IDENTIFICATION OF THE AVIAN PATHOTYPE OF *ESCHERICHIA COLI* ON LAYER FLOCKS BY MULTIPLEX PCR

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Abstract:

Introduction: Colibacillosis in poultry is determined by avian pathogenic *Escherichia coli* (APEC) and represents an important source of economic losses in the poultry industry. APEC's pathogenicity relies on the presence and expression of different virulence factors. The genes *ompA* , *iss* and *fimH*, encoding the outer membrane protein, the protein inducing resistance to complement and the synthesis of type 1 fimbria are present in APEC strains.

Objective: *Escherichia coli* strains isolated from layers were analysed to assess the pathotype they belong to.

Methods: In order to detect the three genes associated with APEC strains, 16 *E. coli* isolates were investigated for virulence associated genes *ompA, iss* and *fimH,* using multiplex PCR.

Results: From the 16 *E.coli* strains submitted, multiplex PCR assessment revealed that 14 (87.5%) of the *E. coli* strains isolated contained at least one virulence gene, while 2 (12.5%) strains did not harbour any of the virulence genes tested. The *fimH* gene was noted in 13 (81.25%) of the strains tested, the *ompA* gene has been present in 12 (75%) strains and the *iss* gene was present in 9 (56.25%) strains. Eight (50%) strains were found to present all three investigated genes.

Conclusion: Presence of these genes is a strong indicatory to consider those strains as belonging to the APEC pathotype.

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Keywords: Avian pathogenic *E. coli*, layers flocks, multiplex PCR

Introduction

The involvement and relevance of the virulence genes in the pathogenicity of avian colibacillosis is still poorly understood and the majority of studies on APEC and APEC-associated virulence genes are descriptive. However, not one or more specific virulence genes have been invariably linked to APEC pathogenicity. It is very rare to find the same set of virulence genes in all APEC, in the same study or in different studies. Nevertheless, APEC harbour several virulent genes that enable the bacteria to enter, colonise, and escape the immune system and cause avian colibacillosis (Awawdeh, 2017).

Well-known virulence factors comprise fimbriae (Type 1 and P) needed for colonization, IbeA involved in the invasion phase, iron acquisition systems, TraT and Iss implicated in serum survival, antigens (K and O) responsible for anti-phagocytic activity, and a hemagglutinin which is sensitive to temperature and yet has an unclear role (Dziva and Stevens, 2008). In order for the infection with APEC to occur, an association of various virulence factors is needed since the sole presence of one such factor is not enough to define the pathogenic potential of the APEC strain. (Vandekerchove, 2004). Intriguingly, these virulence factors are not found universally among APEC and probably multiple alternative mechanisms are needed for pathogenicity mediation (Dziva and Stevens, 2008).

The virulence of APEC varies, and this variation is controlled by specific genes. Examining the expression *in vivo* of the genes coding for the virulence factors is sometimes a difficult task. Furthermore, there are some pathogenic characteristics, such as serum resistance, determined by several genes, which means that lacking one of these genes does not imply automatically the absence of the phenotypic characteristic (Vandekerchove, 2004).

The aim of the study is to search for the presence of 3 genes, *ompA, iss* and *fomH*, that define the major genotypic characteristics of the avian pathogenic *Escherichia coli* strains. The *ompA* gene encodes the synthesis of the outer membrane protein A (ompA), which controls the attachment of APEC onto the respiratory epithelium cells. The *fimH* gene, belonging to the *fim* operon is coding the synthesis of the type 1 fimbria, involved into the attachment of *Escherichia coli* to the respiratory epithelium cells. The synthesis of the external membrane protein Iss is coded by the *iss* gene; this

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protein is responsible for serum (complement) survival, which then facilitates the colonization and multiplication of the bacteria in the bloodstream (Popa *et al*., 2004).

Materials and methods

This study aimed to investigate genotypic characteristics of 16 avian pathogenic *E. coli* strains, isolated from episodes of colibacillosis or routine sampling from layer flocks. Multiplex PCR (Polymerase Chain Reaction) was used to investigate the presence of the *ompA, iss* and *fimH* genes by using the Popa *et al.* (2004) multiplex type.

The samples (organs, corpses, dying birds), were processed in order to isolate and identify *E. coli* strains accordingly to published techniques (cultivation on culture media special for *Enterobacteriaceae* - MacConkey agar - and have been identified based on biochemical characteristics - biochemical tests MIU TSI).

