae strains were identified biochemically using the API 20E system (BioMérieux, Marcy l'Étoile, France).

The antimicrobial susceptibility testing was done by Kirby Bauer disc diffusion method in Mueller Hinton agar (MHA), and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015; CLSI, 2017). Fourteen commercially available antimicrobial disks (Bio-Rad, Marnes la Coquette, France) were tested: amoxicillin/clavulanic acid AMC (20/10 µg), ampicillin (AM, 10 µg), ceftiofur (XNL, 30 µg), cephalotin (CEP, 30 µg), nalidixic acid (NA, 30 µg), norfloxacin (NX, 10 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TE, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1, 25/23, 75 µg), neomycin (N, 30 µg), gentamicin (CN, 10 µg), colistin (CT, 50 µg), chloramphenicol (C, 30 µg) and nitrofurantoin (FT, 300 µg). *E. coli* ATCC 25922 was used as a quality control strain.

In this study, the multidrug-resistant isolate was defined as expressing phenotypic resistance to three or more antibiotic classes (Magiorakos et al., 2012).

## RESULTS AND DISCUSSIONS

From 290 samples received in the Laboratoire Vétérinaire Régional de Mostaganem, Algeria, one hundred forty one non-*E. coli* Enterobacteriaceae strains were isolated. *E. coli* isolates were not included in this study. The most predominant isolated bacteria was *Proteus spp.* followed by *Enterobacter spp.* then *Citrobacter spp.* and *Klebsiella spp.* However, the number of the other isolated Enterobacteriaceae species was very low (figure 1).

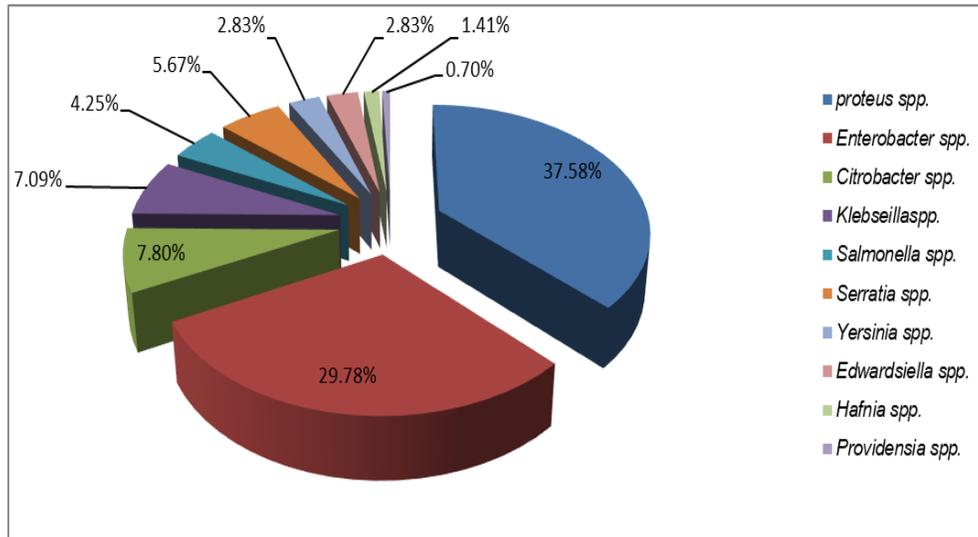


Figure 1. Percentage of non-*E. coli* Enterobacteriaceae isolates recovered from poultry

The percentages of Enterobacteriaceae species isolated in the current study are different to those reported in the previous study conducted in same regions (Benameur et al., 2018). In the last study, it reported that the predominant isolate is *Enterobacter* followed by *Klebsiella* then *Proteus*; these enterobacteriaceae are considered as poultry intestinal microfloras. Nevertheless, they can be implicated as opportunistic pathogens (Adebawale and Adeyemo, 2018). In other hand, they can found in nature, as for *proteus*, which are usually dispersed everywhere as saprophytes and are mostly found in manure, soil, human and animal feces (Senior, 1997); While *Enterobacter* are prototrophic in nature and are commonly found on a number of different plants and seeds (Francine and Patrick, 2006).

