Antimicrobial resistance of 100 *Salmonella* strains isolated from *Gallus gallus* in 4 wilayas of Algeria

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ABSTRACT This study aims at identifying serotypes and surveying the antimicrobial resistance and plasmid support of resistance of 100 *Salmonella* strains, which were isolated from 96 out of 506 (18.97%) samples taken from different production farms in the wilayas (i.e., Algerian states) of Tizi-Ouzou, Bouira, Bejaïa, and Boumerdes in 2007. The highest percentage of *Salmonella* (48%) was recorded in Bouira. Thirteen serotypes were identified among the 100 *Salmonella* strains used in this study. The most prevalent ones were *Salmonella* Heidelberg (24%), *Salmonella* Enteritidis (20%), *Salmonella* Albany (16%), and *Salmonella* Typhimurium (9%). The strains showed resistance to 8 of the 34 antibiotics tested. Fifty-three percent of strains were resistant to at least one antibiotic, among which 15.09% were multiresistant. The most frequently observed resistance was to quinolones (58.49%), with a contribution of 94.74% of *Salmonella* Heidelberg resistant strains. The plasmid transfer performed on 53 strains showed that only 11 exhibited one or more markers of resistance, the most frequent being ampicillin, followed by tetracycline, then cotrimoxazole, sulphonamides, and kanamycin, in that order. The tetracycline characteristics were present in 72.72% of transconjugants, those of the β-lactams and sulphonamides in 27.27% each and those of the aminosides in 9.09%. The incompatibility groups of plasmids belong to the F1me and Com1 classes, and the molecular weight of the plasmid DNA was greater than 100 kb. The phenotypic and genotypic results indicate a clonal dissemination in the *Gallus gallus* species in this particular study; this phenomenon could generate resistant bacteria and transferable genes of resistance to humans.

Key words: *Salmonella*, *Gallus gallus*, antimicrobial resistance profile, plasmid profile, incompatibility group

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INTRODUCTION

The production and consumption of poultry has increased considerably worldwide. Poultry production has left the farmyard and is now industrialized and conducted in a more rational manner (Delpech, 1992). In Algeria, since the 1980s, the emergence of the poultry industries increased the consumption of animal proteins at a much affordable cost (Ferrah et al., 2003). Poultry represents one of the main reservoirs of *Salmonella* that are responsible for a major zoonosis with serious economic and public health consequences (Brisabois, 2001). Antimicrobial agents played a dominant role for the treatment and the control of the salmonellosis. Their overconsumption contributed to the emergence of resistances. In fact, despite the decrease of salmonellosis incidence in the world, multiresistant strains appeared in humans and became increasingly alarming in both industrialized and developing countries (Brisabois, 2001). The surveillance of the current bacterial resistances should not be exclusively based on the determination of the resistant phenotypes. The genetic characterization by conjugation is currently the most used tool in Algeria to better understand the spread of antibiotic resistance in zoonotic bacteria, until the introduction of molecular biology techniques.

Our objectives were the phenotypic characterization of 100 *Salmonella* strains by determining the circulating serotypes in the study's area, the antimicrobial resistance profiles, as well as the genotypic characterization.

MATERIALS AND METHODS

Sampling

As part of official control of poultry in 4 wilayas of Algeria, from January to December 2007, 100 *Salmonella* strains were isolated from 506 avian samples during autopsies of slain live subjects. This was conducted

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at the medical bacteriology department of the Regional Veterinary Laboratory of Draa-Ben-Khedda.

Isolation and Identification of Salmonella Strains

The NF U 47–100/2007 (Afnor, 2007) standard method was adopted for the isolation and the biochemical and serological identification of *Salmonella*. The serotyping was carried out at the enterobacteria service of the Institut Pasteur of Algeria, using *Salmonella* antiserums anti-O and anti-H (BioMérieux, Marcy l'Etoile, France).

