Serogroups and virulence genes of *Escherichia coli* isolated from psittacine birds[1]

Terezinha Knöbl[2,3*], André B.S. Saidenberg[2,4], Andrea M. Moreno[4], Tânia A.T. Gomes[5], Mônica A.M. Vieira [5], Domingos S. Leite[6], Jesus E. Blanco[7] and Antônio J.P. Ferreira[4]

ABSTRACT.- Knöbl T., Saidenberg A.B.S., Moreno A.M., Gomes T.A.T., Vieira M.A.M., Leite D.S., Blanco J.E. & Ferreira A.J.P. 2011. **Serogroups and virulence genes of *Escherichia coli* isolated from psittacine birds**. *Pesquisa Veterinária Brasileira 31(10):916-921*. Faculdade de Medicina Veterinária do Complexo Educacional FMU, Av. Santo Amaro 1239, São Paulo, SP 04505 002, Brazil. E-mail: tknobl@usp.br

Escherichia coli isolates from 24 sick psittacine birds were serogrouped and investigated for the presence of genes encoding the following virulence factors: attaching and effacing (*eae*), enteropathogenic *E. coli* EAF plasmid (EAF), pili associated with pyelonephritis (*pap*), S fimbriae (*sfa*), afimbrial adhesin (*afa*), capsule K1 (*neu*), curli (*crl, csg*A), temperature-sensitive hemagglutinin (*tsh*), enteroaggregative heat-stable enterotoxin-1 (*ast*A), heat-stable enterotoxin - 1 heat labile (LT) and heat stable (STa and STb) enterotoxins, Shiga-like toxins (*stx*1 *and stx*2), cytotoxic necrotizing factor 1 (*cnf*1), haemolysin (*hly*), aerobactin production (*iuc*) and serum resistance (*iss*). The results showed that the isolates belonged to 12 serogroups: O7; O15; O21; O23; O54; O64; O76; O84; O88; O128; O152 and O166. The virulence genes found were: *crl* in all isolates, *pap* in 10 isolates, *iss* in seven isolates, *csg*A in five isolates, *iuc* and *tsh* in three isolates and *eae* in two isolates. The combination of virulence genes revealed 11 different genotypic patterns. All strains were negative for genes encoding for EAF, EAEC, K1, *sfa, afa, hly, cnf,* LT, STa, STb*, stx*1 and *stx*2*.* Our findings showed that some *E. coli* isolated from psittacine birds present the same virulence factors as avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC) and Enteropathogenic *E. coli* (EPEC) pathotypes.

INDEX TERMS: Psittacine birds, *Escherichia coli,* Virulence factors, Septicemia.

RESUMO.- [**Sorogrupos e genes de virulência em *Escherichia coli* isoladas de psitacídeos.**] Amostras de *Escherichia coli* isoladas de 24 psitacídeos doentes foram sorogrupadas e investigadas para a presença de genes que codificam os seguintes fatores de virulência: *attaching e effacing* (*eae*), plasmídeo EAF (EAF), pili associado à pielonefrite (*pap*), fímbria S (*sfa*), adesina afimbrial (*afa*), cápsula K1 (*neu*), curli (*crl, csg*A), hemaglutinina termosensível (*tsh*), enterotoxina termo-estável 1 de *E. coli* enteroagregativa (*ast*A), toxina termolábil (LT) e toxina termoestável (STa e STb), Shiga-like toxinas (*stx*1 e *stx*2), fator citotóxico necrotizante 1 (*cnf*1), hemolisina (*hly*), produção de aerobactina (*iuc*) e resistência sérica (*iss*). Os resultados mostraram que os isolados pertenciam a 12 sorogrupos: O7; O15; O21; O23; O54; O64; O76; O84; O88; O128; O152 e O166. Os genes de virulência encontrados foram: *crl* em todos os isolados, *pap* em 10 isolados, *iss* em sete isolados, *csg*A em cinco isolados, *iuc* e *tsh* em três isolados e *eae* em dois isolados. A combinação dos genes de virulência revelou 11 perfis genotípicos distintos. Todas as amostras foram negativas para os genes que codificam EAF, EAEC, K1, *sfa, afa, hly, cnf,* LT, STa, STb*, stx*1 e *stx*2*.* Estes resultados demonstraram que algumas amostras de *E. coli* isoladas de psitacídeos apresentam os mesmos fatores de virulência presentes nos patotipos de *E. coli* patogênicas para aves (APEC), uropatogênicas (UPEC) e *E. coli* enteropatogênicas (EPEC).

