Genes of Virulence and Phylogenetic Group in Isolates of Avian Pathogenic *Escherichia coli*

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Abstract

Avian pathogenic *Escherichia coli* (APEC) shares some virulence attributes with strains of *E. coli* that cause extraintestinal infections in humans. The APEC is considered a possible cause for a zoonosis. The objective of this work was to determine the prevalence of twelve genes that are associated with virulence in a group of APEC isolates, as well as to identify the phylogenetic groups to which they belong. According to the results, one of these isolates harbors 91.6% of the virulence genes and most of the isolates have 7 or 8 of such twelve genes. The genes *feoB* and *iss* had the highest prevalence, with 95.6%. Genes associated to the acquisition of iron were present in more than 60% of the APEC isolates, while those of the ibeA invasin and vat toxin were detected with the lowest prevalence. A great genetic diversity was observed on the APEC isolates, which suggest that bacterial systems for iron acquisition, and those related to bacterial resistance to the host's defense mechanisms are fundamental virulence factors in these bacteria. On the other hand, the rest of the virulence genes provide valuable information for the development of vaccines against avian colibacillosis. It was also determined that a high percentage of APEC belongs to the phylogenetic group B1, from which mainly commensal and pathogenic *E. coli* strains derive.

Keywords: *E. coli*; Virulence genes; phylogenetic origin

Introduction

Avian pathogenic *Escherichia coli* (APEC) cause a respiratory disease in birds that is usually accompanied by complicating septicemia. It shares virulence attributes with strains of *E. coli* responsible for extraintestinal diseases in humans and it is considered the possible cause a zoonosis [1], hence it is important to analyze these shared attributes of pathogenicity.

The ability of APEC to cause disease depends on numerous pathogenic factors, such as: serogroups, adhesins, and iron acquisition systems, factors to evade the host's defenses, toxins, and invasins, amongst others [2]. Known APEC virulence factors include adhesins, such as type 1 fimbriae and P [2,3], as well as curli and Tsh, that have been related to the colonization of the respiratory tract [4]. Unlike *E. coli* of the intestinal biota APEC expresses enterobactins, aerobactins and yersiniabactins for the uptake and transport of iron [5,6]. Additionally, APEC synthesizes outer membrane proteins as *Iss, Trat* and *OmpA1* that allow it to evade host defenses, as well as another proteins that contribute to the formation of biofilms [7].

Besides, plasmids are important in the pathogenicity of APEC, among them pColV that carries virulence genes and has some operons with unknown function, as well as a great variety of mobile elements [8]. The virulence genes *iucA, tsh, iss, iroN* and *sitA, hlyF, ompT* and the operon *estABC* have been detected in several plasmids, including non-colicinogenic ones [6,9,10].

The phylogenetic origin of *E. coli* is determined based on the combination of three specific markers, the yjaA and chuA genes, as well as TspE.4C2, which have a dichotomous relationship that allows to distinguish the four phylogenetic groups A, B1, B2 and D [11]. Previously, it has been reported that the isolates that cause extraintestinal infections (ExPEC), including urinary infections and sepsis, are derived mainly from the B2 group and in a smaller proportion from the D group, while commensal and intestinal pathogenic *E. coli* (IPEC) are derived of groups A and B [12,13].

The objective of this work was to evaluate the presence of virulence genes in APEC isolates, as well as to determine the phylogenetic group to which they belong.

Material and Methods

Bacterial isolates

Twenty-three (23) APEC isolates were obtained from parenchymal organs of birds with colisepticemia. The isolates were further identified using biochemical testing.

