Characterization and antimicrobial resistance analysis of avian pathogenic *Escherichia coli* isolated from Italian turkey flocks

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ABSTRACT This study investigated the occurrence of avian pathogenic *Escherichia coli* (APEC) in a finishing turkey commercial farm, carrying out longitudinal surveys involving 3 consecutive flocks. The diversity and the distribution of the *E. coli* strains detected during colisepticemia outbreaks were examined. The strains were isolated, serogrouped, assessed for the presence of virulence-associated genes, typed by random amplification of polymorphic DNA (RAPD), and antimicrobial resistance analysis was then carried out. *Escherichia coli* O78 and O2 were predominantly found. Moreover, based on the somatic antigens used in the study, strains were recovered that were nontypeable. On one occasion, an *E. coli* O111 strain was found in turkeys. The *E. coli* isolates differed in terms of antibiotic resistance and RAPD profile. All strains possessed the virulence genes that enabled them to be considered APEC. Strains not only differed between flocks, but also within the same flock. These findings point out the importance of addressing colibacillosis therapy on the basis of a sensitivity test.

Key words: *Escherichia coli*, turkey, antibiotic resistance profile, random amplification of polymorphic DNA, virulence gene

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INTRODUCTION

Colibacillosis is considered one of the leading causes of economic loss in the turkey industry worldwide (Hafez, 2005). Although *Escherichia coli* is usually present in the normal gastrointestinal flora of poultry, it is thought that only specific strains are endowed with virulence factors enabling them to cause disease. These strains are known as avian pathogenic *Escherichia coli* (**APEC**; Dho-Moulin and Fairbrother, 1999). They cause aerosacculitis, polyserositis, septicemia, and other mainly extraintestinal diseases in chickens, turkeys, and other avian species. The most common form of colibacillosis is characterized by respiratory disease, which is usually followed by a systemic infection with characteristic fibrinous lesions (airsacculitis, perihepatitis, and pericarditis) and fatal septicemia. The infection is generally initiated or enhanced by predisposing agents, such as mycoplasmal or viral infections, and environmental factors. The predominant APEC serogroups worldwide are O1, O2, and O78; they account for up to 60% of the isolates depending on the study (Barnes et al., 2008). These serogroups are those most frequently found during turkey colibacillosis cases, as has been frequently reported since the 1960s (Sojka and Carnaghan, 1961; Hemsley et al., 1967; Allan et al., 1993; White et al., 1993; Altekruse et al., 2002; Circella et al., 2009), although O18 and O111 serogroups have been recently found (Olsen et al., 2011).

In the literature, various studies have documented the transmission of APEC from chicken and turkey breeders to their progeny (Rosario et al., 2004; Giovanardi et al., 2005, 2007; Petersen et al., 2006), but the epidemiology of these bacteria in commercial turkeys, from their placement to the slaughterhouse, has not yet been elucidated.

The purpose of this study was to characterize APEC strains occurring in a finishing male turkey commercial farm, carrying out longitudinal surveys over 3 consecutive flocks.

The diversity and the distribution of the *E. coli* strains detected from turkeys showing lesions referable to colibacillosis were examined using microbiological and molecular techniques. The strains were serogrouped,

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assessed for the presence of virulence-associated genes, typed by random amplification of polymorphic DNA (**RAPD**) PCR, and submitted to antimicrobial resistance analysis.

MATERIALS AND METHODS

Farm and Flocks

The farm consisted of a single shed housing 5,000 toms; 3 consecutive flocks were included in the study. The farm was located in northern Italy in a densely populated poultry area. The turkeys were kept on wood shaving litter with a natural ventilation system. All-in, all-out management programs were followed. The study lasted until the turkeys reached 14 wk of age. This farm was selected for the study because during the previous 4 yr it suffered more than one episode of colibacillosis per production cycle and this happened 8 consecutive times.

Clinical Signs and Mortality

On a daily basis, the turkeys were checked for the presence of respiratory signs and colibacillosis-suspected mortality. The weekly mortality rate was recorded.

