Genes associated with pathogenicity of avian *Escherichia coli* (APEC) isolated from respiratory cases of poultry[1]

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ABSTRACT.- Rocha A.C.G.P., Rocha S.L.S., Lima-Rosa C.A.V., Souza G.F., Moraes H.L.S., Salle F.O., Moraes L.B. & Salle C.T.P. 2008. **Genes associated with pathogenicity of avian *Escherichia coli* (APEC) isolated from respiratory cases of poultry.** *Pesquisa Veterinária Brasileira 28(3):183-186.* Centro de Diagnóstico e Pesquisa em Patologia Aviária, Departamento de Medicina Animal, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 8824, Porto Alegre, RS 91540-000, Brazil. E-mail: ana.crocha@terra.com.br

The virulence mechanisms of avian pathogenic *Escherichia coli* (APEC) have been continually studied and are believed to be multi-factorial. Certain properties are primarily associated with virulent samples and have been identified in avian isolates. In this study a total of 61 *E. coli*, isolates from chicken flocks with respiratory symptomatology, were probed by Polimerase Chain Reation (PCR) for the presence of genes responsible for the adhesion capacity, P fimbria (*papC*) e F11 fimbria (*felA*), colicin production (*cvaC*), aerobactin presence (iutA), serum resistance (*iss*), temperature-sensitive hemagglutinin (*tsh*), and presence of K1 and K5 capsular antigens (*kpsII*). The *iss* gene was detected in 73,8%, *tsh* in 55,7%, *iutA* in 45,9%, *felA* in 39,3%, *papC* in 24,3%, *cvaC* in 23% and *kpsII* in18%.

INDEX TERMS: *Escherichia coli*, virulence, PCR, poultry, pathogenicity.

RESUMO.- [**Genes associados à patogenicidade de *Escherichia coli* patogênica para aves (APEC) isoladas de frangos de corte com sintomatologia clínica respiratória**.] Os mecanismos de virulência das amostras de *Escherichia coli* potencialmente patogênicas para aves (APEC) têm sido continuamente estudados e acredita-se ser multifatorial. Certas propriedades são associadas primariamente a amostras virulentas e vêm sendo identificadas em amostras de *E. coli* isoladas de aves. Neste estudo um total de 61 amostras de *E. coli*, isoladas de frangos de corte com problemas respiratórios, foram testadas através da Reação em Cadeia da Polimerase (PCR), para a presença dos genes responsáveis pela capacidade de adesão, fimbria P (*papC*) e fimbria F11 (*felA*), produção de colicinas (*cvaC*), presença de aerobactina (iutA), resistência sérica (*iss*), hemaglutinina temperatura sensível (*tsh*) e presença de dos antígenos capsulares K1 e K5 (*kpsII*). O gene *iss* foi detectado em 73,8%, *tsh* em 55,7%, *iutA* em 45,9%, *felA* em 39,3%, *papC* em 24,3%, *cvaC* em 23% e *kpsII* em 18%.

TERMOS DE INDEXAÇÃO: *Escherichia coli*, virulência, PCR, patogenicidade, frango de corte.

INTRODUCTION

The increasing competition in meat markets gives rise to the necessity of more and more efficient and competitive production. In this scenario, production rates are funda-

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mental to surveillance and competition of poultry processing plants. These rates comprise fundamentally the sanitary aspects of the breeding stock.

Escherichia coli, which is part of the normal microbiota of birds (Bettelheim 1994) in intestinal and respiratory tracts (Morris & Sojka 1985), was forgotten as a potential pathogen. However, lesions in which *E. coli* is the primary and often the secondary agent cause economic damage due to lower corporal development, insufficient feed conversion, increasing mortality, higher cost with medicine, and condemnation of carcasses.

In Brazil, from 2001 to 2005, the condemnation of part of carcasses or entire carcasses because of the presence of lesions, where *E. coli* could be the responsible agent, caused losses estimated to 58 million dollar, 39 millions of which by systemic lesions (Brasil 2006).

Considering that 10-15% of *E. coli* samples can be potentially pathogenic (Barnes & Gross 1997), turned urgent and decisive for the occurrence of the disease the knowledge of interaction of factors connected with handling, nutrition, bird genetics, immunodeficiency, and especially bacterial genetics.

The virulence mechanisms of avian pathogenic *E. coli* (APEC) have been continually studied and are believed to be multifactorial. Certain properties are primarily associated with virulent samples and have been identified in avian isolates. The most frequently mentioned are: adhesion capacity (*pap and fel*), colicin production (*cva*), aerobactin presence (*iut*), serum resistance (*iss*) (Barnes 1997), hemagglutinin sensitive temperature (*tsh*), and the presence of certain capsular antigens (*kps*) (La Ragione & Woodward 2002).