The amplifications were done on raw DNA samples (1 µl ⁄ reaction) obtained by thermal lysis as follows: 1.5 ml of 18 hours bacterial cultures on BHI (brain heart infusion) were centrifuged 17.340g, 4°C, for 10 minutes, removal of supernatant and resuspension of the bacterial pellet in 200 µl ultrapure water (Promega P119C), another one wash in the same conditions, boiling at 100°C for 10 minutes, 1 minute on ice (4°C), centrifuged at 17.340g, 4°C, for 10 minutes, 150-200 µl of supernatant representing the working sample that could be used right away or aliquoted and preserved at -20°C (Popa, 2001).

The primers had a concentration of 25 pmoles each per reaction for *iss* and *fimH*, and 30 pmoles for *ompA*, and were commercially synthesized (NAPS Unit, University of British Columbia, Canada); the sequence of the primers and the amplicons produced, are shown in Table 1.

The amplification reactions were carried out on iCycler (BioRad), in a total volume of 25µl / reaction, with PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) kits. The *E. coli* strain PIB4293 of avian origin (1996; CRb+, ser^r; *iss*+; *ompA*+ and *fimH*+), was used as a positive control. The JM109 strain (Promega P9801) represented the negative control (Popa *et al*., 2004).

Table 1 : Primers and amplicon size used for the amplification studies

Gene	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
Iss	iss-1 iss-2	gtg gcg aaa act agt aaa aca gt cgc ctc ggg gtg gat aa	737	Pfaff-McDonough *et al*., 2000
ompA	ompA-1 ompA-2	ctt gcg gag gct tgt ctg ag agg cat tgc tgg gta agg aa	1421	Pfaff-McDonough *et al*., 2000
fimH	H1 H2	caa aac ctg gtc gtg gat ct ttg ccg tta atc cca gac tc	670	Popa *et al*,. 2004

Source: Popa *et al*., 2004

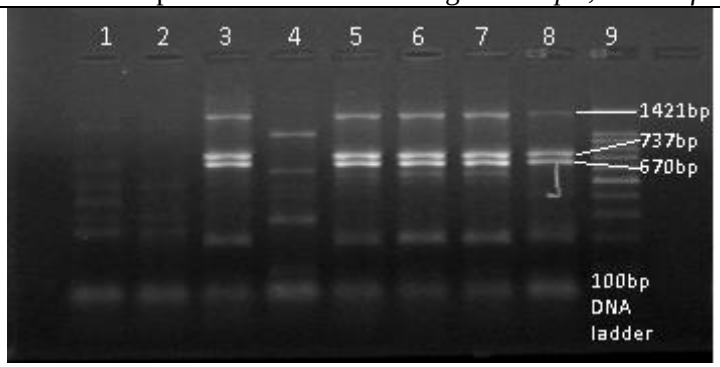
The amplification program (the 'cycling' program) used includes denaturation (94ºC, 5 minutes - 2x or 10 minutes - 1x), annealing (30 cycles of: 94°C, 1 minute + 55°C, 1.5 minutes + 72°C, 2.5 minutes) and elongation (72ºC, 10minutes - 1x; +4°C 10 minutes/unlimited).

The amplicons obtained after PCR were analysed by gel electrophoresis TBE 1.5X. For the image to be digitalized, E.A.S.Y. RH (Herolab GmbH) equipment and ImageWin2PC logical program were used. The resultant size of the amplicons was calculated digitally with the help of UnScanIt (Silk Sci. Ink.) software and further correlated with the DNA commercial standards (PCR marker, Sigma) (Popa *et al*., 2004).

Results

This study followed the presence of the 3 genes (*ompA, iss* and *fimH)* using multiplex PCR screening for 16 *Escherichia coli* strains, as shown on Figure 1 (7 strains), Figure 2 (7 strains) and Figure 3 (2 strains). The multiplex PCR technique allows the simultaneously detection of the three genes mentioned above. After the migration, the amplicons had the following sizes (bp): 1421 bp *ompA*, 737 bp *iss* and 670 bp *fimH*.

