Full Length Research Paper

Serogroups and antibiotics susceptibility pattern of avian pathogenic *Escherichia coli* strains responsible for colibacillosis in broiler breeding farms in the east of Algeria

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In East Algeria, 100 *Escherichia coli* strains were isolated from livers and spleens of 120 broiler carcasses that presented colibacillosis lesions at autopsy. Serogrouping of these strains demonstrated that 83% of their serogroups belong to the most pathogenic serogroups of Avian Pathogenic *Escherichia coli (*APEC): O1 (14%), O2 (53%) and O78 (16%). *In vitro* susceptibility to antimicrobials of veterinary significance was determined by disc diffusion test. Antibiogram demonstrated a high level of resistance to enrofloxacin (82%), trimethoprim-sulfmethoxazole (82%), ampicillin (89%) and amoxicillin/ Ac clavulanic (90%), nalidixic acid (99%) and tetracycline (100%). There were moderate levels of resistance to neomycin (49%) and nitrofurantoin (53%). There were low levels of resistance to gentamicin (2%), chloramphenicol (13%) and no resistance to colistin sulfate (0%). All strains were multi-drug resistant and more than half (58%) of the isolates were resistant to seven antibiotics at least. Thus, thirty one antibiotic resistant patterns of *E. coli* strains were detected, of which 11 were present significantly. Co-resistance was found in eight of the eleven most common multidrug resistant patterns, 62% of the strains expressed this co-resistance. *E. coli* strains isolated expressed resistances to molecules that are routinely used in the field. It is clear that these are less effective against colibacillosis. It is more than ever necessary to perform antibiotic susceptibility testing prior to treatment in order to prescribe the molecule of choice, and set up a monitoring program in Algeria to monitor antimicrobial resistance in pathogenic bacteria that could be potentially transmitted to humans from animal food.

Key words: Algeria, antibiotic resistance, colibacillosis, *Escherichia coli*, serogroups.

INTRODUCTION

Changes in the poultry industry over the past few decades have largely contributed to its economic success. However, they have also created conditions favourable to contagious disease outbreaks. The increase in farm size, in regional farm density, and the increase in alternative poultry productions are among risk

factors that have led to the emergence and re-emergence of poultry diseases (Vaillancourt, 2009). Colibacillosis is the most frequently reported disease in surveys of poultry diseases or condemnations at processing (Saif, 2003). Avian colibacillosis is a bacterial disease, and it is referred to any localized or systemic infection caused entirely or partly by Avian Pathogenic *Escherichia coli* (APEC), including colisepticemia, coligranuloma (Hjarre's disease), air sac disease (chronic respiratory disease, CRD), cellulites (inflammatory process), swollen-head syndrome, peritonitis, salpingitis, osteomyelitis/synovitis (turkey osteomyelitis complex), panophthalmitis, and omphalitis/yolk sac infection (Stordeur and Mainil, 2002; Saif, 2003; Robineau and Moalic, 2010). In broiler chickens, colisepticemia and air sacs disease are the most common forms of colibacillosis (Stordeur and Mainil, 2002; Saif, 2003). Several serogroups of APEC strains are known for their virulence. The first studies on avian *E. coli* by Sojka and Carnaghan (1961) showed that the most common serogroups were O1, O2, O35 and O78. More recently, studies of 112 *E. coli* strains isolated from cases of colibacillosis in Canada by Dozois et al. (1992) showed that 16 serogroups were represented including serogroups O78 (52%) and O1 (6%) which were the most frequent. Other significantly represented serogroups associated with avian colibacillosis are: O8, O15, O18, O35, O88, O109, O115 and O116 (Bree et al., 1989; Dho-Moulin et al., 1990; Babai et al., 1997; Blanco et al., 1997; Dho-Moulin and Fairbrother, 1999). Colibacillosis is an economical devastating disease in the poultry industry; it is the primary cause of morbidity, mortality and condemnation of carcasses in Algeria and many parts of the world (Dho-Moulin and Fairbrother, 1999; Delicato et al., 2003; Ewers et al., 2003; Hammoudi and Aggad, 2008). Colibacillosis is likely the primary cause of antibiotic treatment in poultry house, and the emergence of resistant strains is a legitimate concern (Robineau and Moalic, 2010). Antibiotic usage is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Neu, 1992; Witte, 1998). Resistance to two or more classes of antibiotics is now common in veterinary medicine, and the choice of antibiotic for treatment remains quite arbitrary. In Algeria, very little data are available in epidemiology of antimicrobial resistance of *E. coli* and serogroups from which they belong. Hence, the objective of our study was to get a better knowledge of *E. coli* isolates responsible for colibacillosis in broiler breeding farms. The present study determined serogroups and the sensitivity to 11 antibiotics among a collection of one hundred avian pathogenic *E. coli,* isolates from diseased birds diagnosed with colibacillosis

in five cities in the east Algeria.

