Antibiotic resistance pattern and virulence genes in avian pathogenic Escherichia coli *(APEC) from different breeding systems*

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Antibiotic resistance,
Avian pathogenic *Escherichia coli* (APEC),
Colibacillosis,
PCR,
Virulence gene.

Summary
Colibacillosis is the most frequent bacterial disease in avian species and antimicrobials are the main weapon to reduce incidence and mortality associated to it. However, indiscriminate use of antibiotics may lead to therapy failure and economic losses for the breeder. The aims of this study were to, determine the antibiotic resistance of *Escherichia coli* isolates, evaluate the correlation between *E. coli* isolation and systems of breeding included in this study, and identify the avian pathogenic *E.coli* (APEC) amongst the *E. coli* strains isolated. A total of 51 *E. coli* strains were subjected to antimicrobial susceptibility test and they were screened for the presence of virulence genes through PCR. Resistance was most frequently detected against ampicillin and nalidixic acid meanwhile *E. coli* isolates showed less resistance to the cephalosporins. Overall, 40% of the isolates showed resistance to at least three or more antimicrobials and 16/51 isolates were defined APEC strains. The virulence genes *iucD, cvi/cva, irp2* and *iss* were detected from all 16 APEC strains. The virulence genes *tsh, vat, papC*, and *astA* were detected from 11, 7, 5 and 3 APEC strains, respectively. Results demonstrated the importance of studies on APEC and antibiotic resistance genes in Italy, and it was shown that the systems of breeding might influence the antibiotic resistance.

Resistenza antibiotica e geni di virulenza in E. coli *patogeni aviari (APEC) isolati in varie tipologie di allevamento*

Parole chiave
Antibiotico-resistenza,
Colibacillosi,
Escherichia coli patogeno aviario (APEC),
Geni di virulenza,
PCR.

Riassunto
La colibacillosi è la malattia batterica che si riscontra più frequentemente nelle specie aviari e i farmaci antimicrobici sono l'arma più utilizzata per ridurre sia l'incidenza che la mortalità ad essa legate. L'uso indiscriminato di antibiotici può, tuttavia, portare al fallimento della terapia e a ingenti perdite economiche da parte dell'allevatore. Questo studio ha l'obiettivo di determinare i tassi di resistenza agli antibiotici da parte di ceppi di *Escherichia coli*, valutare la possibile correlazione tra l'isolamento di *E. coli* e le tipologie di allevamento prese in considerazione e rilevare la presenza di *E. coli* patogeni aviari (APEC) tra i vari *E. coli* isolati. Mediante l'impiego di 19 agenti antimicrobici, sono stati sottoposti al test di suscettibilità antimicrobica 51 ceppi di *E. coli*; gli stessi ceppi sono stati analizzati per valutare la presenza di otto geni di virulenza mediante metodica PCR. La resistenza è stata riscontrata più frequentemente verso ampicillina e acido nalidixico, mentre gli isolati di *E. coli* hanno mostrato una minore resistenza nei confronti delle cefalosporine. Complessivamente, il 40% degli isolati ha mostrato resistenza ad almeno tre o più agenti antimicrobici. Sedici di 51 isolati sono stati definiti ceppi APEC poiché in essi sono stati rilevati almeno cinque degli otto geni di virulenza ricercati. Mentre i geni di virulenza *iucD, cvi / cva, irp2* e *iss* sono stati rilevati in tutti i 16 ceppi APEC, *tsh, vat, papC* e *astA* rispettivamente da 11, 7, 5 e 3 ceppi APEC. I nostri risultati dimostrano quanto sia importante approfondire la diffusione del fenomeno dell'antibiotico resistenza e indagare la distribuzione di ceppi APEC in Italia, anche al fine di valutare un'eventuale correlazione tra i due fenomeni, con l'obiettivo di fornire uno strumento preventivo utile. Inoltre, è stato dimostrato come la tipologia di allevamento adottata può influenzare i tassi di antibiotico resistenza.

