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Full Length Research Paper

Molecular typing of Avian pathogenic *Escherichia coli* (APEC) using multiprimer PCR

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A total of 42 *Escherichia coli* strains isolated from broilers and the halls of growth were examined between the years 2010 and 2011, in order to assess the prevalence of avian pathogenic *E. coli* (APEC) strains in the West of Romania, where the majority broiler is represented by the imported ones. Multiplex PCR technique was used in order to detect three genes governing the synthesis of some virulence factors associated with APEC strains. It was observed that *OmpA* gene encodes an outer membrane protein that is responsible for bacterial attachment in 18 strains tested, the *iss* gene encodes an outer membrane protein that induces resistance to complement and thus promotes the APEC strains colonization and this was present in 17 strains tested. *FimH* gene encoding the synthesis of type 1 fimbria was noted in 19 of the strains tested. All isolates had common biochemical characteristics of *E. coli* strains and were multiple resistant to lincomycin, spectinomycin, neomycine, tetracycline, erythromycin, doxycyclin, enrofloxacin. Fixation of Congo red correlated with the presence of three genes is a phenotypic characteristic of *E. coli* strains of avian origin. Presence of these genes is useful in defining the APEC pathotype.

Key words: Avian pathogenic *E. coli*, colibacillosis, multiplex PCR.

INTRODUCTION

Escherichia coli is the most common etiological agent involved in the pathology of broilers (Barnes et al., 2008). It is present in normal microflora of the digestive tract of broilers, while the strains belonging to the APEC pathotype are also present on the respiratory mucosa (Ewers et al., 2003). Since APEC strains enter the body through the intestinal mucosa, the caused infections are considered extraintestinal (Barnes et al., 2008). The strains that cause colibacillar infections in birds and the strains that cause urinary infections in humans are classified in a special group called ExPEC (Rodriguez-Siek et al., 2005).

Iss gene encodes a protein that determines resistance to complement (increased serum survival), *fimH* gene encodes a fimbrial adhesin (type 1 fimbrial adhesion) and the *ompA* gene encodes an outer membrane protein, the last two being involved in the pathogenic process of extraintestinal sequential colonization made by an APEC strain (Pfaff-Mcdonough et al., 2000).

APEC strains are involved in respiratory infections in birds, often triggered in association with some viruses, with losses of 20-40%, or cellulitic infections, leading to the degradation of carcasses (Barnes et al., 2008). Circulating APEC strains are mostly multi-resistant to antibiotics and even to some disinfectants used in poultry industry (Sharada et al., 2010).

This study was to reveal by multiplex PCR, the genotypic characteristics to APEC strains isolated from broilers, from outbreaks of avian colibacillosis.

MATERIALS AND METHODS

The investigations were carried out in broilers' farms in the West of Romania. Epidemiological, clinical and pathological exams were conducted in the studied farms. Laboratory investigations were

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performed in the Laboratory of Molecular Biology of SN Pasteur Institute, Bucharest.

Anatomopathological examination was daily performed by farm's veterinarian, and weekly the broilers' corpses from the respective day were carried to the Bacteriology Laboratory for anatomopathological and bacteriological examinations.

Bacteriological examination was performed in broilers with avian colibacillosis. Long bones from the corpses anatomopathologically examined were sampled. Primary cultivation was made by common methodology in nutrient broth and agar. Then they were on Levine and S - S media from bacterial cultures. The biochemical features were studied by using *API 20E* kit.

Fixing of Congo red dye was made on TSA agar (Trypticase Soy Agar), with an additional 0.15% biliary salts and 0.03% dye, the cultivations were made through exhaustion with the bacteriological dowser in order to obtain isolated colonies (Ewers et al., 2003; Rodriguez-Siek et al., 2005).

The isolates' antibiotic sensitivity was carried out by disc diffusion method, using a total of 12 antibiotics. The microcomprimates being supplied by Oxoid.

Classical PCR method (multiplex PCR) for the detection of APEC

DNA extraction was performed according to the technique published by Germani in 1995.

MultiplexPCR ompA+iss+fimH: 25 µl amplification reaction volume.

The used primers were: *ompA1* and *ompA2* (Pfaff-McDonough et al., 2000), *iss1* and *iss2* (Pfaff-McDonough et al., 2000), *H1* and *H2* (Popa et al., 2004), each of 25 pmole commercially synthesized by Nucleic Acid Protein Service (NAPS) Unit, University of British Columbia (UBC) - Canada.