MATERIALS AND METHODS

Sampling method

From August to October 2014, 120 chicken carcasses were selected from twenty-four poultry houses (five broilers from each poultry house) implemented in the following cities of East Algeria (Setif, Batna, Mila, Constantine and Annaba). Size was 5000 broilers in each poultry house. They were at the end of breeding aged between 49 and 56 day old. The broilers chicken was ISA broiler F15 and Arbor Acres. Autopsies were performed in the slaughter house. Samples were collected weekly every Sunday. Five broilers from each poultry house with growth retardation or generalized carcass congestion were randomly selected and presented to autopsy.

Autopsy of broilers

The liver and spleen of each chicken having colibacillosis lesions (septicemia, airsacculitis, pericarditis and perihepatitis) were removed under sterile conditions. All samples (120 livers and 120 spleens) were placed in separate sterile plastic bags to prevent spilling and cross contamination and immediately transported to the laboratory in a cooler with ice packs (4°C).

Isolation and identification of *E. coli* isolates, media and growth requirements

The organs (liver and spleen) were flamed using a Bunsen burner and cut into small pieces. Enrichment was done by seeding pieces in the tubes of nutrient broth (Pasteur Institute of Algeria) and incubated aerobically at 37°C for 18 to 24 h. A drop of nutrient broth was inoculated with method of exhaustion on Mc Conkey (BiotechLab, Algeria) (selective medium for Gram-negative Enterobacteriaceae) and then incubated aerobically at 37°C for 18 to 24 h. Positive colonies have a diameter of 2-3 mm, light pink color (lactose +) and are surrounded by an opaque halo due to precipitation of bile salts. They are Gram negative, catalase positive and oxidase negative. These colonies were tested by the TSI medium (IPA, Algeria) and urea-indole medium (IPA, Algeria) before being confirmed as *E. coli* by biochemical identification tests using the API 20E system (Bio Mérieux, France).

Serogrouping

Serogrouping was performed by the rapid slide agglutination. The reaction involves the binding of specific antibodies present in the serum with bacterial antigens to form clumps visible to the naked eye. Materials: 1- A clean transparent plate with wells (2x4) (Citoglas, UK), 2– Reagents (Sera): monovalent serum against specific somatic antigen (lipo polysaccharide) of avian *E. coli* strain O1, O2 and O78 (Biovac, France), 3- The tested strains were cultivated on trypto-caseine soja medium (TSA Agar) (labodib, Algeria) overnight at 37°C, 4- Pasteur pipette, 5– Saline (0.9%). All components were brought to room temperature before use, excessive heat was avoided, especially from a light source. The

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Table 1. Antibiotic resistance and serogroups of *E. coli* strains isolated.

Antibiotic	Number of strain (%) n=100				
	O1	O2	O78	Other serogroups	Total
Amoxicillin/Ac clavulanic (AUG,30 µg)	13 (92.8)	48 (90.5)	14 (87.5)	15 (88.2)	90 (90)
Ampicillin (AMP, 10 µg)	11 (78.5)	49 (92.4)	14 (87.5)	15 (88.2)	89 (89)
Tetracyclin (TE, 30 µg)	14 (100)	53 (100)	16 (100)	17 (100)	100 (100)
Nalidixic acid (NA, 30 µg)	14 (100)	52 (98.1)	16 (100)	17 (100)	99 (99)
Enrofloxacin (ENR, 5 µg)	9 (64.2)	44 (83.0)	13 (81.2)	16 (94.1)	82 (82)
Gentamicin (CN, 10 µg)	0 (0)	2 (3.7)	0 (0)	0 (0)	2 (2)
Neomycin (N,30 µg)	5 (35.7)	32 (60.3)	6 (46.1)	6 (35.2)	49 (49)
Colistin Sulfate(CS, 10 µg)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nitrofurantoin (F, 300 µg)	7 (50)	30 (56.6)	7 (43.7)	9 (52.9)	53 (53)
Trimethoprim-sulfmethoxazole (SXT, 25 µg)	12 (85.7)	42 (79.2)	13 (81.2)	15 (88.2)	82 (82)
Chloramphenicol (C, 30 µg)	2 (14.2)	19 (35.8)	1 (6.2)	1 (5.8)	23 (23)
Total number of isolates	14	53	16	17	100 (100)