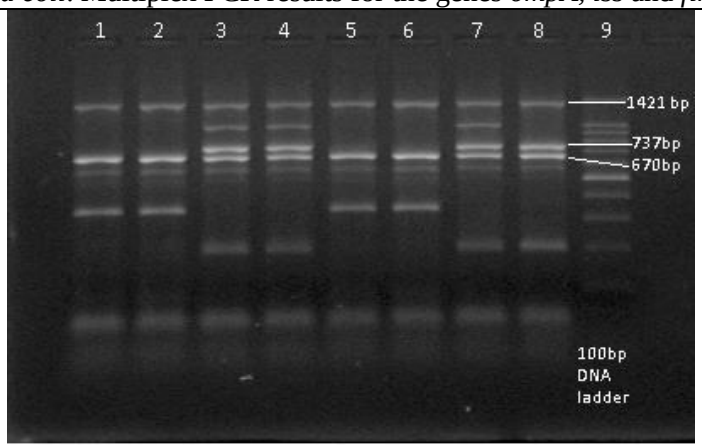
Figure 1: *Escherichia coli*. Multiplex PCR results for the genes *ompA, iss* and *fimH*.



Lanes 1-7: *E. coli* strains; Lane 8: Positive control E. coli PIB4293;
Lane 9: Standard ADN: 100bp DNA Ladder

Source: Author

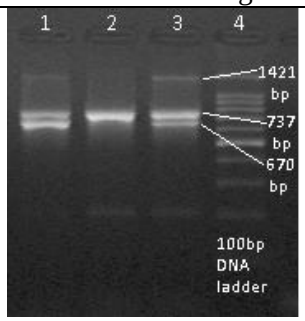
Figure 2: *Escherichia coli*. Multiplex PCR results for the genes *ompA, iss* and *fimH*.



Lanes 1-7: *E. coli* strains; Lane 8: Positive control E. coli PIB4293;
Lane 9: Standard ADN: 100bp DNA Ladder

Source: Author

Figure 3: *Escherichia coli*. Multiplex PCR results for the genes *ompA, iss* and *fimH*.



Lanes 1- 2: *E coli* strains; Lane 3: Positive control E. coli PIB4293;
Lane 4: Standard ADN: 100bp DNA Ladder

Source: Author

The multiplex PCR screening revealed that 50% of the *Escherichia coli* strains presented all 3 genes (*ompA, iss, fimH*). The association of two genes was seen in 25% of the strains, and the presence of only one gene was found in 12.5% of the tested strains (Table 2).

The presence of *ompA* gene was noticed in 75% of the tested strains, the *iss* gene appeared on 56.25% of the tested strains and the *fimH* gene was found in 81.25%.

At least one of the three investigated genes was reported in 14 strains (87.5%). Two strains (12.5%) had only one gene.

Table 2: Multiplex PCR results of *E. coli* strains

Characteristics		No	%
Strains investigated		16	100
mPCR	*ompA*	12	75
	iss	9	56.25
	fimH	13	81.25
1 gene	*ompA*	0	0
	iss	1	6.25
	fimH	1	6.25
2 genes	*ompA+iss*	0	0
	ompA+fimH	4	25
	iss+fimH	0	0
3 genes	*ompA+iss+fimH*	8	50
mPCR		14	87.5

Source: Author

Discussions

At different stages of infection, alternative virulence genes might participate in the pathogenicity mechanism of avian pathogenic *E. coli*, including colonisation (*fimH, fimC, papC, papEF* and *tsh*), invasion (*ibeA, vat*), iron acquisition (*iutA, iroN, IreA, feoB*), serum complement resistance (*iss*) and putative iron transport (*sitA*) (Awawdeh, 2017).

APECs' pathogenicity involves resistance to lysis mediated by complement and phagocytosis initiated by the opsonin (Dziva and Stevens, 2008). Serum resistance virulence genes enable the bacteria to survive outside the gastrointestinal tract and overcome the host defence mechanisms. These virulence genes are frequently associated with septicaemia (Dho-Moulin and Fairbrother, 1999) as they assist the bacteria in avoiding being killed by the defence mechanisms of blood, including complement, antimicrobial peptides and other serum components. Four serum resistance virulence genes, *iss* (increased serum survival, which is a cytotoxic inhibitor), *traT* (surface exclusion protein), *cvaC* (structural genes from the colicin V operon) and *ompA* (outer membrane protein A) relate to the APEC pathogenicity, moreover, *iss* is significantly associated with APEC rather than to AFEC (Avian fecal *Escherichia coli)* (Awawdeh, 2017).

The increased serum survival gene (*iss*), was first characterized in 1979. It has a role in the resistance to complement and it is associated with a ColV plasmid. Iss represents a lipoprotein revealed on the outer membranes of *Escherichia coli* and is encoded by the *iss* gene. Usually it is present on avian pathogenic *E. coli* and more rarely encountered in commensal strains (Nolan *et al*., 2013).