Antimicrobial Sensitivity Testing

The phenotypic study of the antimicrobial resistance was performed at the Bacteriology and Antibiotherapy Department of the Institut Pasteur of Algeria. Thirty-four antimicrobial agents of 9 different chemical classes were used: ampicillin (10 μg), amoxicillin/clavulanic acid (20 μg/10 μg), mecillinam (10 μg), ticarcillin (75 μg), piperacillin (100 μg), latamoxef (30 μg), imepenem (10 μg), aztreonam (30 μg), cefazolin (30 μg), cefalexin (30 μg), cefoxitin (30 μg), ceftazidim (30 μg), cefepim (30 μg), cefuroxim (30 μg), cefotaxim (30 μg), to ceftiofur (30 μg), cephalotin (30 μg), gentamicin (10 μg), amikacin (30 μg), netilmicin (30 μg), kanamycin (30 μg), isepamicin (30 μg), nitrofurantoïne (300 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), levofloxacin (5 μg), tetracyclines (30 μg), chloramphenicol (30 μg), sulphonamides (300 μg), trimethoprim (5 μg), trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg), colistin (10 μg), and fosfomycin (200 μg; Bio-Rad, Hercules, CA). Micrograms indicates the antibiotic load in one disk; the unit of this load (expressed in micrograms) for each antibiotic used for each bacterial species is specified by the supplier (Bio-Rad). The minimum inhibitory concentration was established using the E-test (AB-Biodisk). *Escherichia coli* ATCC 25922 (Institut Pasteur Paris, Paris, France) was used for the control antibiograms. The method adopted for the study of the antimicrobial resistance is based on the diffusion in Mueller-Hinton agar media as described by Kirby-Bauer and interpreted according to the Clinical Laboratory Standards Institute (CLSI, 2001) guidelines, which are recommended by the World Health Organization (WHO, 1999). In our study, the multiresistant strains are the ones that confer a resistance to 2 or more antimicrobials. The study of genetic support required the use of antimicrobial powders: tetracycline (Sigma, Woodstock, VA), nalidixic acid (Serva, Heidelberg, Germany), rifampicin (Life-pharmacy, Tuen Mun, Hong Kong, China), nitrofurantoïnes (Saïdal, Annaba, Algeria), kanamycin (Bristol-Meyers Squibb, New York, NY), trimethoprim (Roche, Basel, Switzerland), injectable sodic ampicillin (Biopharm, Bothell, WA), chloramphenicol sodic ampicillin (Serva Peinbiochemica). Also, several reference strains and plasmids were used (Institut Pasteur Paris): *Escherichia coli* K12Nal (nalidixic acid resistance) and *Escherichia coli* C600Rif (rifampicin resistance) for the plasmid transfer by conjugation and Com1 (K) Rif, Com1 PPED 178 (ACTp) Rif, F1me PPED 181/4(T) Nal, and F1me PPED 181/3(CTp) Rif for the compatibility test, as well as the DNA of the λ phage digested by the Hind III enzyme as a reference to determine the size of the plasmids. The genetic transfer by conjugation that was used for the detection of plasmids carrying the resistance characters was described by Courvalin et al. (1985). After streaking on agar bromocresol purple and 18 to 24 h of incubation at 35°C, a colony of the studied donor strain and that of the recipient reference strain were individually inoculated in buffered glucose broth. After shaking for 4 h in a water bath at 35°C, 1 mL of each culture was mixed with a rake on Mueller-Hinton agar and incubated for 18 h at 35°C. One milliliter of buffered glucose broth was added, and then the supernatant was recovered after mixing. Moreover, 1 mL of an antibiotic for the suspected plasmidic character for the donor strain and 1 mL of another antibiotic for the suspected chromosomal character for the receiving strain were added to 18 mL of Mueller-Hinton agar. The final concentrations of the antibiotics used are Furans, 300 μg/mL; TCY, 33 μg/mL; ampicillin, 200 μg/mL; kanamycine, 25 μg/mL; Nal, 100 μg/mL; TMP, 20 μg/mL; CCC, 25 μg/mL. A quarter of a Petri dish was seeded in lines by the 2 strains and the 3/4 remaining were streaked by the supernatant (mixture of these 2 strains). After 18 h of incubation at 35°C, the appearance of colonies on the part inoculated with the supernatant indicates the presence of the transconjugants.

The resulting transconjugants were tested to determine their resistance profiles and therefore their transferable characteristics. To determine the major incompatibility groups of plasmids the used test was the same as for the genetic transfer; it required a cross between a transconjugant and *E. coli* carrying a plasmid reference. The plasmids were extracted from the transferred strains and their transconjugants, using the alkaline lyse technique (Kado and Liu, 1981), and the plasmid profiles were determined by separating the plasmids extracted on agarose gel.