Extraction of chromosomal DNA

The method described by Sambrook and Russell [14] was used to extract chromosomal DNA. Briefly, each isolate was seeded in LB broth and incubated at 37°C under stirring for 18 hours. After this time 1.5 mL of the culture were harvested and centrifuged at 8,000 xg for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 300 μL of lysis buffer (EDTA 0.05M, NaCl, 0.1M, pH=7.5) and incubated at 80°C in a water bath shaker. After a 5 minutes incubation period, 20 μL of 1% SDS were added to the mix and incubated at 37°C for 30 minutes. Then, 100 μL of 5 M potassium acetate were added and the mixture was centrifuged at 8,000 xg for 3 minutes. The aqueous phase was transferred to a tube and 300 μL of cold isopropanol were added, followed by centrifugation at 8,000 xg for 3 minutes. The supernatant was removed, the precipitate was washed with 300 μL of 70% ethanol and centrifuged at 8,000 xg for 3 minutes. The DNA was air dried for 15 minutes, resuspended in distilled water and stored at 20°C.

Extraction of plasmid DNA

The alkaline lysis method was followed to extract plasmid DNA. Each isolate was inoculated in 5 ml of LB broth and incubated for 18 hours under constant shaking at 37°C. The samples were centrifuged at 8,000 xg for 8 minutes, the supernatant was discarded and the cell pellet was resuspended in 200 μL of GTE (50 mM Glucose, 25 mM Tris-Cl pH 8, 10 mM EDTA), 400 μL of 0.2M NaOH/SDS 1% were added, mixed by five gentle inversions and incubated for 10 minutes. Subsequently, 300 μL of 5M potassium acetate were added, the mixes were stirred vigorously and incubated on ice for 20 minutes. Following a centrifugation at 8,000 xg for 20 minutes, the aqueous phase was transferred to a tube and two (2x) volumes of isopropanol were added. The samples were incubated 45 minutes, which was followed by a 15 minutes centrifugation step at 8,000 xg. The supernatants were removed and the DNA pellets were air dried for 20 minutes. The DNA was resuspended in distilled water and stored at 20°C [14].

Detection of virulence genes

The selection of the virulence genes to study was based in their specific roles the pathogenic process. The expression of these genes was detected by Polymerase Chain Reaction (PCR), using specific oligonucleotides. For *iroN, sitA, feoB, irp-2, iss, ompT* and *tsh* amplification was performed by incubation at 94°C for 4 minutes, followed by 30 cycles of: 94°C for 1 minute, 59°C for 1 minute and 72°C for 2 minute. For amplification of the *ibeA* and *vat*: incubation at 94°C for 4 minutes, 30 cycles of 94°C for 30 sec, 55°C for 30 seconds and 72°C for 1 minute. For *csgA* and *cvaC*: First incubation was at 95°C for 2 minutes, 30 cycles of 94°C for 1:30 minutes, 54°C for 1 minutes and 72°C for 1.5 minutes; while the *fimH* gene amplification conditions were: 95°C for 4 minutes, 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 2 minutes, in all cases there was a final extension of 72°C for 10 minutes.

Identification of phylogenetic groups

The method described by Clermont et al. [11] was used, which is based on the presence or absence of the combination of the genes *chuA, yjaA* and the DNA fragment *TspE4C2*, which were detected by PCR, using oligonucleotides and amplification conditions described by the same author.

Results

Virulence genes are encoded mostly in plasmids

Out of the 12 virulence genes that were analyzed, 75% were detected in plasmid DNA: *ompT, iss, tsh, vat, ironN, sitA, feoB, csgA* and *cvaC*. The other 25% were detected in the bacterial chromosome: *fimH, ibeA* and *irp2*.

Frequency of virulence genes in APEC isolates

The *irp-2, iroN*, and *sitA* genes, which are related to systems of iron uptake were amplified in more than 60% of the isolates, while the *feoB* was found a gene frequency of 95.6%, same for the gene related to mechanisms of resistance to serum: *iss* related. The genes of the adhesins type 1 fimbria and curli were detected in 73% of the isolates, and *tsh* in 39%. The frequency of *ompT*, which encodes the protease that acts against antimicrobial peptides, was 26%. With lesser frequency, *vat* was found in 13% and *ibeA*, which is related to biofilm formation, was present in 8% of the isolates. Finally, the *cvaC* gene indicative of presence of ColV plasmids was detected in 91%.