Several studies reported a correlation of the complement resistance, the virulence and the presence of the *iss* gene in APEC. However, it is unknown whether the *iss* gene alone contributes to the pathogenicity or whether the *iss* gene is a marker for the presence of the plasmids associated with APEC pathogenicity. The *iss* gene is needed for complement resistance, but not for APEC pathogenicity. Other authors reported that the absence of the *iss* gene did not influence the ability of APEC to resist serum complement. In addition to *iss* APEC possess different serum resistance mechanisms that allows the bacteria to survive in the serum (Awawdeh, 2017). *Iss* is supposed to impede the deposition of the membrane attack complex of complement (Dziva and Stevens, 2008).

Lipopolysaccharide and K1 capsule have an obvious role in serum resistance of avian pathogenic *Escherichia coli*, whereas iss plays a subtle but necessary role for full virulence. As mentioned earlier, a correlation exists between complement resistance, the virulence and the presence of the *iss* gene in APEC and, furthermore, complement resistance is highly needed but not enough for virulence (Dziva and Stevens, 2008).

Although colibacillosis is determined by multiple virulence factors of APEC, the *iss* gene seems to be among the most encountered virulence genes in extra-intestinal pathogenic strains (Badouei *et al*., 2015).

In the current study the prevalence of the *iss* gene in *E. coli* isolates was 56.25%. High prevalence of this gene has been reported in Japan (100% in layers) and Germany (82.7% in layers and broilers) (Someya *et al.*, 2007; Ewers *et al*., 2004). Kwon *et al*. (2008) also detected 100% prevalence of the *iss*

gene in layers in Korea. Similar prevalence with this study was found in Brazil (51.4% in broilers, breeders and turkeys) by De Carli *et al*. (2015). However, Van den Westhuizen and Bragg (2012) found a low prevalence (31.4%) of the *iss* gene among APEC isolates from poultry with colibacillosis in South Africa and Zimbabwe.

Other researches have documented the presence of virulence genes including *iss* on *E. coli* isolates from outbreaks of colibacillosis compared with healthy birds (fecal/cloacal swabs). In the USA Johnson *et al*. (2008) found the prevalence of the *iss* gene in 80.5% outbreaks compared to 30% in healthy birds. Lower differences in the prevalence of the *iss* gene among *E. coli* isolates between diseased and healthy chickens were found by Paixao *et al*. (2016) (40.9% vs 24.6%) in Portugal.

Several adhesin genes were associated with avian pathogenic *E. coli*, including Type 1 fimbriae. The F1 fimbriae are encoded by nine *fim* genes and include a major protein known as *FimA* and minor proteins known as *FimF, FimG* and the adhesin *FimH* (Dho-Moulin and Fairbrother, 1999; Awawdeh, 2017). Type 1 fimbriae are expressed only in the upper respiratory tract and not in any other internal organs, which suggests they have a role in initial colonisation (Awawdeh, 2017). When colonizing the lungs and air sacs of birds, Type 1 fimbriae have responsibility in the first phase of this process, indicating an important role of the *fimH* gene in the pathogenesis of avian colibacillosis (Roussan *et al*., 2014). Expression of F1 fimbriae takes place in the course of initial colonization of tracheal epithelial cells. When expressing F1 fimbriae the macrophages will immediately kill the bacteria. Although *in vitro* adhesion to cultured chicken epithelial cells from the pharynx or the trachea require FimH (*F1* fimbrial adhesion), *in vivo* colonization of the chicken's trachea is favoured by the lack of fimH (Nolan *et al.*, 2013).

The *FimH* virulence factor seems to be an essential unit for protecting the APEC isolates against the host immune system, but the exact role of *FimH* in the pathogenicity of APEC isolates remains debatable with incompatible results (Asadi *et al.*, 2018). Multiplication of bacteria is restricted by the activity of heterophils to such a level that it is eliminated more efficiently by the following host's defences. *E. coli* strains that possess FimH, are more successful in combating the heterophilic activity and afterwards invade the host (Melatta, 2003b).

Among *E. coli* strains researched in the present study, the *fimH* gene had the highest prevalence (81.25%) of the genes explored. Similar high prevalence was found in Mexico (95%) (Lopez *et al*, 2017). Nonetheless Mbanga and Nyararai (2015) reported a low prevalence (33.3%) of the *fimH* gene in *E. coli* isolates from collibacillosis cases.