[1] Received on April 30, 2011.

Accepted for publication on August 2, 2011.

[2] Laboratório de Epidemiologia e Doenças Infecciosas das Faculdades Metropolitanas Unidas (FMU), Av. Santo Amaro 1239, São Paulo, SP 04505-002, Brazil. * Corresponding author: tknobl@usp.br

[3] Faculdade de Medicina Veterinária do Grande ABC (Uniabc), Avenida Industrial 3330, Santo André, SP 09080-511.

[4] Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (USP). Av. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270.

[5] Escola Paulista de Medicina, Universidade Federal de São Paulo (Unifesp), Rua Botucatu 740, Vila Clementino, São Paulo, SP 04023-062.

[6] Instituto de Biologia, Universidade Estadual de Campinas (Unicamp), Cidade Universitária Zeferino Vaz, Distrito de Barão Geraldo, Campinas, SP 13081-970, Brazil.

[7] Laboratorio de Referencia de *E. coli* (LREC), Departamento de Microbiologia e Parasitología, Facultad de Veterinaria, Universidade de Santiago de Compostela (USC), Av. Prof. Carballo Calero s/n, 27002 Lugo, Spain.

TERMOS DE INDEXAÇÃO: Psitacídeos, *Escherichia coli,* fatores de virulência, septicemia.

INTRODUCTION

The normal flora of psittacine birds is composed mostly or exclusively of Gram-positive bacteria and includes bacteria of the genera *Lactobacillus*, *Bacillus*, *Corynebacterium*, *Gaffkya* and non-hemolytic strains of *Staphylococcus* spp. and *Streptococcus* spp. For this reason, many authors have suggested that all Gram-negative bacteria are abnormal inhabitants of the psittacine gut and should be considered as pathogens (Hoefer 1997).

There is considerable controversy over the significance of isolating Gram-negative bacteria, in particular *Escherichia coli*, from the cloacae or feces of psittacine birds (Marietto-Gonçalves et al. 2010). The prevalence rates of *E. coli* in feces or cloacal swabs of healthy birds vary among the various psittacine species. For amazon parrots, the recovery rate of *E. coli* was 13.6% according to Graham & Graham (1978), and 18% according to Flammer & Drewes (1988). However, the highest incidence of *E. coli* has been observed in samples collected from sick birds or intestinal tissue at necropsy (Dorrestein et al. 1985).

There is no easy way to distinguish between potentially pathogenic and nonpathogenic *E. coli* isolates. A study on the virulence traits could help in elucidating the clinical importance of infection for psittacine birds, and to determine the genetic similarity between strains isolated from commercial and wild birds (Ron 2006).

Avian pathogenic *E. coli* (APEC) causes several diseases in poultry such as airsacculitis, septicemia, omphalitis, salpingitis, cellulitis, swollen head syndrome and colisepticemia (Monroy et al. 2005). Virulence factors associated with APEC isolated from chickens, turkeys and ostriches include colonization factors (fimbrial and afimbrial adhesins), invasive factors, serum resistance mechanisms, iron acquisition systems, antiphagocytic activity and production of toxins (Knöbl et al. 2001, Monroy et al. 2005, Nakazato et al. 2009). Well-recognized virulence properties include Type 1 (F1) and P fimbriae, IbeA proteases and aerobactin production, Iss for serum survival, K and O antigens for anti-phagocyitc activity, and a temperature-sensitive haemagglutinin of unknown function. These factors do not occur widespread among APEC, suggesting the presence of alternative mechanisms mediating pathogenicity (Dziva & Stevens 2008).