Six isolates were confirmed as *Salmonella* stains in this study, that belonging mainly to four different serotypes (*Kentucky*, *Dublin*, *Worthington* and *Kedougou*). The *Salmonella spp.* rate isolation is very low (4,25%, n=6). This could be explained by the low degree of excretion of *Salmonella* at the time of sampling and / or the intermittent excretion of *Salmonella* by infected birds (Van Immerseel, 2004). It could also be due to competitiveness between different saprophytic germs which inhibit the multiplication of pathogenic germs by modifying the environment because they are more demanding than saprophytic organisms (Valancony et al., 2001).

Isolation of multiple pathogens on different farms in the studied areas also points to the poor sanitary conditions and biosecurity measures on many poultry farms in Algeria.

The traditional first-line available options for treating serious infections caused by Enterobacteriaceae include penicillins, cephalosporins, monobactams, carbapenems, fluoroquinolones, and in certain situations, aminoglycosides. The frequency of resistance to these first-line agents has been rising worldwide in human Enterobacteriaceae isolates during the last years (Mercedes et al., 2013) and now reach high proportions in many areas of the world. In veterinary medicine, many studies have been determined the prevalence of antimicrobial resistance and antimicrobial resistance patterns in *E. coli* and *Salmonella* in many countries around the world. However, few data are available among other Enterobacteriaceae members. The results of the present study revealed that the most of the Enterobacteriaceae isolates were resistant to the majority of tested antibiotics as is summarized in figure 2.

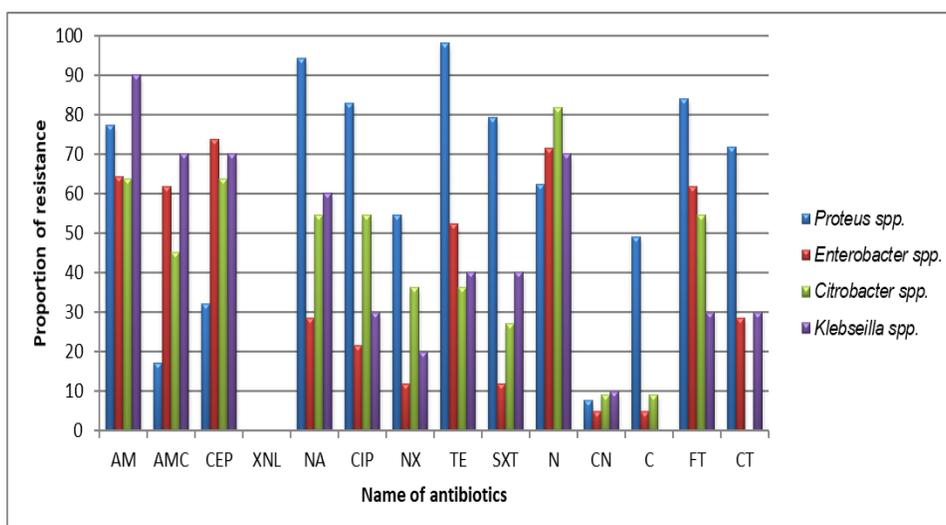


Figure 2. Antibiotic resistance of predominant non-*E.coli* Enterobacteriaceae isolates

AM=Ampicillin, CEP=Cephalothin, AMC=Amoxicillin/clavulanic acid, XNL=Ceftiofur, NA=Nalidixic acid, CIP=Ciprofloxacin, NX=Norfloxacin, TE=Tetracycline, SXT=Trimethoprim-sulfamethoxazole, N=Neomycin, CN=Gentamicin, C=Chloramphenicol, FT=Nitrofurantoin, CT=Colistin.

*Proteus spp.* isolates showed a high resistance level to particular antimicrobials, especially TE 98,94% (n=52), NA 94,93% (n=50), FT 84,90% (n=45), CIP 83,01% (n= 44), SXT 79,24% (n= 42), AM 77,35% (n= 41), CT 71,69% (n= 38). Amid *Enterobacter spp.* isolates, the highest proportion of resistance was toward CEP 73,80% (n=31), followed by N 71,43 % (n= 30), AM 61,24% (n= 27), FT and AMC 61.90% (n= 26), TE 52,38% (n= 22). Also *Citrobacter spp.* isolates exhibited a high resistance to N 81,81% (n= 9), AM and CEP 63,63% (n= 7), FT, NA, and CIP 54,54% (n= 6). Resistance of *Klebsiella spp.* to AM, CEP, N, NA, TE, SXT, and CIP was, respectively, observed in 90% (n=9), 70%(n=7), 70% (n=7), 60% (n=6), 40% (n=4 ), 40%(n=4 ) and 30%(n=3 ) of the isolates. While all the Enterobacteriaceae isolates examined in this work were susceptible to ceftiofur (XNL). The resistance of non *E.coli* Enterobacteriaceae strains to many first-line agents as betalactams (AM, CEF, AMC), quinolone and fluoroquinolone (NA, CIP), Tetracycline (TE), Aminoglycoside (N) is surprising, in contrary about the antibiotic resistance of *E.coli* strains from poultry in the same region (Boutaiba Benklaouz et al., 2020). The results of our study were higher than those previously reported in Algeria (Ahmed Ammar et al., 2017).