bottles of sera were vigorously shaken before use to restore the suspension. Quality control: a - Control of reagents (sera) were deposited in a well with a drop of reagent (about 30 µl). One drop (approximately 30 µl) of saline was added. The reagent is consistent if there is no agglutination reaction after mixing. b - Control of bacterial culture in another well was placed by a drop of saline (about 30 µl). Using a Pasteur pipette or inoculating loop, 3-4 colonies were collected (colonies should be visible to the naked eye). Gently emulsify in the drop of saline. The bacterial culture is compliant if no agglutination occurs after mixing. The test can be done once these two quality controls are compliant.

Antimicrobial susceptibility test

Antibiotic sensitivity was determined by disc diffusion method (Bauer et al., 1966) on solid Mueller-Hinton medium (MH) (Pasteur institute of Algeria) according to the guidelines of the "Clinical Laboratory Standards Institute" CLSI (2008). *E. coli* ATCC 25922 was used as control. Susceptibility was tested against the following antibiotics discs: Ampicillin (AMP, 10 µg), amoxicillin/Ac clavulanic (AUG, 30 µg), chloramphenicol (C, 30 µg), colistin sulfate (CS, 10 µg), Neomycin (N, 30 µg), Nalidixic acid (NA, 30 µg), Enrofloxacin (ENR, 5 µg), tetracycline (TE, 30 µg), Nitrofurantoin (F, 300 µg), Trimethoprim/sulfamethoxazole (SXT, 25 µg) and gentamicin (CN, 10 µg). Commercial antibiotic discs were purchased from Liofilchem, Italy (Ampicillin, amoxicillin/Ac clavulanic, chloramphenicol, colistin sulfate, trimethoprime/sulfaméthoxazole, Nalidixic acid), Bioanalyse, France (Gentamicin, tetracycline, nitrofurantoin), Bio-rad, France (Enrofloxacine and neomycin). The MH plates agar were incubated for 18 to 24 h at 37°C and the diameters of inhibition zones were interpreted by referring to the reading table of *Eenterobacteriaceae* as recommended by the Standardization of Susceptibility to the National Scale Human and Veterinary (2011).

RESULTS

One hundred *E. coli* strains were collected from 120 samples of livers and spleens, the susceptibility of these strains to each antimicrobial agent was tested and their serogroups are shown in Table 1. 83% of the 100 strains

isolated in the study are among the most virulent serogroups of APEC: O1 (14%), O2 (53%), O78 (16%). The antibiotics to which there were very high levels of resistance, were in ascending order: enrofloxacin (82%), trimethoprim-sulfmethoxazole (82%), ampicillin (89%) and amoxicillin/ Ac clavulanic (90%), nalidixic acid (99%) and tetyracycline (100%). The antibiotics to which there were moderate levels of resistance, were in ascending order: neomycin (49%) and nitrofurantoin (53%). The antibiotics to which there were low levels of resistance, were in ascending order: Colistin sulfate (0%), gentamicin (2%), chloramphenicol (13%).

All isolated *E. coli* showed multi-drug resistance were resistant to 2 antibiotics or more as shown in Table 2, the highest multi-resistance rates are 26, 24 and 22% were recorded in strains resistant to 7, 6 and 8 antibiotics, respectively. A total of 31 antibiotic resistance patterns were distinguished. The most important are those designated in Table 3 as D, E, F, I and J. The most common multi-drug resistant profile among these isolates was profile I (13%), which was resistant to tetracycline, nalidixic acid, amoxicillin/Ac clavulanic, ampicillin, trimethoprim/sulfaméthoxazole, Enrofloxacine, nitro-furantoin, neomycin. Co-resistance to tetracycline-nalidixic acid- amoxicillin/Ac clavulanic- ampicillin-trimethoprim/sulfaméthoxazole - enrofloxacine was found in eight of the eleven most common multidrug resistant patterns, 62% of our strains express this co-resistance.