Introduction

Escherichia coli (*E. coli*) is considered a commensal microorganism in people and animals and it is part of normal intestinal microflora in birds (Aarestrup *et al.* 2008, De Carli *et al.* 2015). Some strains might be pathogenic and cause colibacillosis, an extraintestinal disease characterised by pericarditis, air sacculitis, perihepatitis, peritonitis. Colibacillosis is responsible for high economic losses in chicken industry (Matthijs *et al.* 2009, Matter *et al.* 2011, De Carli *et al.* 2015). It is the most frequent bacterial disease in avian species and *E. coli* is considered the first cause of death in poultry sector, even if usually it plays a secondary role during infection (Lutful Kabir 2010).

Colibacillosis is caused by avian pathogenic *E. coli* (APEC) (Matin *et al.* 2017) and its pathogenic ability may be localized or systemic. The APEC pathogenic ability is facilitated by broad range of virulence factors which are coded by virulence-associated genes (De Carli *et al.* 2015). According to molecular criteria, APEC is defined by presence of at least five virulence genes. According to molecular criteria, five genes carried by plasmids were considered as being the most significantly associated with highly pathogenic APEC strains (De Carli *et al.* 2015).

Escherichia coli strains are also considered good indicators of antimicrobial resistance because they are part of the physiological microbiota both in man and animals, and they are also present in the environment (Aarestrup *et al.* 2008).

Antibiotic resistance represents a serious problem to global public health, resulting in a significant impact on animal health and food safety (Aarestrup 2004). The misuse of antimicrobial agents could lead to selection and diffusion of resistant microorganisms with related increase of antibiotic resistance rate (Spellberg 2014). Furthermore, the problem of multi-drug resistance (MDR) can be transmitted and disseminated between animal and human pathogens, leading to treatment problems both animal and human diseases (Collignon *et al.* 2005).

Poultry industries consume wide range of antibiotics, because only few regulations are controlling their use (Hvistendahl 2012). In poultry industries, antibiotics have been used in chicken broilers as growth promoter and disease preventive measures (Bhandari *et al.* 2004, Osti *et al.* 2017, Shrestha *et al.* 2017).

The main goals of this study were (1) to determine the rates of antibiotic resistance of *E. coli* isolated from several avian species, (2) to evaluate possible correlations between *E. coli* isolation and the types of breeding and (3) to detect the presence of APEC among *E. coli* isolates.

Table I. *Origin of* Escherichia coli *strains isolated.*

Progressive number	Type of breeding	Species and/or production class	Type of samples
1	Industrial breeding	Chicken	Intestinal swab
2	Industrial breeding	Chicken	Liver
3	Industrial breeding	Chicken	Liver
4	Industrial breeding	Chicken	Intestinal swab
5	Industrial breeding	Chicken	Liver
6	Industrial breeding	Chicken	Intestinal swab
7	Industrial breeding	Chicken	Lung
8	Industrial breeding	Chicken	Femoral swab
9	Industrial breeding	Chicken	Spleen
10	Industrial breeding	Chicken	Liver
11	Industrial breeding	Chicken	Intestinal swab
12	Industrial breeding	Chicken	Femoral swab
13	Industrial breeding	Chicken	Vertebral swab
14	Industrial breeding	Chicken	Vertebral swab
15	Industrial breeding	Chicken	Pool organs
16	Industrial breeding	Chicken	Yolk sac
17	Industrial breeding	Hen	Liver
18	Industrial breeding	Hen	Liver
19	Industrial breeding	Goose	Intestinal swab
20	Industrial breeding	Goose	Heart
21	Industrial breeding	Goose	Heart
22	Chick dealer	Chicken	Liver
23	Chick dealer	Chicken	Intestinal swab
24	Chick dealer	Chicken	Liver
25	Chick dealer	Chicken	Intestinal swab
26	Chick dealer	Chicken	Liver
27	Chick dealer	Chicken	Intestinal swab
28	Chick dealer	Capon	Intestinal swab
29	Chick dealer	Hen	Intestinal swab
30	Chick dealer	Duck	Intestinal swab
31	Chick dealer	Guinea fowl	Intestinal swab
32	Chick dealer	Guinea fowl	Liver
33	Chick dealer	Goose	Lung
34	Chick dealer	Goose	Intestinal swab
35	Chick dealer	Goose	Lung
36	Chick dealer	Goose	Intestinal swab
37	Rural breeding	Chicken	Intestinal swab
38	Rural breeding	Chicken	Liver
39	Rural breeding	Chicken	Intestinal swab
40	Rural breeding	Chicken	Liver
41	Rural breeding	Chicken	Intestinal swab
42	Rural breeding	Hen	Intestinal swab
43	Rural breeding	Hen	Lung
44	Rural breeding	Hen	Heart
45	Rural breeding	Hen	Liver
46	Rural breeding	Hen	Spleen
47	Rural breeding	Hen	Intestinal swab
48	Rural breeding	Capon	Liver
49	Rural breeding	Capon	Intestinal swab
50	Rural breeding	Pigeon	Liver
51	Rural breeding	Turkey	Intestinal swab