Sampling

All carcasses of spontaneously deceased turkeys or turkeys humanely killed because of lameness underwent necropsy daily. Pericarditis, aerosacculitis, fibrinous pneumonia, hepatomegaly, splenomegaly, and arthrosynovitis were considered indicative of colibacillosis; sampling was carried out on at least one visceral organ per turkey. Moreover, in the lame birds, if osteomyelitis or arthritis were observed macroscopically, the purulent exudate and bone marrow underwent bacteriological testing.

Bacteriology

Viscera or exudates were cultured individually onto 3% sheep blood agar plates (Oxoid, Basingstoke, UK) and incubated aerobically at 37°C from 18 to 24 h. The identification of suspect *E. coli* colonies (at least 2 colonies per plate) was based on biochemical features using the commercial kit RapID E 20 (bioMerièux, Craponne, France); one colony was retained and used for the subsequent testing.

Serogrouping

The serogrouping of the isolates was carried out according to the technique proposed by Blanco et al. (1998). The following monospecific antisera, toward 37 different somatic O antigens, were used: O1, O2, O4, O6, O8, O9, O10, O11, O15, O18, O20, O21, O22, O26, O45, O49, O64, O73, O75, O78, O83, O86, O88, O101, O103, O109, O111, O115, O128, O132, O138, O139, O141, O147, O149, O153, and O157. Briefly, the broth of each *E. coli* strain was heated at 100°C for 1 h and added with 0.5% phenolic physiological solution. Fifty microliters of the culture was mixed with the same volume of each of previously mentioned *E. coli* antisera in a single U-bottom well of a microtiter plate, then incubated at 37°C for 24 h. If seroagglutination occurred, a gray "carpet" appeared covering the bottom of the well, often in a clear fluid. In case of a negative reaction, the bacterial suspension was seen as a small white spot in a clear or a milky turbidity, centered in the well. To detect the auto-agglutinating strains, 50 μL of the broth culture was mixed with the same amount of 0.5% phenolic physiological solution and, without addition of any antisera, incubated as described previously.

Antibiotic Sensitivity Test (Resistotype)

A sensitivity test for antimicrobial agents was carried out on all the *E. coli* strains isolated, using the standard disk procedure proposed by the Clinical and Laboratory Standards Institute (AAVV, 2004). The following antibiotics were used: ampicillin, amoxicillin, oxytetracycline, gentamycin, trimethoprim-sulfamethoxazole, apramycin, aminosidin, colistin (Oxoid, Basingstoke, UK), and enrofloxacin (Bayer, Leverkusen, Germany). The isolates were interpreted as sensitive, resistant, or having intermediate sensitivity.

Resistotypes were determined according to the combinations of antibiotic resistances observed and were identified with capital letters.

Multiplex-PCR for E. coli Virulence Genes (Pathotype)

The *E. coli* were analyzed for the presence of the following virulence genes, identified as being significantly associated with highly pathogenic APEC strains: ferric aerobactin receptor (***iutA***), hemolysin (***hlyF***), increased serum survival (***iss***), membrane siderophore receptor (***iroN***) and outer membrane (***ompT***) according to Johnson et al. (2008), and iron acquisition system (***iucD***, ***fyuA***, and ***irp2***), temperature-sensitive hemagglutinin (***tsh***), fimbrial assembling gene (***fimC***), and P-fimbriae (***papC***) according to Janben et al. (2001).

The strains were inoculated into EC broth (Becton and Dickinson, Franklin Lakes, NJ), incubated aerobically at 37°C for 12 h, and PCR was then carried out. The DNA was extracted with Prepman Ultra (Applied Biosystems, Grand Island, NY). A Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) was used. The PCR products were separated by electrophoresis on 2% agarose gel and were then stained with SYBR safe DNA gel stain (Applied Biosystems).

Random Amplification of Polymorphic DNA

Random amplification of polymorphic DNA (**RAPD**) PCR was applied to analyze the molecular relationship between isolates.