MATERIALS AND METHODS

Bacterial samples

Sixty-one *Escherichia coli* isolates from 58 chicken flocks with respiratory symptomatology and post-mortem lesions compatible with colibacillosis were studied. The samples were stored in BHI (Brain Heart Infusion/ Oxoid, England) with glycerol at 30% in a freezer at -20ºC. The sample motility was verified by use of SIM Medium (Sulfuric acid indol motility/ Merck, Germany).

Virulence genes

E. coli was probed by the Polimerase Chain Reaction (PCR) for the presence of genes responsible for the adhesion capacity, P fimbriae (*papC*) and F11 fimbriae (*felA*), colicin production (*cvaC*), aerobactin presence (*iutA*), serum resistance (*iss*), temperature-sensitive hemaglutinin (*tsh*) and presence of K1 and K5 capsular antigens (*kpsII*).

PCR

Extraction of the DNA. One milliliter of suspension from a bacterial culture in BHI of 24h at 37ºC was collected and centrifuged for 5 minutes. The supernatant was rejected and 800µl of the milliQ water was added. After having been homogenized, the samples were submitted to new centrifugation in the same conditions previously mentioned. The supernatant was rejected and 80µl of milliQ water was added. The samples were then submitted to a temperature of 96ºC for 10 minutes in water bath. The supernatant was removed and maintained frozen until the moment of analysis.

Mix. For the genes *iutA* and *cvaC* were used 11.2µl of milliQ water, 2.5µl of PCR buffer 10x (Lab Trade, Brazil), 2µl of dNTP mix with 2.5mM of each nucleotide (Invitrogen Life Technologies, USA), 2µl of 50mM MgCl2, 1µl of 20pM each primers (Invitrogen Life Technologies, USA), 0.3µl of 5U Taq DNA polymerase (Lab Trad., Brazil) and 5µl of template DNA.

For the genes *felA*, *kpsII*, *papC, tsh* and *iss* were used 11.95µl of milliQ water, 2.5µl of PCR buffer 10x (Lab Trade, Brazil), 2µl of dNTP mix with 2.5mM of each nucleotide (Invitrogen Life Technologies, USA), 1.25µl of 50mM MgCl2 (Lab Trade, Brazil), 1µl of 20pM each primers (Invitrogen Life Technologies, USA), 0.3µl of 5U Taq DNA polymerase (Lab Trade, Brazil) and 5µl of template DNA.

The primers are reported on Table 1.

Amplification. The conditions of PCR, the sequence of the primers and the size of the amplified fragment for each studied gene are described on Table 1. The tests were done in termal cycler PCT-100 (MJ Research) and the amplified DNA was visualized in agarose gel at 1.2% (Invitrogen Life Technologies, USA) contends ethidium bromide (Sigma, USA).

Reference *Escherichia coli* strains. BK 324 (*cvaC, iss, felA, papC, tsh*), IAPAR 1315 (*iutA*) e ATCC 35278 (*kpsII*).

Table 1. Sequence of the primers, size of amplified fragments and conditions used in PCR for the detection of the genes associated to the virulence

Gene	Primer sequence5'- 3'	Fragment size (bp)	PCR conditions
kpsII	gcg cat ttg ctg ata ctg ttg cat cca gac gat aag cat gag ca	272	5 min 94ºC / 30 ciclos 1 min 94ºC, 1 min 63ºC e 2 min 72ºC/ 10 min 72ºC
cvaC	cac aca caa acg gga gct gtt ctt ccc gca gca tag ttc cat	680	5 min 94ºC / 30 ciclos 1 min 94ºC, 1 min 63ºC e 2 min 72ºC / 10 min 72ºC
papC	gac ggc tgt act gca ggg tgt ggc g ata tcc ttt ctg cag gga tgc aat a	328	5 min 94ºC / 30 ciclos 1 min 94ºC, 1 min 63ºC e 2 min 72ºC / 10 min 72ºC
felA	ggc agt ggt gtc ttt tgg tg ggc cca gta aaa gat aat tga acc	270	5 min 94ºC / 35 ciclos 1 min 94ºC, 1 min 63ºC e 2 min 72ºC / 10 min 72ºC
iutA	ggc tgg aca tca tgg gaa ctg g cgt cgg gaa cgg gta gaa tcg	300	5 min 94ºC / 35 ciclos 1 min 94ºC, 1 min 63ºC e 2 min 72ºC / 10 min 72ºC
tsh	ggt ggt gca ctg gag tgg agt cca gcg tga tag tgg	620	5 min 94ºC / 30 ciclos de 1 min 94ºC, 1 min 55ºC e 2 min 72ºC / 10 min 72ºC
iss	gtg gcg aaa act agt aaa aca gc cgc ctc ggg gtg gat aa	760	5 min 94ºC / 30 ciclos de 1 min 94ºC, 1 min 61ºC e 2 min 72ºC / 10 min 72ºC

Table 2. Frequency of the virulence genes and motility of samples of *Escherichia coli*

cvaC (%)	*iss* (%)	*iutA* (%)	*kpsII* (%)	*papC* (%)	*fel A* (%)	*tsh* (%)	Motilidade (%)
14.0 (23.0)	45.0 (73.8)	28.0 (45.9)	11.0 (18.0)	15.0 (24.6)	24.0 (39.3)	34.0 (55.7)	33.0 (54.1)