APEC strains are a subset of extraintestinal pathogenic *E. coli* (ExPEC), a pathogenic category associated with invasive infections in mammals (animals and humans), which also includes uropathogenic *E. coli* (UPEC) and newborn meningitis *E. coli* (NMEC). APEC and UPEC pathotypes presents share virulence associated traits, and have overlapping O serogroups and phylogenetic types (Nakazato et al. 2009). Some of the genes that were found to be widely distributed among both UPEC and APEC have been localized to large transmissible plasmids and pathogenicity islands (PAIs). This similarity supports the hypothesis that birds may act as a reservoir for potentially zoonotic *E. coli* strains (Ewers et al. 2007, Johnson et al. 2007).

The epidemiological link between human and animal disease are well established in some instances but remain unclear in others (Johnson et al. 2007). Recent isolation reports of diarrheagenic *Escherichia coli* in wild birds reinforce the idea of bacterial transmission from humans to birds (Saidenberg 2008, Knöbl & Menão 2010). There are six patotypes of diarrheagenic *Escherichia coli*, based on the genetic background and virulence mechanism: enterotoxigenic *E. coli* (ETEC); enteropathogenic *E. coli* (EPEC); enterohemorrhagic *E. coli* (EHEC); enteroinvasive *E. coli* (EIEC); enteroaggregative *E. coli* (EAggEC) and diffuse adherent *E. coli* (DAEC) (Vidal et al. 2005). The pathotypes ETEC and EPEC have been identified in birds with enteritis (Parreira & Yano 1998, Saidenberg 2008, Knöbl & Menão 2010).

Enterotoxigenic *Escherichia coli* (ETEC) are one of the most prevalent causes of osmotic diarrhea in mammals. ETEC adhere to the small intestinal microvilli and produce one or more of heat labile (LT), heat-stable (STa and STb) and enteroaggregative heat-stable (EAST-1) enterotoxins.

Enteropathogenic *E. coli* (EPEC) are characterized by presence of the large EPEC adherence factor (EAF) plasmid, the cluster of genes encoding bundle-forming pili (*bfp*) and genes that encodes proteins that promote intimate cells adherence leading the attaching and effacement lesions (Vidal et al. 2005).

There are a limited number of publications about the presence of virulence factors in psittacine birds in comparing with commercial avian (Knöbl & Menão 2010). The mere detection of virulence genes is not sufficient to establish a causal relationship, because several factors (management, concomitant infections, contact with humans and others mammals) may alter the course of disease in these birds (Mattes et al. 2005). However, the virulence factors could be a first step in elucidating the pathogenesis of colibacillosis in wild birds. The purpose of this survey was to investigate the serogroups and virulence factors present in *E. coli* isolated from sick psittacine birds.

MATERIALS AND METHODS

The study was conducted with *Escherichia coli* isolated from 24 psittacine birds with enteritis and septicemia, submitted to the Veterinary Medical Teaching Hospital of Faculdades Metropolitanas Unidas (FMU), São Paulo, Brazil, during three consecutive years from 2005 to 2008. Standard bacteriological methods were employed for *E. coli* isolation and identification (Bangert et al. 1988). Ten isolates were obtained from fresh fecal samples, collected by cloacal swabs. Fourteen isolates were obtained from heart or liver of dead birds, immediately collected at necropsy (Table 2).

All isolates were stored at -70ºC in brain heart infusion broth (BHI) (Difco/BBL, Detroit, MI, USA) to which 15% glycerol was added after incubation.

Serogroups were identified by the method described by Guinée et al. (1981) with all available O antisera (O1 to O185). Antisera were obtained and absorbed with corresponding cross-reaction antigens to remove nonspecific agglutinins. O antisera were produced in the Laboratorio de Referencia in *E. coli* (LREC), Universidad de Santiago de Compostela, Lugo, Spain <http://www.lugo.usc.es/ecoli>.