RESULTS

Nineteen percent of the total samples were positive for *Salmonella*. The distribution of *Salmonella* per wilaya showed 48% of cases from Bouira, 21% from Bejaïa, 16% from Boumerdes, and 15% from Tizi-Ouzou. Thirteen different serotypes were identified, among which *Salmonella* Heidelberg was the most prevalent (24%). *Salmonella* Heidelberg, *Salmonella* Enteritidis, *Salmonella* Albany, *Salmonella* Infantis, *Salmonella* Blockley, and *Salmonella* Livingstone were present in all the wilayas. Besides, *Salmonella* Enteritidis prevailed in Bouira, *Salmonella* Albany in Bejaïa and Tizi-Ouzou, and *Salmonella* Heidelberg in Boumerdes (Table 1).

The study of the antimicrobial resistance performed on 100 *Salmonella* strains revealed a resistance to 8 of them from 6 different classes. Fifty-three percent of the strains showed resistance to at least one antimicrobial, among which 15.09% were multiresistant. Forty-seven percent of strains were susceptible to all antimicrobials tested. The resistance to quinolones was the most frequent (58.49%) and included 94.74% of the resistant strains of the prevalent serotype in our study (Table 2). The plasmid transfer assay was performed on 53 strains with resistance to β-lactams (4%), aminoglycosides (2%), tetracyclines (15%), sulphonamides (13%), nitrofurans (19%), and to quinolones (47%). In total, 11 strains have transferred one or several resistance markers, of which the most frequent were ampicillin, tetracycline, cotrimoxazole, sulphonamides, and kanamycin. The tetracycline character was present in 72.72% of transconjugants, those of the sulphonamides and β-lactams in 27.27% each, and that of the aminoglycosides in 9.09%. The compatibility test revealed incompatibility groups of plasmids in Com1 and F1me classes (Table 3). The plasmid DNA analysis resulted in a molecular weight that is greater than 100 kb (Figure 1).

DISCUSSION

Geographical Distribution of Salmonella Serotypes

Bouira was characterized by the most important number of isolated strains because most avian samples were from this region, which has more than one-thousand poultry farms. *Salmonella* Heidelberg prevailed in our study. Our results were supported by the study of Barnhart et al. (1991) in Northern Ireland, on 42 poultry samples (56.5%), and that of Mammina et al. (2003) in Italy, on laying hens and the environment of chicken farms (20.3%). Whereas in a national study, *Salmonella* Heidelberg represents only 1.81% (Elgroud et al., 2009). This serotype is among the 3 most common cases of human salmonellosis in Canada and one of the 5 most prevalent in the USA, according to the Canadian integrated program for monitoring of the antimicrobial resistance (PICRA, 2006). According to this program, international comparisons of human data from 2000 to 2004 indicated that *Salmonella* Heidelberg was more common in North America, whereas Mammina et al. (2003) have shown that this serotype was responsible for only a small proportion of human infection in Italy. According to Usera et al. (1999) and the report of the European Community (EFSA, 2007), *Salmonella* Enteritidis was the most frequently reported, with prevalences of 46.1 and 42%, respectively, and these are higher than those we have recorded. Our present result is higher than that recorded by Elgroud et al. (2008; 3.63%). The isolation frequency of *Salmonella* Infantis and *Salmonella* Albany was comparable to that given in Austria by Berghold and Kornschober (2002). Similarly, the rate that we recorded for *Salmonella* Typhimurium was comparable to that reported by Elgroud et al. (2009) and by Van Immerseel et al. (2005) in poultry farms in Europe between 1991 and 2000 but higher than that noted by these same authors in 2003. The registered prevalence for *Salmonella* Blockley was lower than that found in Saudi Arabia (al-Nakhli et al., 1999). *Salmonella* Hadar, identified in 5% of our strains, was the predominant serotype in Italy (Uyttendaele et al., 1998) and Senegal (Cardinale et al., 2005). *Salmonella* Pullorum, which has no impact on public health, as related to host species has been eradicated from poultry farms in several developed countries, such as the USA and Canada. The declaration of pullorosis in our farms could cause major economic losses, such as those observed from the thirties to the sixties (Villate, 2001). In our study, *Salmonella* Newport and *Salmonella* Montevideo were isolated with a very low prevalence. These results are corroborated by the study of Elgroud