Amplification mixture: illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare 27-9557-01). The amplification reactions of PCR multiplex variant were carried out on PE GeneAmp PCR System 9600 amplifier (PE Applied Biosystems). Amplification program used was: 94°C - 10 min /1x, 94°C - 1 min, 55°C - 1 min 30 s, 72°C - 2 min 30 s / 30x; 72°C - 10 min / 1x, 4°C - 10 min.

Analysis of post-PCR amplicons was performed by TBE gel-electrophoresis 1.5x. Image digitization was made by Easy RH (Herolab GmBH - Germany) equipment and ImageWin2PC logical program.

The positive control used was avian origin strain *E. coli* PIB4293; 1996; CRb+, ser^r; iss+; ompA + and fimH + (Popa et al., 2004).

RESULTS

Results of epidemiological examination

Epidemiological investigation revealed the following:

1. Broilers were derived from farms with permanent bedding system growth.
2. The halls were differently populated, with broilers coming from incubation stations in the country or from import (hybrid ROSS 308 and COBB 500).
3. Ventilation in the halls was realized by fans with adjustable speed and intake air through the obliquely opened side windows and the door, which generated air currents, causing pulmonary congestion in broilers during the first days of life in warmer areas of the hall.
4. Watering system had technical deficiencies, which led to loss of water under watering devices and increased the humidity in these areas.

Preventive treatment with antibiotics or chemotherapy has been applied in the first days of broilers' life in the farm, without an antibiogram being realized in advance. Broilers' density in the halls exceeded the standard hygienic number of chickens recommended.

Losses due to cumulative mortality were an important parameter of epidemiological examination, this parameter varied depending on the age of broilers (Table 1). Analyzing the results in Table 1 it can be seen that the mortality rate increased in broilers' age from 0.61% in the first week of life to 3.4% over the age of 28 days. In this study we can see that the cumulative mortality in the 6 halls was ranged between 5.18% in H3B and 5.95% in H1B.

Anatomopathological observations

Following the anatomopathological examination conducted on 495 broiler corpses of different ages were found the injuries: bleeding diathesis, hyperplasic spleenitis, omphalitis, peritonitis and unabsorbed yolk sac (Figure 1), fibrinous pericarditis and perihepatitis (Figure 2), pulmonary congestion.

Bacteriological observations

From a number of 2485 examined broiler corpses, 495 corpses there were subjected to routine bacteriological examination of which 440 cases, representing 89%, were confirmed as colibacillar infections. *E. coli* cultures were obtained from bacteriological cultivation made by the common methodology in nutrient broth and agar.

Gram negative bacilli and cocobacilli were observed in smears of these cultures. On the S – S medium. Cultures that were lactose positive include those of *E. coli* forming red colonies, while on the Levine medium the colonies were blackish with metallic sheen (Figure 3).

On the Congo red agar the colonies were identified by their dark red color and dry, wrinkled appearence (Figure 4).

From the 440 cases with colibacillar infection 42 strains of *E. coli* were isolated which had APEC characteristic biochemical behavior. Most strains of *E. coli* isolated were resistant to lincomycin, spectinomycin, neomycine, tetracycline, erythromycin, doxycyclin, enrofloxacin and a small percentage to florfenicol (Figure 5).

Results of PCR test

Of the *E. coli* strains isolated, 20 strains were molecularly typified by multiprimer method (multiplex PCR) for genes:

Table 1. The cumulative mortality in broiler chickens with lesions of colibacillosis.

Week	Halls											
	H 1 A		H1 B		H 2 A		H 2 B		H 3 A		H 3 B	
	No of chick died	%	No of chick died	%	No of chick died	%	No of chick died	%	No of chick died	%	No of chick died	%
I	112	0.75	94	0.63	96	0.64	91	0.61	93	0.62	92	0.61
II	121	0.81	103	0.69	121	0.81	114	0.76	121	0.81	99	0.66
III	164	1.09	184	1.23	163	1.09	133	0.89	141	0.94	153	1.02
IV	423	2.82	510	3.4	434	2.89	486	3.24	489	3.26	434	2.89
Total	820	5.47	891	5.95	814	5.43	824	5.5	844	5.63	778	5.18

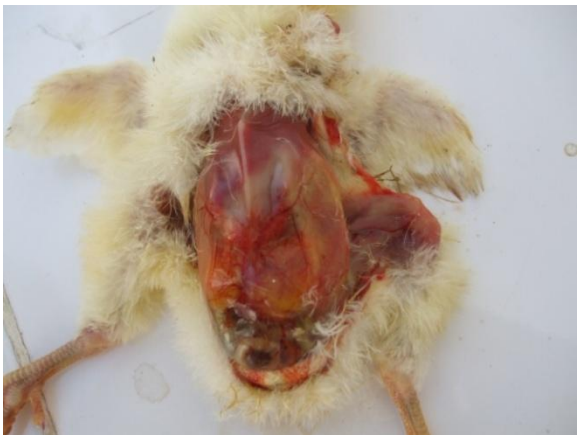

Figure 1. Omphalitis and peritonitis in broiler chicken.