Production of aerobactin was assayed by growing strains in LB medium containing 200 µM of a-a-dipyridyl at 37ºC for 24 h (Monroy et al., 2005). The growth was spun for 3 min (12,000g), the supernatants were filtered through a nitrocellulose membrane (0.22µm) and 50 µL were added to orifices made in LB medium, previously seeded with LG1522 strain. The strains were incubated at 37ºC for 48 h and the production of aerobactin was visualized by the growth of strain LG1522 around the orifices.

The *E. coli* isolates were tested by colony blot hybridization as

Table 1. The primers used for detection of the various genes by PCR, amplicon size, and references

Gene	Oligonucleotide primer pairs (5'-3')	Amplicon (bp)	Reference
*pap*EF	GCAACAGCAACGCTG GTTGCATCATAGAGAGAGCCACTCTTATACGGACA	336	Yamamoto et al. 1995
sfa	CTCCGGAGAACTGGGTGCATCTTACCGGAGGAGTAATTACAAACCTGGCA	410	Yamamoto et al. 1995
*afa*BC	GCTGGGCAGCAAACTGATAACCTCCATCAAGCTGTTTGTTCGTCCGCCG	750	Yamamoto et al. 1995
*hly*A	AACAAGGATAAGCACTGTTCTGGCTACCATATAAGCGGTCATTCCCGTCA	1177	Yamamoto et al. 1995
*iuc*D	TACCGGATTGTCATATGCAGACCGTAATATCTTCCTCCAGTCCGGAGAAG	602	Yamamoto et al. 1995
*cnf*1	AAGATGGAGTTTCCTATGCAGGAGCATTCAGAGTCCTGCCCTCATTATT	498	Yamamoto et al. 1995
*elt*A(LT)	GGCGACAGATTATACCGTGCCCGAATTCTGTTATATATGTC	696	Schultsz et al. 1994
sta	TTAATAGCACCCGGTACAAGCAGGCTTGACTCTTCAAAAGAGAAAATTAC	147	Olsivik et al. 1993
stb	ATCGCATTTCTTCTTGCATCGGGCGCCAAAGCATGCTCC	172	Blanco et al. 1997b
astA(EAST)	ATGCCATCAACACAGTATAT GCGAGTGACGGCTTTGTAGT	110	Vila et al. 2000
*stx*1	GAAGAGTCCGTGGGATTACGAGCGATGCAGCTATTAATAA	130	Pollard et al. 1990
*stx*2	CCGTCAGGACTGTCTGAAACGAGTCTGACAGGCAACTGTC	726	Woodward et al. 1992
crl	TTTCGATTGTCTGGCTGTATGCTTCAGATTCAGCGTCGTC	250	Maurer et al. 1998
*csg*A	ACTCTGACTTGACTATTACCAGATGCAGTCTGGTCAAC	200	Maurer et al. 1998
tsh	GGGAAATGACCTGAATGCTGGCCGCTCATCAGTCAGTACCAC	420	Maurer et al. 1998
iss	GTGGCGAAAACTAGTAAAACAGCCGCCTCGGGGTGGATAA	760	Horne et al. 2000
neuS(kps)	TATAATTAGTAACCTGGGGCGGCGCTATTGAATAAGACTG	927	Tsukamoto 1997

Table 2. Serogroup and virulence properties of *Escherichia coli* isolated from psittacine birds