Table 1. Distribution of *Salmonella* serotypes isolated from *Gallus gallus* on 4 wilayas of the center of Algeria[1]

Salmonella serotype	Wilaya (n)[2]				Total (%)
	Bouira	Bejaïa	Boumerdès	Tizi-Ouzou	
Heidelberg	9	4	**8**	3	24
Enteritidis	**15**	2	1	2	20
Albany	5	**6**	1	**4**	16
Typhimurium	7	2	0	0	9
Blockley	3	1	1	1	6
Infantis	1	3	1	1	6
Hadar	3	1	1	0	5
Livingstone	1	1	1	2	5
Pullorum	2	0	1	1	4
Indiana	1	1	0	0	2
Kedougou	0	0	1	0	1
Newport	0	0	0	1	1
Montevideo	1	0	0	0	1
Total (%)	48	21	16	15	100

[1]Bold numbers indicate the strains by serotype that prevailed in each wilaya.

[2]n = number of serotypes.

Table 2. Rates and antimicrobial resistance profiles of *Salmonella* strains isolated from *Gallus gallus*

Salmonella serotype	n1[1]	β-Lactam	Aminoglycoside	Furan	Quinolone	Cycline	Sulphonamide			n2[2]	Resistance profile[3]
		AMP	K	FT	NAL	TCY	SSS	TMP	SXT		
Heidelberg	19/24	1	0	0	1	1	0	0	0	1	AMP, TCY
		0	0	0	0	1	0	0	0	1	NAL
		0	0	0	17	0	0	0	0	17	TCY, NAL
Enteritidis	12/20	0	0	9	0	0	0	0	0	9	FT
		0	0	0	3	0	0	0	0	3	NAL
Albany	2/16	0	0	0	2	0	0	0	0	2	NAL
Typhimurium	5/9	0	0	1	0	0	1	0	0	1	FT, SSS
		0	0	0	0	1	1	1	1	1	TMP, SXT
		0	0	0	3	0	0	0	0	3	SSS, TCY, NAL
Pullorum	4/4	0	0	0	1	0	0	0	0	1	NAL
		0	0	0	1	0	1	0	0	1	NAL, SSS
		0	0	1	0	0	0	0	0	2	FT
Blockley	3/6	0	0	0	0	0	1	0	0	1	SSS
		0	0	0	0	1	0	0	0	2	TCY
Hadar	3/5	1	0	0	1	1	0	0	0	1	AMP, TCY
		0	1	0	0	0	0	0	0	1	NAL
		0	0	0	0	1	0	0	0	1	K, TCY
Indiana	2/2	0	0	1	0	0	0	0	0	1	FT
		0	0	0	0	1	1	1	1	1	TMP, SXT, SSS, TCY
Infantis	1/6	0	0	0	1	0	0	0	0	1	NAL
Livingstone	1/5	0	0	0	0	1	0	1	1	1	TCY, SXT, TMP
Newport	1/1	1	0	0	1	1	0	0	0	1	AMP, TCY, NAL
Kedougou	0/1	0	0	0	0	0	0	0	0	0	/
Montevideo	0/1	0	0	0	0	0	0	0	0	0	/
%	53/100	5.66	1.89	22.64	58.49	16.98		11.32		53	

[1]n1: total number of resistance strains/total number of isolated strains.

[2]n2: number of strains in different antimicrobial resistance profile.

[3]AMP: ampicillin; K: kanamycin; FT: nitrofurantoïne; NAL: nalidixic acid; TCY: tetracycline; SSS: sulphonamides, TMP: trimethoprim, SXT: cotrimoxazole.

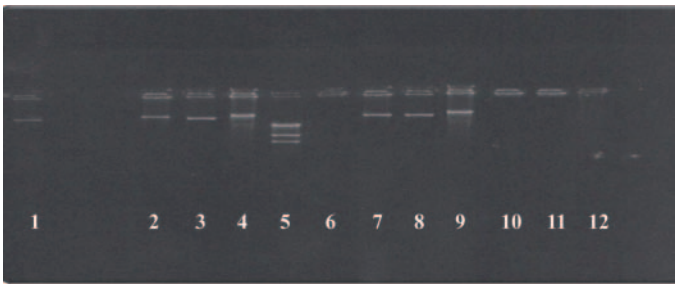

Figure 1. Plasmid profiles of transconjugants: 1: Com1; 5: λ gene digested by Hind III; 10: *Escherichia coli* K_{12}Nal; 11: *E. coli* C_{600}Rif. Transconjugants: 2: 24 (TCY, SSS, TMP) NAL; 3: 36 (TCY, SSS, TMP) NAL; 4: 44 (K) NAL; 6: 41 (TCY) NAL; 7: 37 (TCY) NAL; 8: 52 (TCY) NAL; 9: 75 (TCY) NAL; 12: 21 (TCY, SSS, TMP) NAL. TCY: tetracycline; SSS: sulphonamides; TMP: trimethoprim; NAL: nalidixic acid; K: kanamycin.