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Materials and methods

Fifty-one *E. coli* strains from chickens (n = 27), hens (n = 9), geese (n = 7), capons (n = 3), guinea fowls (n = 2), duck (n = 1), pigeon (n = 1), and turkey (n = 1) originated from chick dealer (n = 15) , rural (n = 15) and industrial (n = 21) breeding were included in the present study.

These isolates were from different types of samples (Table I).

E. coli identification was performed with standard microbiological techniques which include studies of colony morphology, Gram staining, and biochemical tests (API Biomerieux®).

The *E. coli* isolates were tested for antibiotic susceptibility against 19 antimicrobial agents using disk agar diffusion method according to the Clinical Laboratory Standard Institute (CLSI) and EUCAST guidelines, based on available clinical breakpoints. The antibiotics used in this study, belonged to 7 categories of antimicrobial agents, and included ampicillin (AMP 10 µg), amoxicillin/clavulanic acid (AMC 30 µg), cefotaxime (CTX 30 µg), cefepime (FEP 30 µg), cefoxitin (FOX 30 µg), ciprofloxacin (CIP 5 µg), cefazolin (KZ 30 µg), ceftazidime (CAZ 30 µg), chloramphenicol (C 30 µg), enrofloxacin (ENR 5 µg), gentamicin (CN 10 µg), kanamicin (K 30 µg), meropenem (MEM 10 µg), nalidixic acid (NA 30 µg), streptomycin (S 10 µg), compound sulphonamides (S3 300 µg), tetracycline (TE 30 µg), trimethoprim/sulfamethoxazole (SXT 25 µg) and flumequine (UB 30 µg). These antibiotics were selected based on World Health Organization and Regional Pharmacovigilance Center recommendations. Furthermore, these antibiotics were a global representation of all antimicrobial classes.

Subsequently, *E. coli* isolates were investigated for the presence of eight virulence genes with Kylt® APEC ANICON kit. These genes are enteroaggregative toxin (*astA*), increased serum survival protein (*iss*), iron-repressible protein (*irp2*), P fimbrie (*papC*), aerobactin (*iucD*), temperature-sensitive hemagglutinin (*tsh*), vacuolating autotransporter toxin (*vat*), and colicin V plasmid operon genes (*cvi/cva*) (Exers *et al.* 2005).

For DNA extraction the colony was immersed in 500 µl of DNA Extraction-Mix II and it was resuspended carefully. Consequently, the sample vortexed, incubated for 10-15 minutes at 100 °C ± 3 °C, vortexed again and centrifuged at 10,000-12,000 g for 5 minutes.