The high resistance to tetracycline reported in this study can be explained by abusive use of this ancient antibiotic in poultry farms in the study region and in another hand, by the lack of legislative restrictions of its use for therapy, prophylaxis, or growth promotion in Algeria. This percentage is higher than that reported in Indonesia (Yulistiani et al., 2017).

This study also revealed that predominant isolates from poultry showed high resistance properties toward betalactamins as AM, CEP and AMC which are in agreement with other study conducted in Ethiopia (Bushen et al., 2021). These molecules are antimicrobials of first and second generation of betalactamins that have a large use for therapeutic and disease prevention purposes in poultry.

The enterobacteriaceae isolates present also a high level of resistance against quinolones, which concord with the results those previously reported in the same provinces (Benameur et al., 2018). The high resistance rate of quinolone-resistant Enterobacteriaceae from poultry in Algeria was troubling, maybe due to the routine application of the quinolone (NA) at subtherapeutic doses for prophylactic and therapeutic purposes in broilers, and the fluoroquinolones (ciprofloxacin) for the prevention of early chick mortality. The increase in antimicrobial resistance, observed in Enterobacteriaceae isolates, may result from the clonal selection of organisms that tolerate sublethal antimicrobial doses and that present greater fitness under conditions of selective pressure, or from the spreading of resistance genetic determinants through horizontal gene transfer.

Our study recorded moderate resistance levels to some antibiotics which are prohibited in veterinary medicine in Algeria, such as nitrofurantoin and chloramphenicol. Persistence of resistance to these antibiotics has also been observed previously (Senior, 1997); this could be explained by co-selection of mobile resistance elements for the nitrofurantoin and chloramphenicol-resistance (Francine and Patrick, 2006).

Table 1. Multi-drug resistance in non-*E. coli* Enterobacteriaceae strain isolates from poultry

Enterobacteriaceae strains	Number of antibiotics									Multi-drug resistant Strains (%)
	0	1	2	3	4	5	6	7	≥8	
<i>Proteus spp.</i> (n=53)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	8 (15,09)	2 (3,77)	7 (13,20)	6 (11,32)	30 (56,60)	53 (100)
<i>Enterobacter spp.</i> (n=42)	0 (0,00)	1 (2,38)	3 (7,14)	6 (14,28)	4 (9,52)	12 (28,57)	3 (7,14)	4 (9,52)	9 (21,42)	38 (90,47)
<i>Citrobacter spp.</i> (n=11)	0 (0,00)	1 (9,09)	1 (9,09)	1 (9,09)	1 (9,09)	2 (18,18)	0 (0,00)	1 (9,09)	4 (36,36)	9 (81,81)
<i>Klebsiella spp.</i> (n=10)	0 (0,00)	0 (0,00)	0 (0,00)	2 (20,00)	3 (30,00)	0 (0,00)	0 (0,00)	2 (20,00)	3 (30,00)	10 (100)
<i>Serratia spp.</i> (n=8)	0 (0,00)	0 (0,00)	2 (25,00)	0 (0,00)	1 (12,50)	1 (12,50)	1 (12,50)	1 (12,50)	2 (25,00)	6 (75,00)
<i>Salmonella spp.</i> (n=6)	0 (0,00)	0 (0,00)	1 (16,66)	1 (16,66)	1 (16,66)	2 (33,33)	1 (16,66)	0 (0,00)	0 (0,00)	5 (83,33)
<i>Yersinia spp.</i> (n=4)	0 (0,00)	1 (25,00)	0 (0,00)	1 (25,00)	0 (0,00)	0 (0,00)	0 (0,00)	1 (25,00)	1 (25,00)	3 (75,00)
<i>Edwardsiella spp.</i> (n=4)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	1 (25,00)	0 (0,00)	3 (75,00)	4 (100)
<i>Hafnia spp.</i> (n=2)	0 (0,00)	0 (0,00)	1 (50,00)	0 (0,00)	0 (0,00)	0 (0,00)	1 (50,00)	0 (0,00)	0 (0,00)	1 (50,00)
<i>Providencia spp.</i> (n=1)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)	1 (100)	1 (100)
<b>Total (n=141)</b>	<b>0</b> (0,00)	<b>3</b> (2,12)	<b>8</b> (5,67)	<b>11</b> (7,80)	<b>18</b> (12,76)	<b>19</b> (13,47)	<b>14</b> (9,92)	<b>15</b> (10,63)	<b>53</b> (37,58)	<b>130</b> (92,19)