et al. (2009) but refuted by those of Uyttendaele et al. (1998) in Belgium and Foley et al. (2008) in the USA, who have ranked it among the 6 most common serotypes. The study of the distribution of *Salmonella* serotypes within 4 wilayas taught us about their dynamic expansion across the adjacent wilayas, or even their possible spread in the national territory. Bouira was the wilaya that counted most of the serotypes, with a high prevalence of *Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Typhimurium. Bejaïa had less diversity than that recorded in Bouira; the predominant serotypes were *Salmonella* Albany, *Salmonella* Heidelberg, and *Salmonella* Infantis. Although Tizi-Ouzou and Boumerdes had only 15 and 16 strains, diversity was observed with a predominance of *Salmonella* Albany and *Salmonella* Heidelberg, respectively. The homogeneous distribution of all serotypes in the study's area could be the consequence of the uncontrolled movement of poultry products.

Resistance of Antimicrobial Agents Tested

There is a positive correlation between antimicrobial use and bacterial resistance rates; indeed, each exposure eliminates the susceptible bacteria and promotes the growth of resistant strains (Jaecklin, 2002; Fluit, 2005). Even though the treatment of avian salmonellosis is banned in Algeria by ministerial order (JORA, 2003), the high rate of antimicrobial resistance that we recorded (53%) could be explained by the indiscriminate use of these drugs for therapeutic, prophylactic purposes or as growth promoters. Our result was significantly higher than that reported by Al-Bahry et al. (2007; 23.7%) and lower than that recorded by Elgroud et al. (2008; 80%) and that reported by the PICRA (2006; 63%). Multidrug resistance observed in our study was also described by Bornert (2000) and Al-Bahry et al. (2007). Moreover, resistance was found for 6 of the 9 antimicrobial classes tested. The observed resistance to first generation quinolones (nalidixic acid) was the most frequent. This is likely due to their repeated use in poultry production. Fortunately, there is no resistance to fluoroquinolones tested (ciprofloxacin, enrofloxacin, levofloxacin) as reported by Mc Kenzie and Nadeau (2006). These results corroborate several international studies (Heurtin-Le Corre et al. 1999; San Martin et al., 2005; PICRA, 2006). *Salmonella* isolates were found to be resistant to ciprofloxacin in previous French (Brisabois et al., 1997) and Algerian studies (Elgroud et al., 2009; Bouzidi et al., 2011), which is more worrying as fluoroquinolones should be reserved for the treatment of invasive human salmonellosis (Davies et al., 1999). Although furans have been removed from the Algerian nomenclature, we recorded a high rate of resistance to nitrofurantoïnes. This result was lower than that noted in 2004 in Poland by Wasyl and Hoszowski (2004; 48.2%). The rates of resistance to cyclines reported by several authors were higher than those we recorded and ranged from 20 to 91% (Brisabois et al., 1997; Dinh Nam Lâm et al., 2000; Mc Kenzie and Nadeau, 2006; PICRA, 2006). As for sulphonamides, our result was higher than that reported by the national monitoring network to antimicrobial resistance (7%; Aboun, 2005) and lower than that noted by Brisabois et al.

