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Clonal study of avian *Escherichia coli* strains by *fli*C conserved-DNA-sequence regions analysis[1]

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ABSTRACT.- Campos T.A., Nakazato G., Stehling E.G., Brocchi M. & Silveira W.D. 2008. **Clonal study of avian *Escherichia coli* strains by *fli*C conserved-DNA-sequence regions analysis.** *Pesquisa Veterinária Brasileira 28(10):508-514.* Departamento de Microbiologia e Imunologia, Instituto de Biologia, Cx. Postal 6109, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz s/n, Barão Geraldo, Campinas, SP 3081-862, Brazil. *Corresponding author: wds@unicamp.br

The clonal relationship among avian *Escherichia coli* strains and their genetic proximity with human pathogenic *E. coli*, *Salmonela enterica, Yersinia enterocolitica* and *Proteus mirabilis,* was determined by the DNA sequencing of the conserved 5' and 3'regions *fli*C gene (flagellin encoded gene). Among 30 commensal avian *E. coli* strains and 49 pathogenic avian *E. coli* strains (APEC), 24 commensal and 39 APEC strains harbored *fli*C gene with fragments size varying from 670bp to 1,900bp. The comparative analysis of these regions allowed the construction of a dendrogram of similarity possessing two main clusters: one compounded mainly by APEC strains and by H-antigens from human *E. coli*, and another one compounded by commensal avian *E. coli* strains, *S. enterica,* and by other H-antigens from human *E. coli.* Overall, this work demonstrated that *fli*C conserved regions may be associated with pathogenic clones of APEC strains, and also shows a great similarity among APEC and H-antigens of *E. coli* strains isolated from humans. These data, can add evidence that APEC strains can exhibit a zoonotic risk.

INDEX TERMS: APEC, clonal analysis, *fli*C gene.

RESUMO. - [**Estudo clonal de *Escherichia coli* aviário por análise de seqüências de DNA conservadas do gene *fli*C.**] A relação clonal entre linhagens de *Escherichia coli* de origem aviária e sua proximidade genética com *E. coli* patogênica para humanos, *Salmonella enterica, Yersinia enterocolitica* e *Proteus mirabilis* foi determinada através da utilização das seqüências conservadas 5' e 3' do gene *fli*C (responsável pela codificação da flagelina). Entre as 30 linhagens comensais de *E. coli* aviária e as 49 linhagens patogênicas de *E. coli* para aves (APEC), 24 linhagens comensais e 39 APEC apresentaram o gene *fli*C, que foi encontrado em tamanhos que variam de 670pb a 1900pb. Um dendrograma representando similaridade genética foi obtido a partir do seqüenciamento das regiões 5' e 3' conservadas do gene *fli*C das linhagens de *E. coli* de origem aviária, das seqüências dos antígenos H de *E. coli* de origem humana, de *S. enterica, Y. enterocolitica* e de *P. mirabilis.* A análise do dendrograma demonstrou que este apresenta dois grupos principais: um composto principalmente por isolados APEC e por antígenos H de *E. coli* de origem humana e outro formado por isolados comensais de *E. coli* aviária, *S. enterica* e por antígenos H de *E. coli*. No geral, o presente trabalho demonstrou que as regiões conservadas do gene *fli*C podem estar associadas à diferenciação clonal de linhagens de *E. coli* aviária, e que existe uma grande similaridade genética entre estas linhagens e antígenos H de *E. coli* humana. Estes dados podem adicionar evidências de que linhagens APEC podem apresentar riscos zoonóticos.

TERMOS DE INDEXAÇÃO: APEC, análise clonal, gene *fli*C.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) strains cause a variety of diseases in poultry, including respiratory tract

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infection, septicemia, omphalitis, swollen-head syndrome and enteritis, being responsible for significant economic losses in the poultry industry (Gross 1994). The pathogenesis and role of virulence factors present in these *E. coli* strains were not fully elucidated yet, although considerable efforts have been made in recent years to establish the mechanisms of pathogenesis (Dho-Moulin & Fairbrother 1999). Besides, clonal population studies based on REP and ERIC-PCR sequences, PFGE, and MLEE have demonstrated that APEC strains present a complex structure that could represent the reason for the absence of a clear definition of pathotypes in these strains (White et al. 1993, Ngeleka et al. 1996, Moura et al. 2001, Silveira et al. 2002, Brito et al. 2003, Ewers et al. 2004, Brochi et al. 2006).