Other studies compared the frequency of the *fimH* gene among *E. coli* strains isolated from clinical colibacillosis and from healthy chickens. Pfaff-McDonough *et al*. (2000) found a large difference between *fimH* presence in colibacillosis cases (77%) and healthy chickens (19%). Schouler *et al.* (2012) investigated 460 *E. coli* isolates from France, Spain and Belgium collected over a period of eight years and found a similar prevalence between pathogenic (86.4%) and non-pathogenic (71.3%) *E. coli* isolates.

The presence of outer membrane protein A (OmpA), encoded by the chromosomal gene *ompA*, has been correlated to the complement resistance of *E. coli* (Pfaff-McDonough *et al*., 2000). This protease also contributes to APEC's pathogenicity in embryonated chicks and to cleaving colicins (Trampel *et al*., 2007). The outer membrane of *Escherichia coli* comprises a major protein, OmpA. The serum resistance of avian pathogenic *E. coli* may be possible due to the contribution of this outer membrane protein by impeding the deposition of C3 on the surface of the bacteria or by blocking the membrane attack complex (Li *et al*., 2011).

OmpA plays an essential part in APEC environmental adaptation, survival and virulence (Wang *et al*., 2017). It performs as a physical connection between the outer membrane and peptidoglycan. It is contributing to the *E. coli* virulence by the mechanism of increased serum resistance, invasion of the cell, playing a role in immune activation of the host and resistance to antimicrobials. In neonatal meningitis *E. coli* (NMEC), OmpA plays a crucial role in the adhesion of bacteria onto the microvascular endothelial cells of the brain (Van Goor *et al.*, 2017).

Expression of OmpA in *E. coli* contributs to macrophages attaching, to phagocytosis, and to survival inside the macrophages. Opsonisation of the bacteria is not necessary for up-take and survival of the *E. coli* inside macrophages. *E. coli* strains possessing OmpA will survive and multiply inside the macrophages, whereas *E. coli* strains that doe not have OmpA don't survive or replicate inside the cell (Sukumaran *et al*., 2003).

Our study found a high prevalence of the *ompA* gene (75%) among *E. coli* isolates from layer flocks. Van Goor *et al*. (2017) found 84% prevalence of the *ompA* gene. Ronco *et al*. (2017) found the *ompA* gene among all *E. coli* analysed isolates, both from healthy and diseased birds, suggesting its limited value of association to pathogenicity.

Studies on the three virulence genes (*ompA, iss* and *fimH*) were also conducted in Romania. Iancu *et al.* (2015) found a high prevalence of the genes *ompA* (83.1%), *iss* (88.1%) and *fimH* (83.9%) in a recent study on broiler flocks. Gurau *et al.* (2018) investigated *E. coli* virulence genes *iroN* and *iucC* among broiler and layer flocks in Romania. Therefore, it could be said that this is the first study analysing the above-mentioned genes in layer flocks.

Conclusions

The multiplex PCR used for investigating the virulence genes *ompA, iss* and *fimH* in APEC isolates from layer flocks showed a high frequency of these genes, which could be considered a strong indication that those strains belong to the APEC pathotype.

87.5% of *E. coli* strains investigated in the present study possessed at least one of the three targeted genes. Eight strains (50%) possessed all three genes, 4 strains (25%) possessed two genes (*ompA+fimH*). The association of *ompA+iss* or *fimH+iss* was not found in any of the tested strains. Out of the *E. coli* strains isolated, in 75% of the cases two or three of the tested genes were found.

The limitation of this study is the paucity of epidemiological data that would allow us to emphasise the link between the diseased flock and the genetic pattern of strains. Each of the genes, alone, registered different frequencies: the *fimH* gene had the highest frequency (81.25%), followed by the *ompA* gene (75%) and the *iss* gene (56.25%). Based on these values, we could fall into the trap of minimizing the impact of the gene with the lowest frequency, but in the absence of a known association to the clinical state of the flock, it may prove to be not only a simplistic but mostly un erroneous approach. The study of different (different as the presence of pathogenicity genes) APEC strains in a case-control trial would allow the identification of genes association more closely with the APEC phenotype.

The results of our study, as well as that of other investigators, highlight the need for a Cascade Investigation Protocol of the E-genome isolates to indicate APEC membership.

The multiplex PCR technique could serve as a useful mean for colibacillosis survey in poultry.

This is the first study that analysis the prevalence of the three virulence genes (*ompA, iss* and *fimH*) in layer flocks in Romania.

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