Genetic diversity in the APEC isolates

The type and number of virulence genes varied among APEC isolates: in YA15, eleven of the twelve genes studied were detected, in YA1 ten virulence genes, while YA16 and YA17 presented nine of them. Thirty (30%) of the isolates carried eight virulence genes, and 34.8% of the APEC isolates contained seven genes. Two isolates presented six virulence genes and one isolate, five. Finally, the isolate RS10 had only two virulence genes corresponding to 4.3%. It is important to note that 64.8% of the isolates contained between 7 and 8 of the virulence genes analyzed (Table 1).

Table 1: Genetic diversity of 23 APEC isolates in relation to virulence attributes

Strain	Irp2	iroN	sitA	feoB	fimH	tsh	csgA	ibeA	iss	vat	cvaC	ompT	Number of genes
YA15	+	+	+	+	+	-	+	+	+	+	+	+	11
YA1	+	+	-	+	+	+	+	-	+	+	+	+	10
YA16	+	+	+	+	+	+	+	-	+	-	+	-	9
YA17	+	+	+	+	+	+	+	-	+	-	+	-	9
YA6	-	+	+	+	+	+	+	-	+	-	+	-	8
YA8	-	+	-	+	+	+	+	-	+	-	+	+	8
YA10	+	+	+	+	-	-	+	-	+	-	+	+	8
YA11	+	-	+	+	+	-	+	-	+	-	+	+	8
YA13	+	+	+	+	+	-	+	-	+	-	+	-	8
RS14	+	+	+	+	+	-	+	-	+	-	+	-	8
RS17	+	+	-	+	+	-	+	-	+	-	+	+	8
YA4	-	+	+	+	-	+	-	-	+	+	+	-	7
YA5	+	-	+	+	-	+	+	-	+	-	+	-	7
YA9	-	+	+	+	+	-	+	-	+	-	+	-	7
YA12	-	+	+	+	+	-	+	-	+	-	+	-	7
YA18	-	+	+	+	-	+	-	-	+	-	+	+	7
YA21	+	+	+	+	+	-	-	+	+	-	-	-	7
YA23	+	+	+	+	+	+	-	-	+	-	-	-	7
RS16	+	+	+	+	+	-	-	-	+	-	+	-	7
YA2	-	-	-	+	+	-	+	-	+	-	+	+	6
RS11	-	+	+	+	-	-	+	-	+	-	+	-	6
YA14	-	-	-	+	+	-	+	-	+	-	+	-	5
RS10	+	-	-	-	-	-	-	-	-	-	+	-	2

Phylogenetic groups in the APEC isolates

About 8.6% of the isolates are from the phylogenetic group A, 34.7% belong to the B1, while 34.7% correspond to the B2 group and 21.7%, to group D. The range of virulence genes per phylogenetic group is shown in (Table 2).

Table 2: Phylogenetic group and virulence genes in 23 APEC isolates

Phylogenetic group	Number of isolate	Range of virulence genes	Average virulence genes
A	2	7 a 8	7.50%
B1	8	6 a 8	7.00%
B2	8	7 a 11	8.00%
D	5	2 a 8	6.25%

Discussion

The majority of the virulence genes were detected with a frequency higher than 50% and it was shown that the APEC isolates have high genetic variability regarding them. *fimH* was detected with a frequency of 73%, coinciding with the 76.6% observed by Ghanbarpour et al. [15]. The frequency of the fimbria curli was 73%, while Maurer et al. [16] found it in all of the strains studied and Knobl et al. [17] reported a frequency of 26%. Tsh has shown adhesion capacity to Caco-2 cells [18] and a prevalence between 10% to 90% was been reported for this gene [2,17,19,20]. In this work, it was detected in 39% of the strains, which coincides more closely with the 39.5% reported by Delicato et al. [21]. The discrepancy in the reported prevalence shows the heterogeneity of the APEC isolates.