The genes expressing proteins located on the surface of bacterial cells are candidates as potential biomarkers to assess intra-species genetic variation, because such proteins show a much greater rate of divergence in the amino acid sequence than those located internally. Among surface bacterial cell proteins, flagella proteins have been used as a target to population variation studies (Winstanley & Morgan 1997).

The flagellum is the cell structure responsible for motility in the majority of bacterial species. Flagellar activity is coupled to a sensory apparatus in a control system that allows movement of a cell towards attractive environments and away from repellent ones. The basic structure of bacterial flagella can be sub divided into three parts: (i) the basal body, which consists of a series of rings and a central rod, is anchored in the inner and outer membranes of the cell; (ii) the hook is located external to the cell and provides the link between the basal body and filament; and (iii) the flagellar filament, the largest portion of the flagella, consists of repeating sub-units of the protein flagellin in a helical arrangement and that often extends many times the length of the cell (Macnab 1992, Winstanley & Morgan 1997).

Flagellin proteins have a distinctive domain structure, comprising conserved N- and C-terminal regions, and a central domain that may vary considerably in both amino acid sequence and size (Macnab 1992, Winstanley & Morgan 1997). In *Escherichia coli*, flagellin proteins are encoded by the *fli*C gene that has conserved 5' and 3' regions and a high polymorphic central region (Kuwajima et al. 1986, Winstanley & Morgan 1997, Fields et al. 1997, Wang et al. 2003, Botelho et al. 2003, Amhaz et al. 2004, Tominaga 2004, Beutin et al. 2005, Moreno et al. 2006). Joys (1985) suggested that the accumulation of random mutations maintained by functional pressure absence is responsible by the polymorphism presented in the central region of *fli*C gene. However, Honda et al. (1999) demonstrated a primordial function for the central region of flagellin. They proposed the hairpin model, suggesting that flagellin monomers fold into a hairpin-like conformation, with the conserved N- and C- domains located prevalently on the inside and being responsible for defining the basic filament structure, whilst the central, variable domain is exposed on the surface. Reid et al. (1999) and Strauch & Beutin (2006) suggested that host immune selective pressure, recombination and lateral genetic transfer would be responsible for the polymorphism of the *fli*C central region and, consequently, by the flagellin polymorphism. The genetic variation of the *fli*C central region reflects the restriction fragment length polymorphism (RFLP) variability presented by *E. coli* strains belonging to the same H antigen. Such variability renders difficult the development of primers and molecular test to detect H antigens associated with pathogenic clones of *E. coli* (Fields et al. 1997, Wang et al. 2003, Tominaga 2004, Beutin et al. 2005, Moreno et al. 2006). The *fli*C gene has been used in bacterial systematic and population genetic studies because its variability reflects the selective procedures involved on the bacterial flagella antigen (H antigen) diversity. For phylogenetic trees and intra-specific population variation analysis the *fli*C sequences are divided in three regions: two corresponding to the 5' and 3' conserved regions and one corresponding to the central region (Winstanley & Morgan 1997).

The purpose of the present study was to establish the clonal relationship of commensal and pathogenic avian *E. coli* strains (APEC) by the DNA sequencing and comparison of the *fli*C conserved 5' and 3' regions. The *fli*C of the *E. coli* H-serotypes, of nine *Salmonella enterica* serovars and of the flagellin gene sequences of *Proteus mirabilis* and *Yersinia enterocolitica* were also used to assess the clonal population analysis of the commensal strains and APEC strains herein studied and to verify the genetic relationship of these avian *E. coli* strains with human *E. coli* and enterobacteria.