Bacterial DNA was extracted with Prepman Ultra and quantified by spectrophotometer. Twenty nanograms of DNA was used as a template for the RAPD kit Ready-to-Go beads (GE Healthcare, Piscataway, NJ). The kit was used as described by the supplier with primer 1290 (Maurer et al., 1998). The conditions for DNA amplification were 5 min at 95°C, 45 cycles of 1 min at 95°C, 1 min at 36°C, and 2 min at 72°C. The amplification products were resolved by electrophoresis on 2% agarose gel stained with SYBR safe DNA gel (Applied Biosystems). The image was captured using Gel Doc 2000 (Bio-Rad, Hercules, CA). The fingerprinting was analyzed with GelCompar II (version 2.0, Applied Maths, Sint-Martens-Latem, Belgium), and the measure of the similarity was based on densiometric curves using a Pearson correlation. A dendrogram was generated using the unweighted pair group method with arithmetic mean. To test the reproducibility of the RAPD technique, the samples were analyzed in 2 independent reactions, the results of which could not show any loss or shift in the position of the banding patterns.

RESULTS

The results of the serogrouping and the antibiotic sensitivity test are shown in Figure 1 and Table 1.

Flock I

During wk 4, the turkeys showed respiratory signs (conjunctivitis, rhinitis, and sinusitis), and mortality was recorded (0.53%), the highest being observed during the first longitudinal survey. At 14 wk of age, some birds showed lameness.

Dead or culled birds underwent necropsy, and sampling for bacteriology was carried out from 15 birds showing macroscopic lesions referable to colisepticemia and 3 birds with joint lesions. *Escherichia coli* was isolated from 13 carcasses, including one with tibiotarsal joint lesions. All 13 strains were nonhemolytic. Eight isolates belonged to serogroup O78 (7 from the viscera and 1 from the joint), 1 to serogroup O111 and 4 were nontypeable (**NT**; based on the somatic antigens used in the study; Table 1, Figure 1). All *E. coli* O78 strains showed resistotype A, characterized by resistance to ampicillin, amoxicillin, oxytetracycline, and sensitivity to trimethoprim-sulfamethoxazole, gentamycin, apramycin, colistin, aminosidin, and enrofloxacin. The *E. coli* O111 isolate was sensitive to all the antibiotics tested except for oxytetracycline (resistotype E). The NT strains belonged to 3 different resistotypes (B, C, and D) and shared sensitivity to colistin, apramycin, gentamycin, and enrofloxacin, and resistance to oxytetracycline.

Both the *E. coli* O78 and O111 strains were found to be positive for all virulence genes studied except for the *papC* gene in the O78 strains and the *hlyF* gene in the O111 strain. The NT strains possessed various patterns of virulence genes (data not shown).

Flock II

The mortality rate reached the highest rate during wk 12 of life (0.68%); the turkeys showed clinical signs similar to those observed in flock I. Between wk 13 and 14 of age, few new cases of lame turkeys were noticed.

Viscera from 20 carcasses showing lesions consistent with colisepticemia, and exudates from the coxo-femoral and tibiotarsal joints (affected by arthritis and osteomyelitis) collected from 6 lame turkeys underwent bacteriological testing.

Twenty-three *E. coli* strains were isolated (17 from the viscera and 6 from the joints). All strains were nonhemolytic. Eighteen isolates belonged to serogroup

Flock	I	II	III
Week of age 2	-	O2/F (1)	-
3	*O78/A (1)	O2/F (1) O2/G (2) O78/A (1)	-
4	§O78/A(2) NT/B (1) NT/C (1)	-	-
5	O78/A (1)	O78/A (1)	-
6	-	-	O2/I (2)
8	-	-	O2/I (1)
9	NT/D (1)	O78/H (1)	O2/I (2) NT/L (2)
10	NT/B (1)	O78/I (1) O78/A (4)	§O2/I (3)
11	O78/A (1)	-	-
12	O111/E (1)	§NT/I (1) O78/I (4)	-
13	O78/A (1)	-	-
14	O78/A (2)	O78/A (4) O78/I (1)	-

Figure 1. *Escherichia coli* strains isolated in flocks I, II, and III: number and week of detection, serogroup, and resistotype. *Serogroup/resistotype (no. of strains isolated). §The highest mortality rate in the flock.