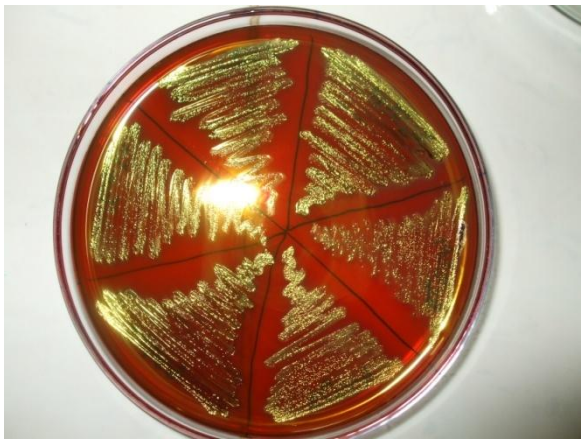

Figure 3. *Escherichia coli* on the Levine medium.

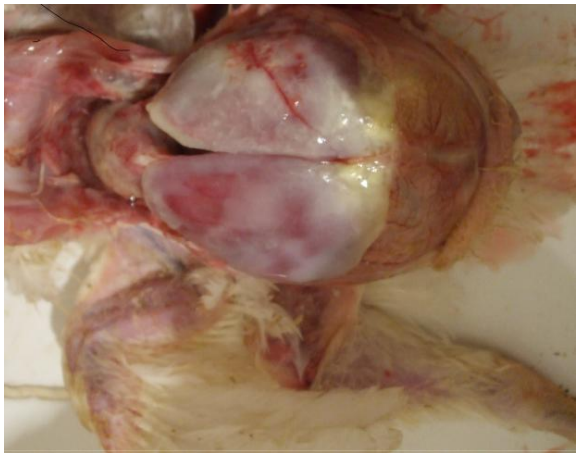

Figure 2. Fibrinous pericarditis and perihepatitis in broiler chicken. This is a typical of *E. coli* infections.

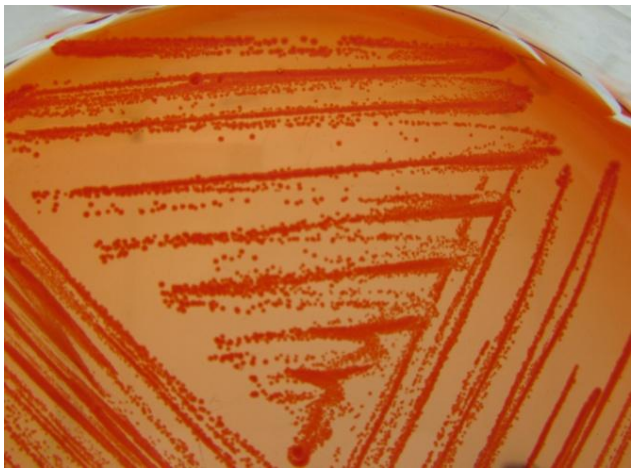

Figure 4. *Escherichia coli* on the Congo red medium.

omph, iss and fimH, according to Table 2 and Figure 6. Of the analyzed *E. coli* strains, 16/20 was clearly classified in APEC pathotype because they were positive for all three specific genes in multiprimer PCR amplification assay (Table 2).

Of the other strains, 2/20 (S3 and S12) were positive

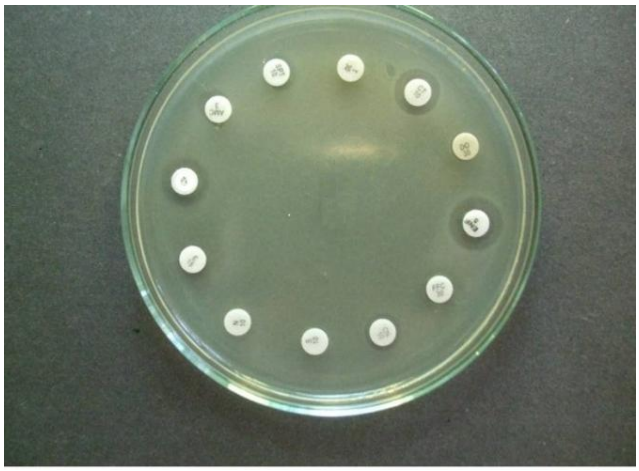

Figure 5. Antibiotic sensitivity of *E. coli* strain.