MATERIALS AND METHODS

Bacterial strains

Twenty-four septicemic (S), 14 swollen head syndrome (SHS), and 11 omphalitis (O) *Escherichia coli* strains isolated from different outbreaks, and 30 commensal strains (C) isolated from foals showing no signs of any of the above mentioned diseases and belonging to the Laboratory of Microbial Molecular Biology, DMI, UNICAMP, were studied in the present work. Strains from septicemic cases were isolated from liver, air sac and lung; swollen head syndrome strains were isolated from infra-orbitary sinuses and omphalitis strains were isolated from the yolk sacs of embryos chickens; commensal strains were collected from the cloacae region. All strains were kept at -80ºC in LB medium containing 15% glycerol final concentration.

Motility assay

All avian *E. coli* strains had their motility tested. Each strain was cultivated on MacConkey agar plates for 18 hours at 37ºC. One colony forming unit (CFU) of each strain was inoculated in LB medium added of 0.3% agar with a sterile needle and incubated for 18 hours at 37ºC. Motility was determined as the cloudy growth of each strain in LB (Sambrook & Russel 2001) medium containing 0.5% agar.

Genomic DNA extraction and detection of the *fli*C gene by PCR

Genomic DNA was extracted and purified as described previously (Ausubel et al. 1988). Extracted DNA was carefully harvested in sterilized deionized water and its integrity was determined by using 0.7% agarose gels in TE buffer as described by Sambrook & Russell (2001).

The detection of the *fli*C gene on each strain was accomplished with polymerase chain reactions (PCR) by using the primers *fli*C1 (5'- ATGGCACAAGTCATTAATACCCAAC-3') and *fli*C2 (5'- CTAACCCTGCAGCAGAGACA-3') described by Fields et al. (1997). *E. coli* K12 HB101 strain was used as a positive control for the detection of *fli*C. The PCR reactions were prepared to contain 20ng of DNA, 10pmol of each primer, 10mM of the four deoxynucleoside triphosphates (*Invitrogen)*, PCR buffer (*Invitrogen*), and 1 unit of Taq-polymerase high fidelity (*Invitrogen*). All amplification reactions were performed at a "Mastercyle" thermocycle (*Ependorff)*. PCR were performed in 35 cycles of amplification as follow: 1min at 95°C, 1min at 61ºC, and 2min at 72ºC. PCR products were analyzed by submersed gel agarose (1.0%) electrophoresis as described by Sambrook et al. (1989).

*fli*C gene sequencing

All *fli*C genes fragments amplified by PCR were purified by using the GFX purification kit (GE - Healthcare Amershan). After purification, 600ng of PCR products were submitted to the DNA sequencing reaction after adding 4µL of ET (Dye Terminator, GE, Healthcare Amershan), 10pmol of primer, and sterile deionized water to a 10µL final volume. The PCR-sequencing reactions, performed at the "Mastercyle" thermocycle (*Ependorff*) in 96 wells sequencing-plates, consisted of 30 cycles as follow: 20sec at 90ºC, 15sec at 50ºC, and 1.20min at 60ºC. Each *fli*C fragment was sequenced forward (primer: 5'-ATGGCACAAGTCATTAATACCCAAC-3') and reverse (primer: 5'- CTAACCCTGCAGCAGAGACA-3') at least three times.

Precipitation of the PCR-sequencing products

After PCR-sequencing, each product was precipitated as follow: 200µL of Ammonium acetate 7.5M was added on each well of the sequencing plate. The sequencing plate was mixed in vortex and spun at 900rpm. 55µL of ethanol 100% (room temperature) were added in each well, and the plate was mixed and maintained at room temperature with absence of light for 30 minutes. The plate was centrifuged for 60 minutes (4,000 rpm at 20°C), and the supernatant discarded. 100µL of ethanol 70% (4ºC) were added in each well and the plate was again submitted to centrifugation (4,000 rpm at 4ºC) during 10 minutes. The supernatant was discarded, and the plate maintained at 4ºC during 2 hours in the absence of light. 10µL of loading solution were added in each well, the plate was carefully mixed (900 rpm) and maintained at 4ºC light protected, during 3 hours. The sequencing samples were then processed on the MegaBace apparatus (GE Healthcare Bio-Sciences, Little Chalfont, UK) to sequence reading.