RESULTS AND DISCUSSION

Many researchers have shown that mechanisms for pathogenicity of APECs are directly related to the interaction of several factors. Several authors dedicated themselves to these studies, and in spite of frequency variations, the following main factors are significant: adhesion capacity, colicin production, aerobactin presence, serum resistance, sensitive temperature hemagglutinin, and the presence of certain capsular antigens (Dho & Lafont 1982, Naveh et al. 1984, Rocha et al. 2002, Ngeleka et al. 2002, Brito et al. 2003, Delicato et al. 2003, McPeake et al. 2005).

In this study the presence of some genes, which are determinant in virulence and motility of litter isolates, was found. The frequency is shown on Table 2.

In colicin producing samples, the genetic determiners and the proteins that accompany them are located in plasmids, which are called Col factors (Luria 1987). Colicin V, unlike the other ones, is found mainly in virulent bacteria involved in extra-intestinal infections affecting humans and animals (Gilson et al. 1987, Lior 1994) and it inhibits the bacterial growth, interfering with the potential of membrane formation (Yang & Konsky 1984).

In this study *cvaC* gene and *ColV* plasmid structural gene were detected in 23% of the samples. Only Blanco et al. (1997) found similar results (22%), while other authors obtained higher percentage. McPeake et al. (2005) reported 99.1% and Rodriguez-Siek et al. (2005) 66.8%. In Brazilian samples the gene has been detected in 35% of them (Delicato et al. 2003).

Serum resistance is mediated by the lipopolysaccharid structural surfaces (LPS) of the capsule and other proteic membranes, the presence of outer membrane protein (Gross 1994) and K1 and K5 antigens, which are codified by *kps* genes (Johnson et al. 2005). Some plasmids are able to transmit serum resistance to sensitive receptive cells. *Iss* genes were identified in pColV-I-K94 plasmids, the protein of which is related to cytotoxic complex inhibition (Bins at al. 1979). The results of the present study are similar to the 72.8% reported by McPeake et al. (2005), the77% reported by Pfaff-McDonough et al. (2000), and the higher percentage of 81.5% found by Rodriguez-Siek et al. (2005).

The property of invading and multiplying presented by pathogens is influenced by iron availability, which is essential for growth in living cells (Neilands et al. 1985). The aerobactin system enables microorganisms to grow in iron free media at low concentration. *E. coli* especially uses this way of capture and transport (Rohrbach et al. 1995, Braun 2003).

The presence of operon aerobactin is in general related to ColV plasmids, although it can be chromosomal (Linggood et al. 1987, Johnson 1991). Table 2 shows that *iut A* gene, which codifies outer membrane protein aerobactin receptor, was detected in 45.8% of the samples. Gomis et al. (2001) reached similar results (46.8%). Rodriguez-Siek et al. (2005) and Delicato et al. (2003) got 80.2% and 63%. Vandekerchove et al. (2005) presented a lower result (23%). Rodriguez-Siek et al. (2005) detected higher results, 41.2%.

A serologic variant of P fimbriae (F11) is codified by the *fel*A operon (De Ree et al. 1985). In samples analyzed in the present study, 39.3% were positive for the presence of this gene. Controversial results are reported by other authors, of them Delicato et al. (2003) who got 12%, while Rodriguez-Siek et al. (2005) found 78%.

Provence & Curtiss (1994) reported hemagglutination in *E. coli* samples which was detected only at 26-30ºC, and therefore was called sensitive temperature hemagglutinin and identified as the *tsh* gene. Its role in the pathogenicity of avian isolates still demands investigation, although its presence in isolated samples obtained from diseased birds has been reported by various authors. In the present work 55.7% of the samples analyzed from litter were positive. Ewers et al. (2004) found 53.3%, while other authors reported 85.3% (Janben et al. 2001), 93.95% (McPeake 2005), and 99% (Ngeleka et al. 2002).

The flagella, which are thin surface appendixes, give motility to Gram positive and negative bacteria in aqueous media. Their rotating movements allow microorganisms to approach adjacent epithelial cells, crossing the mucus barrier and causing adhesion, multiplication, colonization and infection (La Ragione & Woodward 2002). In the present study motility was detected in 54.1% of the samples, higher than 36.8% reported by McPeake et al. (2005).

The diversity of genes associated with pathogenicity, which was found in the present study and in other scientific articles, suggests the existence of a real interaction among APEC virulence factors. However, despite the technological progress, the contribution to their pathogenicity has not been established so far. Consequently, no significant advance and objective answers to professionals have been obtained.

The growing limitations for the use of antimicrobics in avian production, do not allow professionals to be informed about the *E. coli* pathogenic potential, to direct their procedures. For this to happen, new technology is necessary to detect the pathogenicity of isolates in an objective, fast and efficient way.

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