The PCR was performed in 20 µl volume containing 18 µl Master-Mix (10 µl 2x PCR-Mix, 2 µl 10x Loading Dye, 6 µl Primer-Mix) and 2 µl DNA template. The cycling conditions were as follows: 94 °C for 3 minutes, 35 cycles of 30 sec at 94 °C, 30 sec at 57 °C, and 1 minute at 72°C.

The amplicons were analyzed by 2% agarose gel electrophoresis (Euroclone®) prepared in 1x TBE buffer (Biorad®). All the PCR products were stained with appropriate intercalating dye and the bands were visualized and photographed under UV light. The amplified product was considered to contain virulence gene if it produced band of the expected size. The amplicon size of the toxin genes of APEC is described on the manufacturer's instructions.

Data related to resistance and virulence genes rates were analyzed by the Fisher exact test. A value of $P < 0.05$ was considered significant.

Results

The *E. coli* resistant strains found in this study are displayed in Table II.

The resistance most frequently observed was against ampicillin and nalidixic acid (23/51, 45%) followed by tetracycline (22/51, 43%), sulphonamide (21/51, 41%), flumequine (17/51, 33%). *Escherichia coli* isolates exhibited lower resistance to cefotaxime and cefepime (0%) followed by cefoxitin and ceftazidime (1/51, 2%).

Overall, 41% (21/51) of the isolates showed resistance to at least three or more antimicrobial agents with a significantly ($P > 0.05$) higher number of isolates from animal of industrial breeding (13/21) than isolates obtained from animals of rural breeding (2/21). No significant differences ($P > 0.05$) were observed between the number of resistent isolates obtained from samples collected from animals of dealer and those from industrial and rural breeding.

An *E. coli* strain isolated from liver of an animal of industrial breeding was resistant to 11 antimicrobial molecules, including all antimicrobial categories.

The rates of resistance according to the type of breeding were illustrated in Figures 1, 2 and 3.

Of the 51 *E. coli* isolates, 16 (31%) were found to be APEC strains because they contain at least five virulence genes. Out of 16 APEC strains, 7 were isolated from samples collected from animals of industrial breeding (6 chicken and 1 hen), 5 from samples collected from animals of dealer (3 chicken and 2 geese), and 4 from samples collected from animals of rural breeding (2 geese, 1 capon and 1 hen). Seven virulence genes were present in 2 APEC strains, 6 in six strains and 5 in 8 strains. The virulence genes *iucD, cvi/cva, irp2* and *iss* were detected in all 16 APEC strains. The virulence genes *tsh, vat, papC*, and *astA* were detected in 11, 7, 5 and 3 APEC strains, respectively.

The virulence-associated genes *iucD, iss*, *irp2*, and *cvi/cva* were found in both APEC and non-APEC

Table II. *Number of resistant, intermediate and susceptible strains according to the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.*

Antimicrobial	Antimicrobial acronym	Concentration (µg)	Resistant (%)	Intermediate (%)	Susceptible (%)
Ampicillin	AMP	10	45	0	55
Amoxicillin/clavulanic acid	AMC	30	12	0	88
Cefotaxime	CTX	30	0	0	100
Cefepime	FEP	30	0	0	100
Cefoxitin	FOX	30	2	0	98
Ciprofloxacin	CIP	5	18	6	76
Cefazolin	KZ	30	16	18	66
Ceftazidime	CAZ	30	2	2	96
Chloramphenicol	C	30	22	0	78
Enrofloxacin	ENR	5	18	22	60
Gentamicin	CN	10	6	6	88
Kanamicin	K	30	6	8	86
Meropenem	MEM	10	0	0	100
Nalidixic acid	NA	30	45	4	51
Streptomycin	S	10	24	12	64
Compound sulphonamides	S3	300	41	0	59
Tetracycline	TE	30	43	4	53
Trimethoprim/sulphametoxazole	SXT	25	25	0	75
Flumequine	UB	30	33	12	55