E. coli strains	Avian specie	sample	serogroup	Virulence factors eaeA	pap	crl	csgA	iuc	iss	tsh
01	*Amazona aestiva*	feces	ONT	-	-	+	+	-	+	-
02	*Amazona aestiva*	feces	ONT	-	-	+	+	-	+	-
03	*Guarouba guarouba*	feces	O88	-	-	+	+	-	+	-
04	*Guarouba guarouba*	feces	ONT	-	-	+	-	-	-	-
05	*Amazona amazonica*	feces	O21	-	-	+	-	-	-	-
06	*Amazona aestiva*	feces	ONT	-	-	+	-	-	-	-
07	*Amazona amazonica*	feces	ONT	-	+	+	+	-	+	-
08	*Amazona aestiva*	feces	O84	-	+	+	-	-	-	-
09	*Amazona aestiva*	feces	O84	-	+	+	-	-	-	-
10	*Amazona aestiva*	feces	O84	-	+	+	-	-	-	-
11	*Amazona amazonica*	feces	O7	-	-	+	+	-	-	-
12	*Amazona aestiva*	Liver	ONT	-	+	+	-	-	+	-
13	*Amazona aestiva*	Liver	O152	-	-	+	-	-	-	-
14	*Amazona aestiva*	Heart	O64	-	+	+	-	-	+	-
15	*Amazona aestiva*	Heart	O23	-	-	+	-	+	+	+
16	*Amazona amazonica*	Liver	O128	+	-	+	-	-	-	-
17	*Amazona aestiva*	Liver	O76	+	-	+	-	+	-	-
18	*Amazona aestiva*	Liver	O54	-	+	+	-	+	-	+
19	*Amazona amazonica*	Heart	O152	-	-	+	-	-	-	-
20	*Aratinga aurea*	Liver	ONT	-	+	+	-	-	-	-
21	*Ara ararauna*	Heart	O15	-	+	+	-	-	-	-
22	*Ara ararauna*	Liver	O15	-	-	+	-	-	-	-
23	*Ara chloroptera*	Heart	ONT	-	+	+	-	-	-	-
24	*Melopsittacus undulatus*	Liver	O166	-	-	+	-	-	-	+
Total of positives				2	10	24	5	3	7	3

*All isolates were negative for genes encoding for EAF, *neu*S (*kps*), *sfa, afa, hly, cnf,* LT, STa, STb, *ast*A, *stx*1 and *stx*2. ONT = O not typeable.

described previously by Maas (1983) using specific cloned probes for: AA (aggregative adherence) (Baudry et al. 1990); *eae*A (*E. coli* attaching and effacing) (Jerse et al. 1990) and EAF (EPEC adherence factor) (Baldini et al. 1983). Specific DNA sequences were labeled with [a-d-^{32}P]-dCTP using the Ready-To-Go™ DNA Labelling Beads (GE Healthcare, São Paulo, SP, Brazil).

The prototype wild-type strains from which DNA probes are derived were used as positive controls of each probe. *E. coli* K12 C600/pBR322 was used as negative control in all hybridization tests.

The primer sequences were used to detect genes encoding pili associated with pyelonephritis (*pap*), haemolysin (*hly*), aerobactin (*iuc*), cytotoxic necrotizing factor 1 (*cnf*1), S fimbriae (*sfa*), afimbrial adhesin I (*afa*I), heat labile (LT) and heat stable (STa and STb) enterotoxins, Shiga-like toxins (*stx*1 and *stx*2), temperature-regulated adhesin, curli (*crl, csga*) and temperature-sensitive hemagglutinin (*tsh*), increased serum survival (*iss*) and K1 capsule (*neu*). Amplicon sizes and the relevant literature are given in Table 1.

The DNA extraction was performed as described by Boom et al (1990). The standard PCR amplification mixture consisted of 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200mM each of the four deoxynucleoside triphosphates, sets of primers, and 0.5 U of Taq DNA polymerase in a final volume of 25ml. Amplified products were separated in 1.5% agarose gel and examined after ethidium bromide staining. A 100-bp DNA ladder was used as a molecular size marker.

RESULTS

As shown in Table 2, twelve different serogroups were detected: O7; O15; O21, O23; O54; O64; O76; O84; O88; O128; O152 and O166. Eight isolates did not react with the antisera used.

Table 3. Genotypic profiles of psittacine *Escherichia coli* strains

Profiles	Combination of genes	Nº of trains	Sample
G1	*crl+*	6	Feces/Liver/Heart
G2	*crl+ csg*A+	1	Feces
G3	*crl+ pap+*	6	Feces/Liver
G4	*crl+ tsh+*	1	Liver
G5	*crl+ eae*A+	1	Liver
G6	*crl+ csg*A+ *iss+*	3	Feces
G7	*crl+ pap+ iss+*	2	Liver/Heart
G8	*crl+ eae*A+ *iuc+*	1	Liver
G9	*crl+ iuc+ iss+ tsh+*	1	Heart
G10	*crl+ pap+ iuc+ tsh+*	1	Liver
G11	*crl+ csg*A+ *pap+ iss+*	1	Feces
Total		24	

The presence of *pap* genes was detected in 10 isolates in the serogroups O84, O64, O54 and O15. Four *pap+* strains were not serotypable (Table 2). All isolates were positive for curli (*crl*), although only five contained the *csg*A gene.