DNA-sequence analysis

The *fli*C sequences obtained were analyzed by the Bioedit software (Hall 1999). For each strain, it was obtained one *consensus* sequence resulting from the three sequencing reactions realized. All *concensus* sequences from avian *E. coli* strains, *fli*C sequences described for the *E. coli* H-antigen (Wang et al. 2003), *fli*C sequence described for *Salmonella enterica* serovars (Popoff et al. 1997), and flagellin DNA sequences described for *Yersinia enterocolitica* (Kapatral & Minnichi 1995) and *Proteus mirabilis* (Belas & Flaherty 1994) were aligned to obtaining the final dendrogram of similarity.

DNA-similarity analysis and dendrogram obtaining

The dendrogram was generated with the Mega 3.1 software (Kumar et al. 2004) by the UPGMA algorithm.

RESULTS

Forty-two avian *Escherichia coli* strains presented positive motility after 18 hours of growth into LB 0.3% agar at 37ºC. Among these strains, 15 were commensal, 11 were isolated from septicemic cases, 11 were isolated from swollen head syndrome cases (SHS), and 6 were omphalitis strains (Table 1).

The *fli*C gene was detected in 62 strains by the PCR assays (Table 1). Fragments varying from 670 bp to 1,900 bp were detected (Table 1 and Fig.1). Among *fli*C$^+$ strains, 24 were commensal, 16 were septicemic, 14 were SHS, and 9 were omphalitis strains.

Thirty-six mobile strains harbored the *fli*C gene, 26 *fli*C$^+$ strains showed to have negative motility and seven mobile strains were negative for *fli*C amplification.

Table 1. Motility and *fli*C detection by PCR among avian *Escherichia coli* strains

Strain	Motility	*fli*C (bp)	Strain	Motility	*fli*C (bp)
C1	+	1020	S11	-	-
C2	+	-	S12	-	-
C3	+	1740	S13	+	1240
C4	-	1415	S14	-	1240
C5	-	1515	S15	+	1240
C6	-	1740	S16	+	-
C7	+	1415	S17	-	-
C8	+	1515	S18	+	-
C9	-	1620	S19	-	1740
C10	+	1400	S20	-	-
C11	+	1300	S21	-	1740
C12	-	1750	S22	-	1740
C13	-	1750	S23	-	1325
C14	+	1500	S24	-	1740
C15	-	1500	SHS1	+	1435
C16	-	1750	SHS2	+	1435
C17	+	1500	SHS3	+	670
C18	-	1900	SHS4	+	670
C19	-	1900	SHS6	+	2025
C20	-	-	SHS7	+	670
C21	+	1500	SHS8	+	670
C22	+	-	SHS9	+	1225
C23	-	1750	SHS10	+	1225
C24	+	-	SHS11	-	1435
C25	+	1400	SHS12	+	2025
C26	-	1620	SHS13	-	2025
C27	+	-	SHS14	-	1435
C28	-	1400	SHS15	+	-
C29	+	1400	O1	-	-
C30	-	-	O2	-	1740
S1	+	1420	O3	-	1740
S2	+	1420	O4	+	1300
S3	+	1325	O5	+	1620
S4	-	1950	O6	+	1500
S5	+	1420	O7	+	1620
S6	+	1325	O8	-	1500
S7	-	-	O9	+	1620
S8	+	1325	O10	-	-
S9	+	1420	O11	-	1620
S10	-	-			

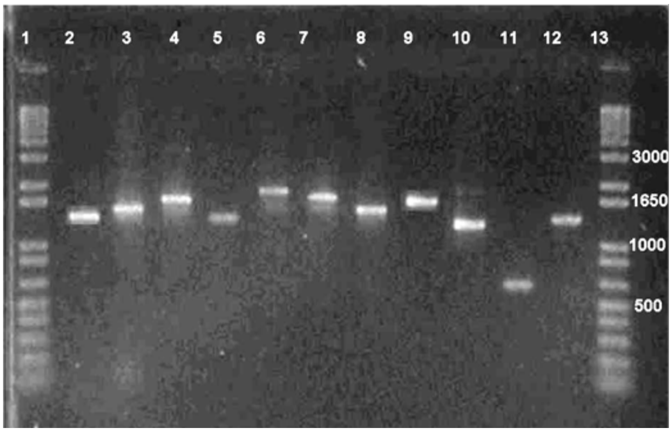

Fig.1. Agarose gel (1%) with the *fli*C PCR products from avian *Escherichia coli* strains. 1: 1Kb ladder (bp); 2: C11 strain; 3: C15 strain; 4: C26 strain; 5: S3 strain; 6: C19 strain; 7: C23 strain; 8: S1 strain; 9: S19 strain; 10: S14 strain; 11: SHS2 strain; 12: SHS8 strain; 13: 1Kb ladder (bp).