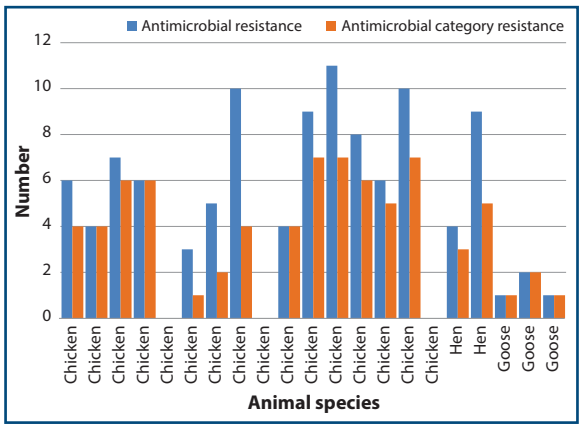

Figure 1. *Number of antimicrobials and antimicrobial categories in animals of industrial breeding.*

strains but their detection rates were significantly ($P < 0.05$) higher in APEC isolates. Conversely, the virulence associated gene *papC* was found in the APEC isolates only.

Discussion

Colibacillosis caused by APEC results in huge economic losses in poultry industry throughout the world (Roy *et al.* 2006, Barnes *et al.* 2008, Dziva *et al.* 2008). Antimicrobials are the main weapon to fight both the incidence and the mortality associated with colibacillosis (Harisberger *et al.* 2011). Antibiotics were also used as feed additives to improve weight gain (Bower *et al.* 1999). However, indiscriminate

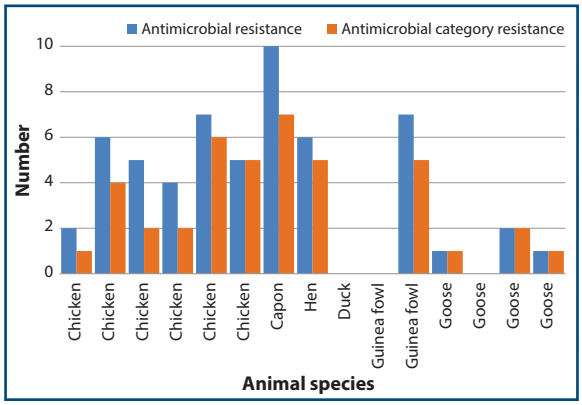

Figure 2. *Number of antimicrobials and antimicrobial categories in animals of chick dealer.*

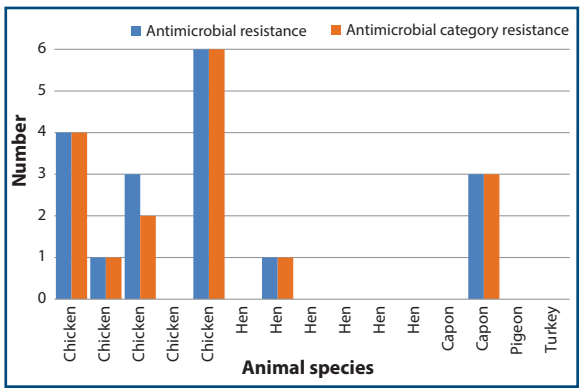

Figure 3. *Number of antimicrobials and antimicrobial categories in animals of rural breeding.*

use of antibiotics has provided selective pressure for the emergence of drug resistance strains which may lead to therapy failure and potential economic losses for breeders (Scioli *et al.* 1983, Quednau *et al.* 1998, Bower *et al.* 1999, Oosterik *et al.* 2014).

Of the nineteen antibiotics tested in this study, only two showed 100% efficacy against all *E. coli* strains. The highest rates of resistance (45%) were found with ampicillin and nalidixic acid. Other studies have also shown similar resistance but with higher rates (Yang *et al.* 2004, Li *et al.* 2010, Shrestha *et al.* 2011, Yassin *et al.* 2017, Matin *et al.* 2017, Shrestha *et al.* 2017, Bakhshi *et al.* 2017, Subedi *et al.* 2018). Similarly, for quinolones such as ciprofloxacin and enrofloxacin which in this study showed moderate resistance (18%) in other studies resistance rates higher than 50% were reported (Yassin *et al.* 2017, Subedi *et al.* 2018).