Table 1. Strains of *Escherichia coli* isolated during the study: serogroups and resistotypes[1]

E. coli serogroup (no. of isolates)	Antibiotics used[2]								Resistotype
	CT	GM	APR	AX AP	AM	SX	EN	OT	
Flock I									
O78 (8)	S	S	S	R	S	S	I	R	A
NT (2)	S	S	S	R	S	R	S	R	B
NT (1)	S	S	S	R	R	R	S	R	C
NT (1)	S	S	S	S	S	S	S	R	D
O111 (1)	S	S	S	S	S	S	I	R	E
Flock II									
O78 (11)	S	S	S	R	S	S	I	R	A
O2 (2)	S	R	S	R	S	S	S	I	F
O2 (2)	S	S	S	R	S	R	I	R	G
O78 (1)	S	I	S	R	S	S	R	R	H
O78 (6) NT (1)	S	S	S	R	S	R	I	R	I
Flock III									
O2 (8)	S	S	S	R	S	R	I	R	I
NT (2)	S	S	S	R	S	R	R	R	L

[1]NT: nontypeable, S: sensitive; I: intermediate sensitivity; R: resistant.

[2]CT: colistin; GM: gentamycin; APR: apramycin; AX: amoxicillin; AP: ampicillin; AM: aminosidin; SX: trimethoprim-sulfamethoxazole; EN: enrofloxacin; OT: oxytetracycline.

O78, 4 to serogroup O2, and 1 was nontypeable (Table 1, Figure 1).

The *E. coli* O78 strains (11 from colisepticaemic lesions and 6 from joints) were isolated throughout the lifespan beginning with wk 3. The majority of the O78 strains (11) showed resistotype A (see previous paragraph), 6 showed resistotype H (with resistance to ampicillin/amoxicillin, oxytetracycline, and trimethoprim-sulfamethoxazole), and 1 showed resistotype I (with resistance to ampicillin/amoxicillin, oxytetracycline, and enrofloxacin).

Two *E. coli* O2 isolates showed resistotype F (with resistence to ampicillin/amoxicillin and oxytetracycline) and 2 showed resistotype G (with resistance also to trimethoprim-sulfamethoxazole and gentamycin).

Both the *E. coli* O78 and O2 strains isolated in flock II were found to be positive for all the virulence genes studied except for *papC*.

Flock III

The highest percentage of mortality (0.42%) was recorded during wk 10 of life. No episodes of lameness were observed.

Viscera from 10 carcasses with lesions of colisepticemia underwent bacteriological testing. From these samples, 8 *E. coli* O2 and 2 NT strains were isolated (Table 1, Figure 1). All O2 strains showed resistotype I (see previous paragraph) and were positive for all virulence genes (Figure 2). The NT strains belonged to resistotype L (with resistance to ampicillin/amoxicillin, oxytetracycline, trimethoprim-sulfamethoxazole, enrofloxacin) and showed positivity only for *iutA*, *hlyF*, *iss*, *iroN*, and *ompT* virulence genes.

Molecular Relationship Between Isolates

Phylogenetic analysis, obtained by RAPD-PCR, was used to determine if there were genetic similarities between the *E. coli* strains isolated from the flocks.

The *E. coli* isolated from flock I were grouped into 3 different clusters (Figure 3): cluster I, which consisted of 1 *E. coli* O78 isolate, 1 *E. coli* O111 isolate, and 1 NT strain; cluster II, which included the remaining *E. coli* O78 strains (with more than 80% of DNA similarity); and cluster III, which included the remaining 3 NT strains.

In Figure 4, the RAPD-PCR profiles of selected *E. coli* O78 isolated from flocks I and II were compared. The strains analyzed fell into 4 different clusters I, II, III, and IV (90, 86, 87, and 98%, respectively, high internal genetic similarity). Cluster I included 3 strains from flock I and 2 strains from flock II. Cluster II included strains from both joints and viscera. Cluster III included only strains from flock II. Cluster IV included only strains isolated from joints.

In Figure 5, all *E. coli* O2 strains isolated during our study (flocks II and III) were compared.