About 400 bp of the *fli*C 5'region and 500bp of the *fli*C 3' region were sequenced by the described primers. All obtained sequences were analyzed with the Bioedith (Hall 1999) and BLAST (http://www.ncbi.nlm.nih.gov/blast) to verify the homology of the *fli*C gene. After the homology confirmation, all sequences were aligned together with the *fli*C sequence of H-antigen from human *E. coli*, from 9 serovars of *S. enterica,* and with the flagellin sequences from *Y. enterocolitica* and *P. mirabilis.* The conserved sequences were used to obtain the dendrogram showed in Figure 2.

The dendrogram obtained presented four main clusters, named 1, 1A, 1A.1 and 1A.2 (Fig.2). Cluster 1 was compounded by four strains, two SHS (SHS1, SHS2), one commensal (C1), and one omphalitis (O6). Cluster 1.A possessed three commensal strains (C12, C21, C9), one SHS strain (SHS3), and the flagellin genes from *P. mirabilis* and *Y. enterocolitica.* Cluster 1.A.1 was compounded by 12 commensal (C14, C7, C15, C5, C28, C29, C25, C8, C17, C4, C10, C22) strains, two septicemic (S1, S5), one omphalitis strain (O8), by six H-antigens from human *E. coli* (H21, H11, H27, H16, H8, H2) and by the *fli*C from the nine *S. enterica* serovars. Finally, cluster 1.A.3 was compounded by 38 H-antigens from human *E. coli* and by 38 avian *E. coli* strains. Among these, 8 strains were commensal (C11, C18, C23, C13, C6, C26, C19, C3), 14 were originated from septicemic cases (S24, S19, S15, S23, S9, S8, S3, S22, S21, S13, S6, S14, S2, S4), 9 were from SHS cases (SHS6, SHS8, SHS14, SHS13, SHS7, SHS11, SHS12, SHS10 e SHS4), and 7 were from omphalitis cases (O5, O4, O11, O3, O9, O7, e O2) (Fig.2).

DISCUSSION

In recent years, several works demonstrated the presence of genetic similarities among APEC strains, human *Escherichia coli* and other *Enterobacteriaceae* species, which suggested that APEC strains present a zoonotic risk (Stocki et al. 2002, Johnson et al. 2003, Mokady et al. 2005, Rodriguez-Siek et al. 2005). The aim of the present study was to verify if there is a genetic similarity, as determined by the DNA sequencing and comparison of the *fli*C gene among avian pathogenic *E. coli* and other strains of human bacterial pathogens such as *E. coli*, *Salmonella enterica, Yersinia enterocolitica,* and *Proteus mirabilis*. At the same time, the DNA sequencing and comparison of the *fli*C gene allowed us to insert the different APEC strains into clusters of similarity.

The PCR-amplified DNA-fragments of the different avian *E. coli* strains presented variable molecular weights (Table 1 and Fig.1). These data are in agreement with previous studies that demonstrated the high genetic polymorphism of *fli*C gene from *E. coli.* This polymorphism was attributed to the duplication of DNA sequences, genetic recombination and the presence of insertion elements in the central region of the *fli*C gene. All together, these genetic events would be responsible by the H-antigen variability (Fields et al. 1997, Reid et al. 1999, Tominaga 2004, Beutin et al. 2005, Strauch & Beutin 2006). However, the fragment variability observed in our study was different from that observed by Moreno et al. (2006), where *fli*C fragments varying from 1,300 bp to 1,980 bp, among immobile *E. coli* strains, and from 1,190 to 4,170 among mobile *E. coli* strains were detected. Among the avian *E. coli* strains analyzed in the present work, *fli*C presented fragments varying from 670 bp to 1,900 bp (Table 1).