It is important to highlight the high frequency found for the genes related to iron uptake, particularly *feoB*. This gene is part of the Feo system, which has the function of transporting ferrous iron and has been associated with virulence in *E. coli*, *Helicobacter pylori*, *Legionella pneumophila* and *Campylobacter jejuni* [22]. Iron uptake appears to be one of the main virulence factors in strains of APEC. Jeong et al. [23], attribute the presence of redundant iron uptake systems in APEC to the fact that these could function in different host's niches.

In relation to *iss*, whose function is to mediate bacterial resistance to serum's complement, Pfaff-McDonough et al. [24] highlighted its importance in *E. coli* isolated from sick birds (77%), compared to the 19% found in *E. coli* isolated from healthy birds. In this work, the frequency of *iss* was 95%, in agreement with that reported by Janben et al. [25] with 94%, and with 100% according to Kwon et al. [19]. This high frequency reveals the importance of *iss* function that allows *E.coli* to evade host defenses, multiply and disseminate, thus promoting the development of the disease.

In this work, *ibeA* it was detected in 8% of the isolates, while Rodríguez-Siek et al. [2] and Zhao et al. [26], reported a frequency of 14.7% and 17% respectively. The participation of *ibeA* in APEC is related to the formation of biofilms in vitro and to the capacity of invasion in APEC strains, although the exact mechanism by which *ibeA* contributes to the development of colibacillosis is unknown.

In this work the vat gene in APEC induces the formation of intracellular vacuoles with cytotoxic effects similar to those caused by the VacA toxin of *Helicobacter pylori*. The frequency of vat has been reported as 10%, 34% and 95% [19,20,23]. In this work, vat was detected in 13% of the isolates and it is located in pathogenicity island (PAI). It has been proposed that PAI acquisition is the main mechanism of the evolution of pathogens, under this pattern one could explain the genetic diversity of APEC.

The pathogenicity in APEC is multigenetic, the number of genes involved and the combinations between them is what probably increases the virulence, so it is important to analyze these combinations and define molecular pathotypes.

Mobile genetic elements such as PAIs and some plasmids contribute to bacterial virulence because they harbor specific genes. In this study, the ColV plasmid was detected in 91% of the isolates, in other studies it has been reported from 53 to 67% [2,26,27]. The combined detection of the *tsh, iss, iroN* and *cvaC* genes suggests the presence of *ColV* plasmids; in this study the iss, iroN and cvaC combination was 69.5%, while *iss, tsh* and *cvaC* decreased to 34.7%. This could be explained by the fact that *iss* and *iroN*, along with other genes, are located in the conserved region in APEC's pColV, while *tsh* is located in the variable region and is flanked by elements of IS1, suggesting its high mobility. The isolates YA21 and YA2 did not show *cvaC*, but they showed the *iss-iroN* or *tsh-iss* combinations, which were amplified from plasmid DNA, suggesting the presence of non-ColV virulence plasmids. The importance of the presence of virulence genes in plasmids lies in the development of new strains due to the transfer of mobile genetic elements.

Based on the phylogenetic classification of *E. coli*, it was expected that most of the APEC isolates belonged to the phylogenetic groups B2 and D, however it should be noted that 34.7% of the isolates are derived from group B1, in the same proportion as from group B2. In addition, the number of virulence genes was similar in both groups with an average of seven genes, which is not in agreement with that described for group B1, which is mainly formed by commensal strains. Zhao et al. [26], reported that 40% of APEC isolates analyzed belong to group A, which has also been classified as commensal strains and IPEC; likewise, Jeong et al. [23] found high percentages of APEC isolates within groups A and B. Under this, it is assumed that the classification of *E. coli* in phylogenetic groups per se, does not allow differentiating the commensal strains of the ExPEC strains and suggests that the phylogenetic origin of the APEC isolates is diverse.

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