DISCUSSION

Among the 120 chicken carcasses autopsied, expressing lesions of colibacillosis, bacteriological analysis of livers and spleens got 100 positive cultures of *E. coli* (83.3%). In the remaining 20 cases (16.7%), bacterial cultures were negative. The negative cultures may result from drug intervention before referring the cases to the

Table 2. Strains of *E. coli* showing multi-drug resistance.

Number of antibiotics	Number of strains (n=100)	Percentage of strains resistant out of 11 tested
0	0	0
1	0	0
2	1	1
3	3	3
4	6	6
5	8	8
6	24	24
7	26	26
8	22	22
9	8	8
10	2	2
11	0	0

Table 3. The most frequent antibiotic resistance patterns of *E. coli* isolates (n=76).

Resistance patterns	Designation	Number of strains (%)
TE-NA-AUG-AMP-SXT	A	5 (5)
TE-NA-AUG-AMP-SXT-N	B	4 (4)
TE-NA-AUG-AMP-ENR-F	C	5 (5)
TE-NA-AUG-AMP-SXT-ENR	D	9 (9)
TE-NA-AUG-AMP-SXT-ENR-N	E	10 (10)
TE-NA-AUG-AMP-SXT-ENR-F	F	12 (12)
TE-NA-AUG-AMP-SXT-ENR-N-C	G	4 (4)
TE-NA-AUG-AMP-SXT-ENR-F-C	H	4 (4)
TE-NA-AUG-AMP-SXT-ENR-F-N	I	13 (13)
TE-NA-AUG-AMP-SXT-ENR-F-N-C	J	8 (8)
TE-NA-AUG-AMP-SXT-ENR-F-N-C-CN	K	2 (2)

TE: Tetracyclin   NA: Nalidixic acid AUG: Amoxicillin/Ac clavulanic AMP: Ampicillin SXT: Trimethoprim-sulfmethoxazole F: Nitrofurantoin C: Chloramphenicol   N: Neomycin ENR : Enrofloxacin CN: Gentamicin

laboratory, probably the chickens may be on treatment so there's no importance for the timeout of antibiotic treatment like that reported by Saberfar et al. (2008). The APEC are responsible for many forms of colibacillosis in chickens, and it is increasingly recognized that the possession of certain genes chromosomal or plasmid encoding the virulence factors gives APEC strains pathogenicity own due to their ability to survive in the host like that reported by Blanco et al. (1997), Stordeur and Mainil (2002) and Stordeur et al. (2003). Among the 100 *E. coli* isolated in our study, 83% belong to three serogroups O1, O2, O78 most virulent among the APEC, with the dominance of O2 with a rate of 53% followed by O78 (16%) and O1 (14%) while (17%) of our strains belong to other serogroups. Our observations are correlated with those reported by Blanco et al. (1997) and Dozois et al. (2000) and Mellata et al. (2003). Lafont et al.

(1984) and Chulasiri and Suthienkul (1989) reported that characteristics of virulent *E. coli* in birds and other animals are often shared, and avian strains potentially can be a source of genes and plasmids that encode for antimicrobial resistance and virulence factors.

For the antibiotic resistance, these high rates of resistance to amoxicillin/clavulanic acid (90%) and ampicillin (89%) are probably related to the excessive and indiscriminate use of β-lactams antibiotics, and the diversity of mechanisms of resistance of *E. coli* as reported by Quintiliani and Courvalin (1995). Tetracycline (100%) is the oldest drug used, especially in therapy preventively and even as "growth factors", resulting in very high resistance in poultry as reported by Abdennebi (2006). For quinolone: Nalidixic acid (99%) and enrofloxacin (82%), these very high levels of resistance can be explained on one hand by the extensive use of