The resistance rates of the *E. coli* strains included in this study to ceftazidime and cefoxitin were very low. In contrast with a previous study of Younis and colleagues (Younis *et al.* 2017), that found 95.8%, 90.4% and 76.7% resistance rates against cefepime, cefoxitin and cefotaxime, respectively, all the *E. coli* isolates included in this study were susceptible to cefotaxine and cefepime..

Tetracycline resistance in our isolates was quite high (43%) which is consistent with what found by other authors (Vandemaele *et al.* 2002, Smet *et al.* 2008, Salehi *et al.* 2010, Persoons *et al.* 2012, Younis *et al.* 2017).

The high levels of resistance to sulfonamides (41%) revealed in this study, was not unexpected. Indeed sulfonamides were widely and continuously used for a long time and resistance was already described before 1950 (Yassin *et al.* 2017).

The resistance to aminoglycosides was quite low in our study (gentamicin and kanamicin 6%, streptomycin 24%), that is in disagreement with other authors which reported higher levels of resistance (Yassin *et al.* 2017, Yousin *et al.* 2017).

In this study, the frequency of eight virulence genes and their correlation with phenotypic antibiotic resistance were evaluated. *E. coli* strains are defined APEC when at least five virulence genes are detected. The virulence genes *iucD, iss, irp2*, and *cvi/cva* were found in both, APEC and non-APEC strains; the presence of these genes in both groups of strains might indicate that they are not associated with virulence. However, *iss* gene has been described as a virulence gene of recognised importance in *E. coli* of chickens (Ellis *et al.* 1988). Furthermore, *irp2* and *iucD*, both related to iron acquisition system, tend to be present on the same strain; in this study all the APEC strains contained either iron acquisition systems. Although at lower frequency, the *astA* gene was also detected in both the APEC and non-APEC isolates. In contrast, the virulence gene *papC* was detected in APEC strains only. This finding confirms what reported in other similar studies and supports the hypothesis that it is an important virulent gene. The *vat* and *tsh* genes were distribuited in both the APEC and non-APEC strains.

In this study, the frequency of virulence genes *iucD, cvi/cva, irp2* and *iss* observed in APEC strains was higher than that found by Subedi and colleagues (Subedi *et al.* 2018) which reported frequency of *irp2* and *cvi/cva* of 73.3% and 57.8%, respectively. In other studies the frequency of *irp2* observed (59.3% and 67%) was also much lower (Sadeghi Bonjar *et al.* 2017, Kwon *et al.* 2008). Furthemore, Kwon and colleagues reported 16% frequency of *cvi/cva* virulence gene (Kwon *et al.* 2008). The frequency of *tsh* virulence gene (69%) was comparable with literature data (Subedi *et al.* 2018). Lower frequencies were found for *vat, papC* and *astA* virulence genes (44%, 31% and 19%, respectively).

Four APEC strains from rural breeding showed no resistance (2 strains) or only resistance to nalidixic acid (other 2 strains). However, it should be highlighted that 3/7 isolates from industrial breeding resulted susceptible to all drugs. Out of 9 resistant APEC strains, 6 showed multidrug resistance to at least three or more antimicrobials agents (4 from industrial breeding and 2 from dealer). Although four strains from rural breeding were identified as APEC, they did not show high level resistance. Conversely, the strains from industrial breeding showed multidrug resistance suggesting a possible excessive use of antibiotics in poultry industries (Subedi *et al.* 2018).

These results demonstrate the importance of studies on APEC, antibiotic resistance rates in our country and its correlation with poultry breeding, aiming to acquire preventive measures to minimize losses due to APEC and multidrug-resistance that plays an important role and have high significance to public health.

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