Table 3. Determination of incompatibility groups and plasmid profiles of *Salmonella* strains that transferred resistance markers[1]

Salmonella serotype	n1[2]	Resistance profile[3]	n2[4]	Transferred resistance	Incompatibility group	DNA extraction
Heidelberg	19	AMP, TCY, NAL	1	(AMP)Rif	ND	/
		TCY	1	TCY(NAL)	Com1	+
Typhimurium	5	SXT, TMP, SSS, TCY	1	(TCY, SSS, TMP)NAL	Com1	+
Blockley	3	TCY	2	(TCY)NAL	Com1	+
				(TCY)NAL	Com1	+
Hadar	3	AMP, TCY, NAL	3	(AMP)Rif	ND	/
		TCY		TCY(NAL)	Com1	+
		K		K(NAL)	ND	/
Livingstone	1	TCY, SSS, TMP, SXT	1	(TCY, SSS, TMP)NAL	Com1	NV
Indiana	2	TCY, SSS, TMP, SXT	1	(TCY, SSS, TMP)NAL	Com1	+
Newport	1	AMP, TCY, NAL	1	(AMP, TCY)Rif	F1me	NE

[1]ND: not determined; NV: not visualized; NE: no extract.

[2]n1: number of donor strains on which tests conjugation were performed.

[3]AMP: ampicillin; TCY: tetracycline, NAL: nalidixic acid; FT: nitrofurantoïne; SXT: cotrimoxazole, TMP: trimethoprim; SSS: sulphonamides, K: kanamycin.

[4]n2: number of strains that transferred one or more resistance markers.

(1997; 88%). Resistance to β-lactams involved ampicillin only; our result was lower than those reported by Mc Kenzie and Nadeau (2006), Al-Bahry et al. (2007), and Dinh Nam Lâm et al. (2000), which were between 36 and 88.89%. Acquired resistance to aminoglycosides is not common in *Enterobacteriaceae* (Courvalin and Philippon, 1989); the only restraint in our study was resistance to kanamycin, although this antimicrobial agent is not used in poultry. Our result, probably due to the cross resistance with neomycin, was in agreement with those obtained by Brisabois et al. (1997), Millemann (1998), Dinh Nam Lâm et al. (2000), and Mc Kenzie and Nadeau (2006). The susceptibility to polypeptides was confirmed by Aboun (2005); on the other hand, the susceptibility to chloramphenicol was reversed by Brisabois et al. (1997), Dinh Nam Lâm et al. (2000), and Al-Bahry et al. (2007), who noted 91, 25, and 14% resistance rates, respectively. Out of 24 *Salmonella* Heidelberg strains tested, only 5 were susceptible to all antimicrobials used. The 19 remaining were mostly resistant to nalidixic acid. In Canada, this serotype would be particularly resistant to the third generation cephalosporins (PICRA, 2006). The resistance of *Salmonella* Enteritidis to furans was confirmed by the results of Espigares et al. (2006). In our study, *Salmonella* Typhimurium was resistant to quinolones, furans, cyclones, and sulphonamides. This multidrug resistance could facilitate the spread of this serotype and the development of other mechanisms of resistance to other antimicrobial classes. Indeed, in the literature, *Salmonella* Typhimurium was resistant to several antimicrobial agents with a special surge of resistance to nalidixic acid (Brisabois et al. 1997; Heurtin-Le Corre et al., 1999). *Salmonella* Hadar, resistant to quinolones and tetracycline, was multiresistant in the study of Espigares et al. (2006).

Genetic Support of Resistance

The *Salmonella* strains resistant to nalidixic acid and nitrofurantoïnes were not transferable, confirming the chromosomal support of these resistances. The majority of resistance markers were transferred; the most common were ampicillin, tetracycline, cotrimoxazole, sulphonamides, and kanamycin. In our study, a plasmid grouping identified the same classes (Com1 and F1me) as described by Chaslus-Dancla (1980) in France and found in a national study on enterobacteria isolated in western Algeria (Barka, 2002). The results obtained by physical analysis of plasmid content of transconjugants implied that the spread of resistance characteristics of studied *Salmonella* strains is provided by high molecular weight plasmids. This study allowed us to highlight diverse serotypes, with a predominance of *Salmonella* Heidelberg. The free flow of the same *Salmonella* serotypes and the dissemination of antimicrobial resistance and plasmid groups, observed in a limited area of study, is an alarming reality. The fight against avian salmonellosis requires the application of control measures at various levels of the production chain. It would also require the development of an effective plan of epidemiological surveillance, and for this, similar and complementary studies must be conducted in Algeria and will include the identification of different phage types and their distribution, monitoring the geographical spread and resistance evolution of identified serotypes, the spread of resistant *Salmonella* strains from animals to humans, and the identification of resistance genes. The use of different techniques in molecular biology is essential, particularly those based on the analysis of the restriction pattern of plasmids should be generalized.

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