Table 2. Results of multiplex PCR.

No.	Strain code	Results of multiplex PCR		
		***ompA* gene Amplicon 1421 bp**	***iss* gene Amplicon 737 bp**	***fimH* gene Amplicon 670 bp**
1	S1	+	+	+
2	S2	+	+	+
3	S3	+	-	+
4	S4	-	+	+
5	S5	+	+	+
6	S6	+	+	+
7	S7	+	+	+
8	S8	+	+	+
9	S9	+	+	+
10	S10	+	+	+
11	S11	+	+	+
12	S12	+	-	+
13	S13	-	-	-
14	S14	+	+	+
15	S15	+	+	+
16	S16	+	+	+
17	S17	+	+	+
18	S18	+	+	+
19	S19	+	+	+
20	S20	+	+	+
21	Positive control *E. coli* PIB4293	+	+	+

for attachment factors *ompA* and *fimH* (Table 2), 1/20 was positive for one of the attachment factors and the resistance to complement factor and 1/20 was negative for all three specific genes (S13 strain).

The classification of the isolates as APEC is based on detection of some virulent factors. In this case, the multiplex PCR technique applied highlighted three genes that encode three APEC specific virulent factors. *Iss* gene encodes a protein that determines the resistance to complement, *fim*H gene encodes a fimbrial adhesin ("type 1"), and *omp*A gene encodes an outer membrane protein, the last two being involved in the pathogenic process of extraintestinal sequential colonization made by an APEC strain.