these molecules due to their large availability on the Algerian market, and especially by the presence of generics at affordable prices, and on the other hand with the fact that the quinolones share one and the same mechanism of action. Therefore, acquired resistance to one automatically confers resistance to other members of this family (cross-resistance). Baucheron et al. (2003) reported that two mutations in the gyrA gene and one or two mutations in the parC gene at the quinolone resistance determining region (QRDR) in *E. coli* strains of avian origin, give a high level of resistance to nalidixic acid and enrofloxacin. For sulfonamides, Trimethoprim/sulfamethoxazole (82%) is probably the consequence of the very important requirement of this anti-infective, used especially in the prevention of salmonella, and also during coccidiosis almost systematically used in combination with the anticoccidial treatment and prevention of the latter, leading to ineffectiveness against coliform. The abusive and anarchic use of the whole range of antibiotics available in Algeria is probably the major cause of the high percentages of resistance. In addition, lack of legislative restrictions of their use for therapy, prophylaxis, or growth promotion can be seen in the study of Hammoudi and Aggad (2008). Despite the fact that administration of chloramphenicol (13%) and nitrifurantoin (53%) is forbidden in veterinary, resistance to these antibiotics was high. This is probably due to persistence of previous resistances, a "cross" resistance or more likely to illegal use of these agents. The sensitivity of all strains to colistin sulfate could be explained by the moderate use of this molecule in poultry and these characteristics. In effect, this molecule does not cross the intestinal barrier and is inactive orally on systemic colibacillosis. Moreover, the resistance of Gram-negative bacteria is uncommon to colistin, even exceptional, and is chromosome-type. However, Garnacho-Montero et al. (2003) reported that chromosomal mutation is rare. The high sensitivity of *E. coli* strains to gentamicin is due to the non-use of this antibiotic in poultry farms and therefore no selection of resistant strains. In practice, there are constraints on its use, it is not available for poultry in Algeria and is used only for humans injectable form. Uninteresting for the farmers: the administration of this product requires a skilled workforce which can lead to stress and make the situation very delicate. The injections are not tolerated in chickens, especially with colibacillosis, as reported by Saberfar et al. (2008). Resistance was observed in all of the examined strains. This is similar to the findings of previous studies done in other countries by Blanco et al. (1997), Zahraei and Salehi (2006) and Saberfar et al. (2008). But our results were higher than those reported by Hammoudi and Aggad (2008) and Aggad et al. (2010) in Western Algeria.

Multi-drug-resistance appears as a veritable problem, as 100% of *E. coli* strains were resistant to at least two antibiotics, while over three quarters (82%) were resistant to at least 6 antibiotics, more than half (58%) were resistant to at least 7 antibiotics and more than a third (32%) were resistant to at least 8 antibiotics. Multi-resistance is probably due to the self-medication by breeders and alternating molecules before the first treatment gives these results. There is no importance attached to the processing delay. Indeed, numerous antibiotics are administrated often concomitantly for prophylaxis or infections. This indicates that the abusive and indiscriminate use of antibiotic is probably the genesis of the high incidence of antibiotic resistance and multi-resistances of *E. coli* in poultry breeding in Eastern Algeria. Such practices, especially 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 *E. coli* clones, as reported by Hamoudi and Aggad (2008). The results of this multi-resistance were treatment failures, and consequently reduced production due to high levels of morbidity and mortality in poultry flocks.

In our study, 31 *E. coli* patterns were isolated, the most important are presented in Table 3 and designated A to K. the danger of *E. coli* strains expressing antibiotic resistant patterns D, E, F, G, H, I, J and K, with rates of 9, 10, 12, 4, 4, 13, 8 and 2%, respectively were must considered. These strains show resistance to 6, 7, 8, 9, 10 antibiotics, even to antibiotics regarded as the most active on *E. coli* strains, such as gentamicin, chloramphenicol and nitrofurans and neomycin. These strains could transfer their wide antibiotic resistant pattern via the exchange of genetic material. Bebora et al. (1994) and Davies (1994) indicated that antibiotic resistances are frequently encoded by conjugative plasmids or transposons, thus *E. coli* of avian origin could act as a possible source for the transfer of antibiotic resistances to other bacterial species, including human pathogens. Thus, an increase in the reservoir of antibiotic resistant bacteria could heavily impair the treatment of human diseases. Indeed, Van Den Boogard et al. (2001) identified similar antibiotic resistant patterns present in *E. coli* isolated from people who worked with these birds, and, in some instances, specific strains were shared among the birds and workers. These findings indicate that transmission of antibiotic resistance by organisms that affect chickens to humans is common.

In this study, co-resistance to tetracycline - nalidixic acid- amoxicillin/Ac clavulanic-ampicillin- trimethoprim/ sulfaméthoxazole - enrofloxacine was found in *E. coli* strains expressing antibiotic resistant patterns D, E, F, G, H, I, J and K. This co-resistance is present in 11 out of 13 most important multidrug resistant profiles and more than half 62% of our strains express it. The consequence of this genetic organization is the co-selection: a class of antibiotics to which the bacteria are resistant may select resistance to unrelated classes of antibiotics, thus generating a wide resistant phenotype of bacterium like that reported by Courvalin (2008).

Conflict of interests

The authors have not declared any conflict of interest.

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