Although bacterial motility is considered as an indicative for the *fli*C gene presence, several *fli*C^{+} bacterial strains did not present positive motility, and seven mobile strains did not have the *fli*C fragment amplified (Table 1). The absence of motility among *fli*C^{+} strains may be attributed to the *fli*C non expression or to the non expression of other genes needed to bacterial motility or even the inexistence of these genes. Bacterial motility is a result from the expression of about 40 genes organized as a regulon (Macnab 1992). By the other hand, mobile strains with *fli*C^{-} genotype would suggest that *fli*C gene, at least for these strains, is not responsible by the expression of the flagellar filament or is the result of sequence variations in the primers annealing regions. We believe that the former hypothesis is more reliable since the sequences choose for the primers annealing are very much conserved. In addition, studies realized by Raitner (1998) and by Tominaga (2004) demonstrated that genes *flm*A and *flk*A are responsible by the flagellin expression in *E. coli* strains belonging to serogroups H53 and H54.

As it was previously observed by other research groups (Kuwajima et al. 1986, Fields et al. 1997, Reid et al. 1999, Botelho et al. 2003, Wang et al. 2003, Amhaz et al. 2004, Beutin et al. 2005, Moreno et al. 2006, Strauch & Beutin 2006), our results also demonstrate, as determined by DNA-sequencing, that the *fli*C gene from avian *E. coli* strains presented conserved extremities and central regions with high polymorphism and as proposed by