All *Salmonella* serotypes exhibited resistance to quinolone (NA) but only the serotype *Kentucky* was resistant to fluoroquinolone (CIP) (figure 3). This result is in line with findings for *Salmonella* isolates from food animals and humans in Eastern Algeria (Djeffal et al., 2017). In other hand, several reports have mentioned *S. Kentucky* CIP-R isolates that have been identified from different sources (animals, food, the environment and humans) and geographic locations (Le Hello, 2013). Moreover, a *Kentucky* ciprofloxacin-resistant serotype was isolated in France from a patient who had previously visited Algeria (Weill and Le Hello, 2011). The presence of *salmonella* isolates resistant to fluoroquinolones in poultry is much more worrying, as these antibiotic molecules are among the last resort for the treatment of severe human salmonellosis.

Emergence of MDR bacteria, especially Enterobacteriaceae, has increased in recent years. In this study, all the Enterobacteriaceae strains were resistant to at least one antibiotic. High level of Enterobacteriaceae isolates 130 (92.19%) demonstrated multidrug resistance to at least three unrelated antimicrobial agents. Our result concord with other study in the same region (Benameur et al., 2018).

Resistance to three or more antibiotics was found in 53 (100%) isolates of *Proteus spp.*, followed by 38 (90.47%), 10 (100%), 9 (81,81%), 6 (75%), 5 (83,33%), 3 (75%), 4 (100%), 1(50%) and 1(100%) isolates of *Enterobacter spp.*, *Klebsiella spp.*, *Citrobacter spp.*, *Serratia spp.*, *Salmonella spp.*, *Yersinia spp.*, *Edwardsiella spp.*, *Hafnia spp.* and *Providencia spp.* respectively, as shown in table 1.

This indicates that the abusive and indiscriminant use of these antibiotics, such as wrong indication, wrong duration and wrong antibiotic combinations, is probably the origin of the high incidence of MDR strains (Harris et al., 2015; Reygaert, 2018). In addition to the above, their use without prior antibiotic sensitivity testing of bacterial isolates, may lead to the development of a pool of antibiotic-resistant genes and to the selection of increasing numbers of resistant Enterobacteriaceae clones.

Antibiotics resistance patterns of the predominant Enterobacteriaceae strains isolated in this study were frequent and divers as shown in table 2 and 3.

Table 2. The most frequent antibiotic resistance patterns of *Proteus spp.* isolates (n=53)

Resistance patterns	Designation	Number of strains (%)
TE NA	A	2(3,77)
TE NA CIP	B	8(15,09)
TE NA CIP SXT FT	C	1(1,88)
TE NA CIP SXT FT CT	D	7(13,20)
TE NA CIP SXT FT CT AMP	E	2(3,77)
TE NA CIP SXT FT CT AMP N NX	F	4(7,54)
TE NA CIP SXT FT CT AMP N NX C CEF	G	3(5,66)
TE NA CIP SXT FT CT AMP N NX C CEF	H	2(3,77)
TE NA CIP SXT FT CT AMP N NX C CEF CN	I	1(1,88)
TE NA CIP AMP FT CT	J	1(1,88)

Table 3. The most frequent antibiotic resistance patterns of *Enterobacter spp.* isolates (n=42).