Two clusters with high internal genetic similarity (more than 80%), clearly separated between them, were obtained. Cluster I included O2 strains from flock III; cluster II included O2 strains from flock II.

DISCUSSION

The objective of the present investigation was the study of APEC occurrence-incidence during the production cycles of commercial turkeys because it is well known that several episodes of colibacillosis may appear subsequently in the life span of these birds.

These outbreaks are often treated immediately with antibiotics, without waiting for the sensitivity test results, which are available only at least 48 h after the birds were tested. The *E. coli* strains found in our study belonged to different resistotypes, even if of the same serogroup. This finding strongly suggests to address the choice of the antibiotic to use on the basis of a sensitivity test. High resistance to oxytetracycline, ampicillin, and amoxicillin (96%) was detected. The rise of

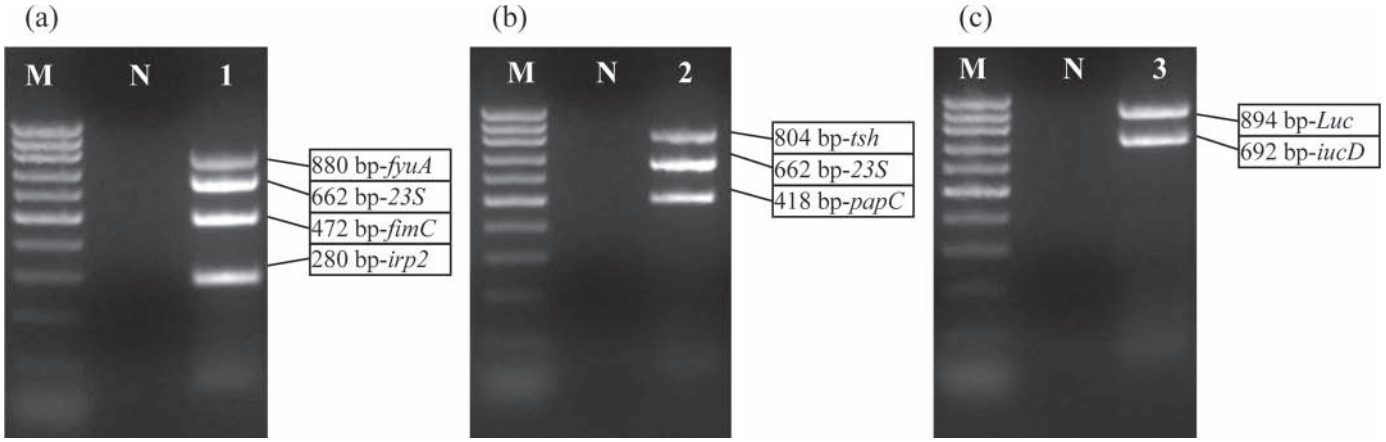

Figure 2. Agarose gel electrophoresis of multiplex PCR products of a representative isolate (avian pathogenic *Escherichia coli* O2, flock 3) carrying virulence genes. Lane M: 100-bp DNA ladder (Fisher Molecular Biology, Rome, Italy); lane N: negative control; (a) lane 1: *fyuA*, internal positive control *23S*, fimbrial assembling gene (*fimC*), *irp2*; (b) lane 2: temperature-sensitive hemagglutinin gene (*tsh*), internal positive control *23S*, P-fimbriae (*papC*); (c) lane 3: external positive control *Luc* (pGL3-basic Vector, Promega, Madison, WI), *iucD*.

antibiotic-resistance to *E. coli* is of great concern for the poultry industry worldwide. Our results confirm previous reports that showed various degrees of antibiotic resistance in *E. coli* strains isolated from poultry (Yang et al., 2004; Zhao et al., 2005; Gosling et al., 2012; Russo et al., 2012).

All the strains isolated during the study, including the nontypeable strains, should be considered as highly pathogenic APEC isolates because they were harboring the majority of the virulence genes coding for structures useful for attachment on epithelia and survival in the bloodstream according to Janben et al. (2001) and Johnson et al. (2008).