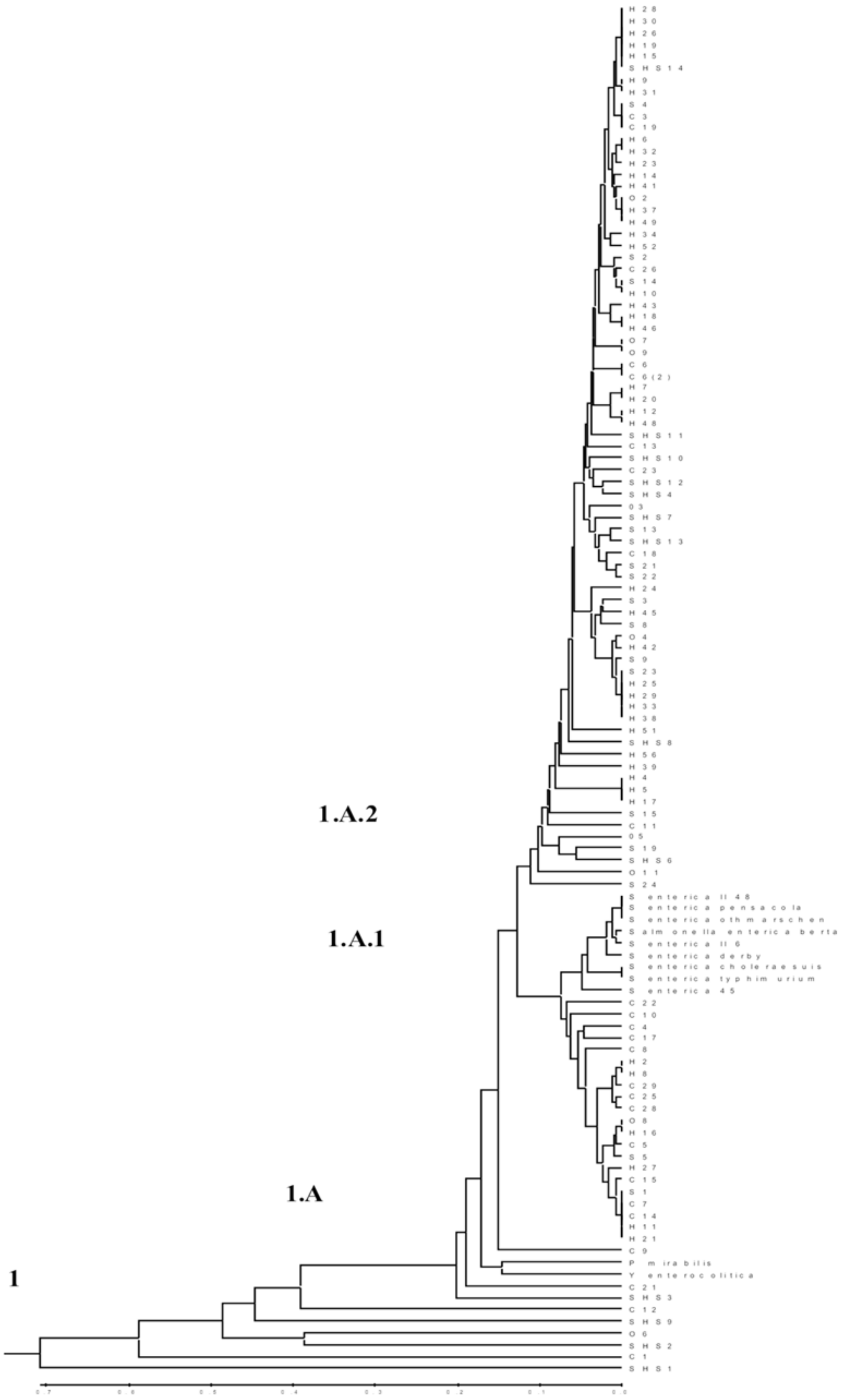

Fig.2. Dendrogram generated by 5' and 3' *fli*C gene regions sequencing of avian *Escherichia coli* strains, of *fli*C gene from H-antigen from human *E. coli,* of *fli*C from *Salmonella enterica* serovars, from flagellin gene from *Proteus mirabilis* and from *Yersinia enterocolitica,* by using UPGMA algorithm (MEGA 3.1 software).

Winstanley & Morgan (1997) and Wang et al. (2003), we used the conserved *fli*C regions (5' and 3') to establish possible phylogenetic proximity among avian *E. coli* strains, *E. coli* H-antigen, *S. enterica, Y. enterocolitica,* and *P. mirabilis.*

The dendrogram obtained by the comparison of these regions (Fig.2) demonstrated two distinct main groups: one (1.A.2) compounded, in its majority, by APEC strains (82%) and other (1.A.1) compounded mainly by commensal avian *E. coli* strains (Fig.2). Thirty-eight (38) human H- *E. coli* (Wang et al., 2003) were also grouped in cluster 1.A.2. The genetic proximity of the *fli*C genes among APEC and human *E. coli* strains may suggests that the flagellar filament of these bacterial have a similar ancestor origin with the occurrence of divergence in the central region of *fli*C gene probably collaborating to differentiate the flagellar filament of these groups of bacteria. In this way, the genetic proximity of *fli*C conserved regions among APEC and human *E. coli* strains could indicated genetic similarity among these strains and may suggest a possible zoonotic risk to be present on APEC strains.

Clusters 1.A and 1.A.1 were compounded by 35 different strains. From these 14 were commensal avian *E. coli* strains, 6 were human *E. coli* H-antigen, 9 were different *S. enterica* serovars, and three were APEC strains (one omphalitis, one septicemia and one swollen head syndrome) (Fig.2). All strains from cluster 1.A.1 presented 90% similarity. These data suggest that flagellar antigen from commensal avian *E coli* presents similarity with *S. enterica* flagellar antigen, which, again, may indicate a similar genetic origin of commensal avian *E. coli* and *S. enterica.* These data are also corroborated by those published by Wang et al. (2003) who proposed, based on the same approach, to exist a common ancestor *fli*C gene for *E. coli* of human origin and *S. enterica*. Inside the same line of thought, genetics events probably occurred to differentiate among the APEC strains and *E. coli* H-antigen strains that were grouped in the cluster 1.A.2 (Fig.2). In this late case, the proximity among these strains supports the status of zoonotic bacteria to APEC, a hypothesis that is reinforced by the sharing of pathogenic traits among avian and human *E. coli.*

In conclusion, this work demonstrated that there is a genetic proximity among APEC and human *E. coli* strains, as assessed by the DNA sequencing and comparison of the *fli*C conserved regions of these strains. These data add evidences that APEC strains exhibits a zoonotic risk.

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