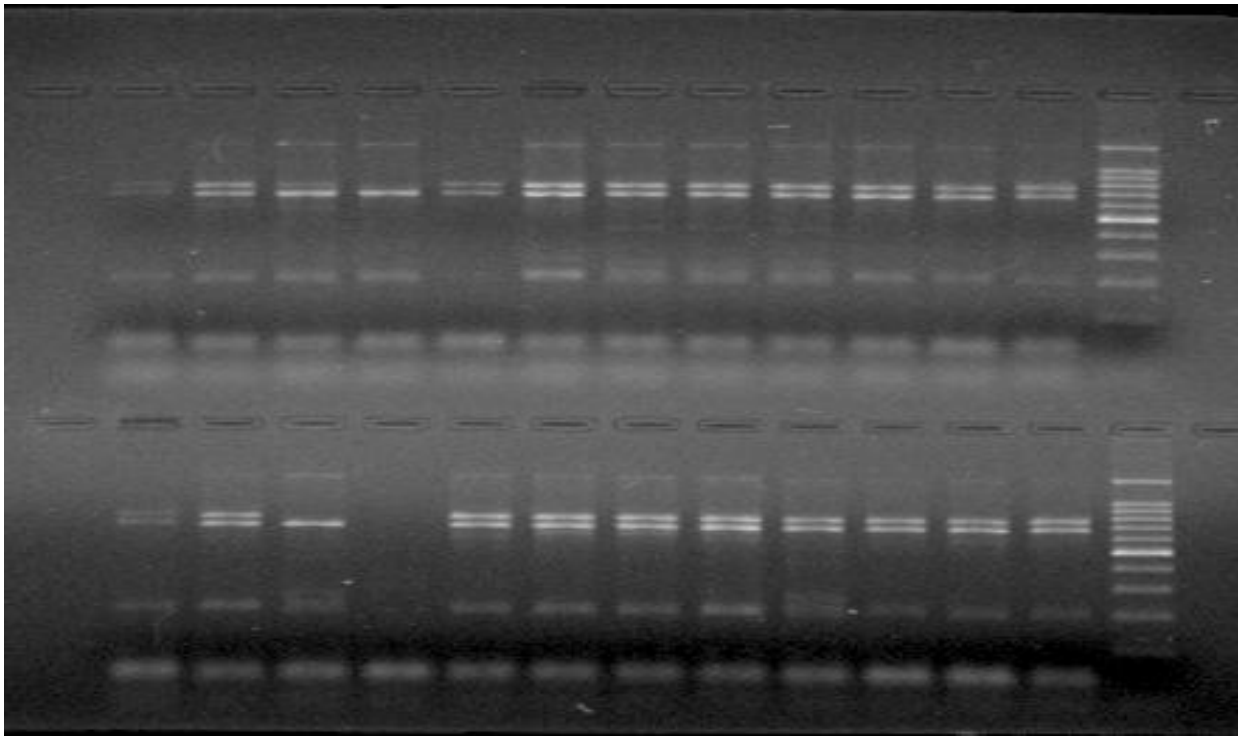

Figure 6. *Escherichia coli*. Molecular typifying by multiprimer PCR (multiplex PCR), as APEC pathotype. Control by gel-electrophoresis post PCR Row 1: Lane 1: Positive control Ec PIB4293 (positive ompA; positive iss; positive fimH); Lane 2: S1 (positive ompA; positive iss; positive fimH); Lane 3: S2 (positive ompA; positive iss; positive fimH); Lane 4: S3 (positive omp; negativ iss; positive fimH); Lane 5: S4 (negative ompA; positive iss; positive fimH); Lane 6: S5 (positive ompA; positive iss; positive fimH); Lane 7: S6 (positive ompA; positive iss; positive fimH); Lane 8: S7 (positive ompA; positive iss; positive fimH); Lane 9: S8 (positive ompA; positive iss; positive fimH); Lane 10: S9 (positive ompA; positive iss; positive fimH); Lane 11: S10 (positive ompA; positive iss; positive fimH); Lane 12: Positive control Ec PIB4293 (positive ompA; positive iss; positive fimH); Lane 13: Standard ADN: 100bp DNA Ladder (Promega). Row 2: Lane 1:Positive control Ec PIB4293 (positive ompA; positive iss; positive fimH); Lane 2: S11 (positive ompA; positive iss; positive fimH); Lane 3: S12 (positive omp; negative iss; positive fimH); Lane 4: S13 (negative ompA; negative iss; negative fimH); Lane 5: S14 (positive ompA; positive iss; positive fimH); Lane 6: S15 (positive ompA; positive iss; positive fimH); Lane 7: S16 (positive ompA; positive iss; positive fimH); Lane 8: S17 (positive ompA; positive iss; positive fimH); Lane 9: S18 (positive ompA; positive iss; positive fimH); Lane 10: S 19 (positive ompA; positive iss; positive fimH); Lane 11: S 20 (positive ompA; positive iss; positive fimH); Lane 12: Positive control Ec PIB4293 (positive ompA; positive iss; positive fimH); Lane 13: Standard ADN: 100bp DNA Ladder (Promega).

DISCUSSION

Avian pathogenic specific *E. coli* (APEC) is one of the extraintestinal pathotypes classified as colibacilli strains (ExPEC).

Mortality losses are an important parameter of epidemiological examination (Ewers et al., 2003), varying according to age of broilers 0.61% in the first week of life and 3.4% in the last week of life. This percentage increase may be due to unfavorable microclimate conditions.

Cumulative mortality in the studied flocks ranged between 5.18% in H3B and 5.95% in H1B, this data is similar with those presented by Zanella et al. (2000), which reported a mortality of 5 - 10% in *E. coli* infections, although (Omer et al., 2008) reported a mortality rate of 1.8%.

Our results are similar to those in the scientific literature that indicates that all studied strains ferment lactose on both culture media, this biochemical feature being considered constant at APEC strains.

Rodriguez-Siek et al. (2005) found that 99% of the 451 strains studied fermented lactose in the two media, confirming that the *APEC* strains have this biochemical characteristic, unlike other *E. coli* strains belonging to other pathotypes.

The multiple antibiotic resistances observed in this study were similar to results obtained by other researchers in other countries (Sharada et al., 2010).

The obtained results show the presence of APEC strains in broilers, which can be transmitted through direct contact or indirectly through various sources from housing, as confirmed by the identification of 8 APEC strains in the air of household.

The economic importance of *E. coli* infections in broilers associated with the increasing frequency of the

strains involved in these infections' etiopathogenesis requires monitoring of strains and genotypic characterization on which to shape the APEC pathotype. Multiplex PCR technique allows an early diagnosis on the pathogenicity of APEC strains.

Conclusions

1. Mortality percentage increased with age of broilers from 0.61% in the first week of life to 3.4% at the age of 28 days, and cumulative mortality ranged between 5.18 and 5.95%.
2. Bacterial examination confirmed 89% cases of colibacillosis from which 42 strains of *E. coli* were isolated.
3. All isolates had characteristic biochemical features of *E. coli* strains: lactose fermentation on both selective culture media (SS and Levine).
4. The isolated strains were multiple resistant to lincomycin, spectinomycin, neomycine, tetracycline, erythromycin, doxycyclin, enrofloxacin.
5. The presence of the three genes is correlated with fixation of Congo red, a characteristic phenotypic feature of *E. coli* strains of avian origin.
6. Multiplex PCR technique leads to an early diagnosis on the pathogenicity of APEC strains, reducing the time of 3-7 days to achieve by conventional techniques to maximum 24 hours.

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