The majority of *E. coli* strains found in flock I and II were O78, whereas *E. coli* O2 strains were in the majority in flock III. Serogroups O78 and O2 are among the most common reported worldwide (Altekruse et al., 2002; D'Incau et al., 2006; Giovanardi et al., 2007, 2011; Barnes et al., 2008; Circella et al., 2009). To our knowledge, the detection of APEC O111 represents an uncommon finding in turkeys, where it has been only recently reported (Olsen et al., 2011). In the past, it was reported by Zanella et al. (2000) and Trampel et

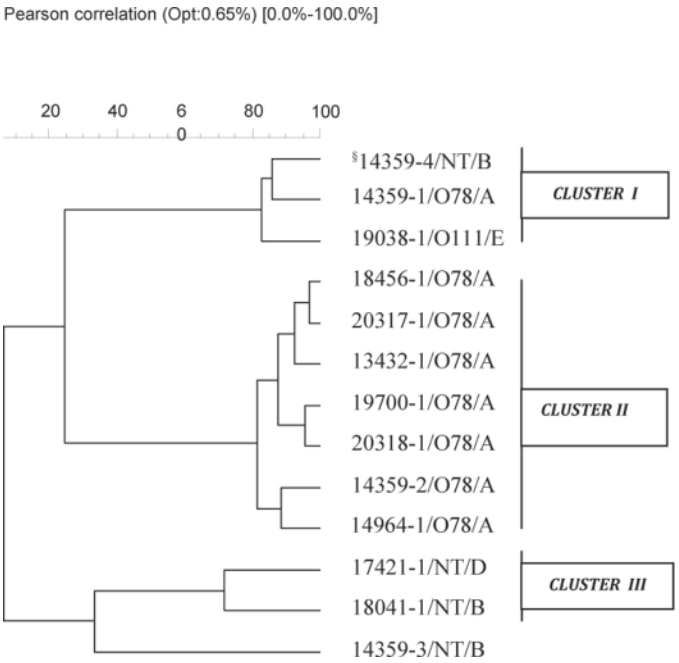

Figure 3. Molecular relationship between the avian pathogenic *Escherichia coli* strains isolated in flock I, obtained with random amplification of polymorphic DNA analyses. §Isolate identification number/serogroup/resistotype. NT = nontypeable.

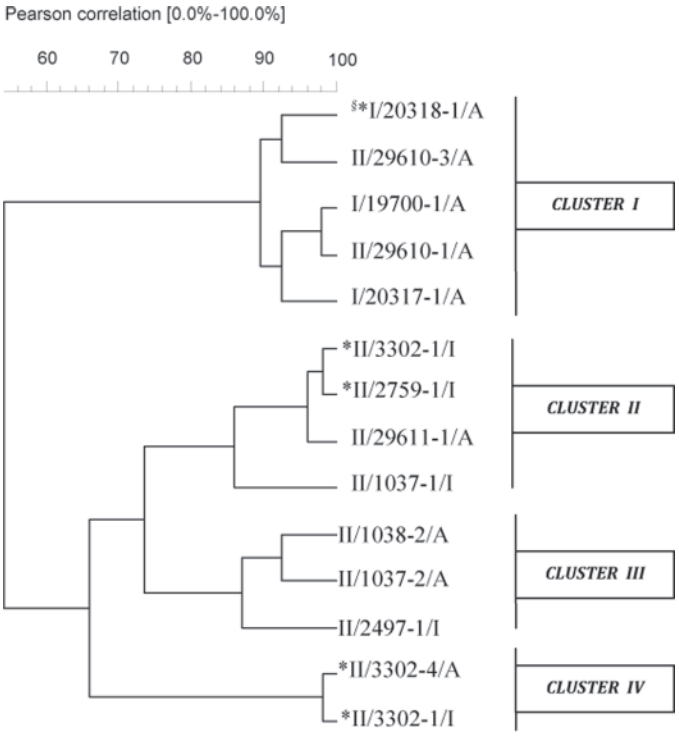

Figure 4. Molecular relationship between avian pathogenic *Escherichia coli* O78 strains isolated in flocks I and II, obtained by means of random amplification of polymorphic DNA analyses. §Flock of origin/isolate identification number/resistotype. *Isolate coming from the joint.