Resistance patterns	Designation	Number of strains (%)
CEF AMP	A	1(2,38)
CEF AMP N	B	2(4,76)
CEF AMP N FT	C	5(11,90)
CEF AMP N FT TE	D	7(16,66)
CEFAMP N FT TE AMC NA	E	2(4,76)
CEF AMP N FT AMC	F	1(2,38)
CEF AMP NX N AMC SXT TE CIP	G	3(7,14)
AMP NX TE CN NA AMC N CIP	H	1(2,38)
CEF N TE C	I	2(4,76)
N CT	J	2(4,76)

The most common multidrug resistant profiles among *proteus spp.* were profile B and D with percentage of 15,09% and 13,20% respectively; which were resistant to tetracycline, nalidixic acid, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and colistin. Concerning *enterobacter spp.* strains were profile C and D with percentage of 11,90% and 16,66% respectively; which were resistant to tetracycline, cephalotin, ampicillin, neomycin and nitrofurantoin. Resistance of predominant MDR enterobacteriaceae isolates to first line-antibiotic such as penicillins and fluoroquinolones in poultry in western Algeria is constantly increasing. This rising in resistance to various last-resort agents makes difficult to select an empirical therapy (Livermore et al., 2008; Canton et al., 2012). Prudent use practices should include restricting the access for use of antimicrobials that are considered to be important for human medicine in animal production (WHO, 2017).

Multidrug resistant poultry pathogens may result in treatment failure, leading to economic losses, but also be a source of resistant bacteria/genes (including zoonotic bacteria) that may can be a risk to public health.

The development of new antimicrobial agents is among the urgent recommendations proposed to take action in the public health and veterinary farming domains by limiting the inappropriate exposure of bacteria to antibiotics in order to slow down a natural evolution toward resistance and its spread in the downstream environment in a One Health context (WHO, 2015; WHO, 2017).

Few data are available on the pattern of antimicrobial resistance and multidrug resistance in Enterobacteriaceae other than *E. coli* and *Salmonella spp.* from food producing animals. Regional and local descriptions of antibiotic resistance add to the global view of antimicrobial resistance. The study provides an insight into the prevalence of antibiotic resistance in a largest country in Africa.

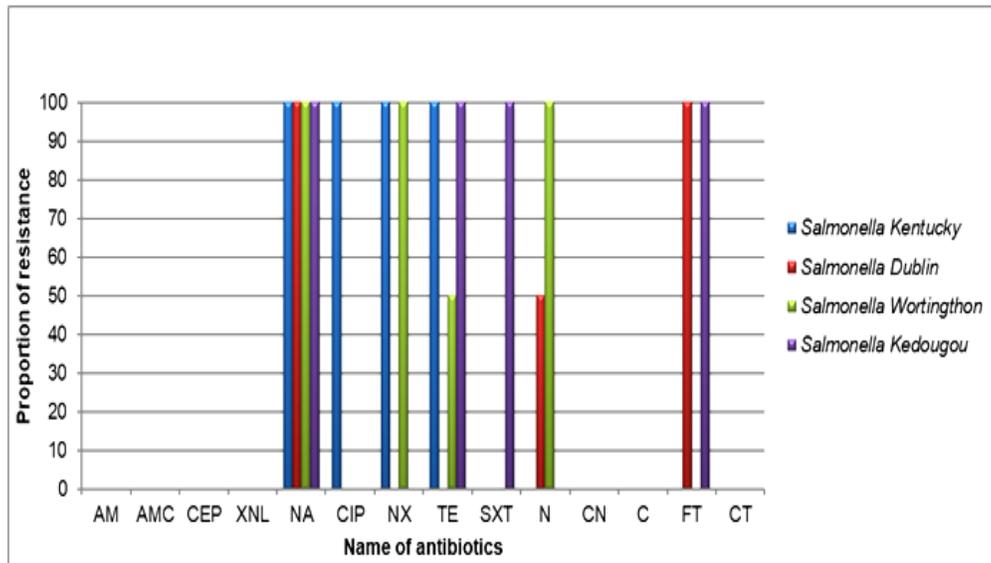


Figure 3. Antimicrobial resistance among *Salmonella serotypes*

## CONCLUSION

- In summary, the present study reported the presence of various potentially pathogenic and multidrug resistance non-*E. coli* Enterobacteriaceae from poultry in western Algeria.
- We believe that such this alarming rate of resistance to various first-line antimicrobial agents in poultry in the region is linked to the abusive and uncontrolled use of antibiotics in poultry that cause the increasing treatment failure.
- Therefore, the more information on the role of environmental microbiomes in the rise of antibiotic resistance, the creative approaches to the discovery of novel antibiotics and their controlled introduction to therapy in poultry management are among the solutions that are becoming urgent and mandatory.

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