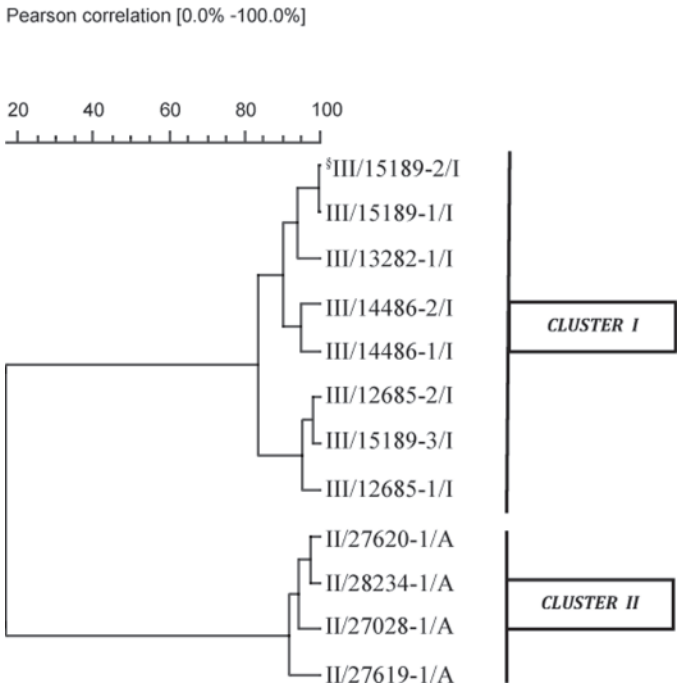

Figure 5. Molecular relationship between avian pathogenic *Escherichia coli* O2 strains isolated in flocks II and III, obtained by means of random amplification of polymorphic DNA analyses. §Flock of origin/isolate identification number/resistotype.

al. (2007) during severe episodes of infection in laying hens. In our study, the O111 strain did not seem to cause any severe disease in turkeys; therefore, additional studies are needed to better understand its pathogenicity.

The 7 APEC strains isolated from the coxofemoral or tibial-tarsal joints all belonged to the O78 serogroup. In our study, the tropism for articular tissues appeared to be typical for this serogroup. The RAPD molecular analysis, carried out on the strains isolated in flock II, revealed that 2 of these strains grouped into a specific genetic cluster clearly separated from the other clusters, which included the colisepticemic isolates. This finding confirmed previous studies. Nairn (1973) described *E. coli* of different serotypes, including O78, for the first time as a cause of arthritis and osteomyelitis in turkeys. Van den Hurk et al. (1994) observed an elevated incidence of arthritis of the femoral-tibial and tibial-tarsal joints in commercial turkeys after inoculation of the hemorrhagic enteritis virus followed by inoculation with *E. coli* O78.

An interesting finding regarding the presence of APEC O78 strains sharing the same resistotype was the same pathotype and high genetic similarity in consecutive production cycles (flocks I and II). It could be hypothesized that specific bacterial clones survived, between the production cycles, in the environmental dust during the all-in, all-out procedures (such as litter disposal and cleaning and disinfection of the premises). Singer et al. (2000) demonstrated that an *E. coli* strain, associated with cellulitis in chickens, can persist over successive flocks in the same house for up to 191 d regardless of the cleaning and disinfection strategy used.

Our study clearly demonstrated that great heterogeneity can be found between APEC isolates coming from 3 consecutive production cycles in a turkey flock. The APEC strains isolated not only differed between flocks but also within the same flock. They belonged to various resistotypes and possessed the virulence genes responsible for colisepticemia or arthritis. Their variety in RAPD molecular profiles indicated that serogrouping is not sufficiently useful in epidemiology studies if not used in combination with information derived from these molecular techniques. Also, only in this way has it been possible to demonstrate that different APEC strains could coexist simultaneously in the same productive cycle.

In summary, our findings pointed out the practical importance of prompt evaluation of the antibiotic resistance profile of APEC isolated during colibacillosis outbreaks in turkeys, and the relevance of the procedures of cleaning and disinfection of the premises and equipment between different production cycles.

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