Three isolates presented the gene encoding for aerobactin siderophore (*iuc*), although growth on the iron-deficient medium was observed for all isolates in biological assay. The increased serum survival gene (*iss*) was present in seven isolates and the temperature-sensitive hemagglutinin (*tsh*) gene was detectable in three isolates.

Two isolates were positive for the *eae* gene. These isolates belonged to serogroups O128 and O76, and were isolated from liver of psittacine birds, at necropsy.

The strains were grouped into 11 distinct genotypic patterns; according to the virulence factors presence (Table 3).

All *E. coli* isolates were negative when tested for the presence of genes encoding for EAF, EAST, LT, STa, STb, Stx1, Stx2, Hly, CNF1, S fimbriae, afimbrial adhesion I and K1 capsule.

DISCUSSION

It is very difficult to differentiate potentially pathogenic from nonpathogenic strains, because *Escherichia coli* are frequently secondary invaders in birds, associated with stress, malnutrition, poor hygiene and hypovitaminosis A (Mattes et al. 2005, Marietto-Gonçalves et al. 2007). The potentially pathogenic *E. coli* strains can be screened by different tests, like phenotypic assays as Congo red binding (Styles & Flammer 1991), serotyping (Schremmer et al. 1999), and genotypic assays (Pakpinyo et al. 2002, Knöbl et al. 2008, Nakazato et al. 2009).

Several surveys have revealed that many avian septicemic *E. coli* belong to a limited number O serogroups (O1, O2, O18, O35 and O78) (Menão et al. 2002, Dziva & Stevens 2008). However, some studies showed that a wide antigenic diversity exists among isolates from avian colibacillosis (Blanco et al. 1998). Furthermore, the involvement of a particular O serogroup in disease appears to vary with geographic location. The present study revealed that *E. coli* isolated from psittacine was classified in 12 distinct serogroups: O7, O15, O21, O23, O54, O64, O76, O84, O88, O128, O152 and O166.

The serogroup O15:H8 was reported as an important agent of diarrhea in ostriches, due to type 2 heat-labile enterotoxin (LT II) production (Nardi et al. 2005). There are only a few reports demonstrating that *E. coli* strains isolated from avian can produce toxins (Tsuji et al. 1990, Blanco et al. 1997a, Parreira et al. 1998, Salvatori et al. 2001, Parreira & Gyles, 2003). None of the isolates analyzed here possessed the genes that encoded toxins LT, STa, STb, Stx1, Stx2, Hly or CNF.

Schremmer et al. (1999) examined *E. coli* isolated from psittaciformes and emphasized the presence of seven strains belonged serovars O63:H10, O110:H6, O131:H-, O153:H10 and ONT:H6, that were positive for the *eae* gene, four of which were also positive for the *bfp*A gene. The authors concluded that EPEC should be considered as potential pathogens in psittaciform birds, and may be a reservoir of human EPEC infections. Likewise, in our study two isolates of serogroups O128 and O76 were positive for *eae*A gene, and thus were considered atypical enteropathogenic *E. coli* (aEPEC), a diarrheagenic pathotype characterized by the absence of the *stx* gene and EAF region.

Table 3 shows 11 distinct patterns with a predominance of genes associated with systemic infections (*pap, iss, tsh* and *iuc*). Among the various genotypes observed, only *tsh* and *iuc* genes were restricted to isolated of organs. The others were also found in stool samples. One should be cautious in interpreting these results, because the clinical manifestations of colibacillosis in psittacine birds are hyperacute and cannot be ruled out sepsis in birds with diarrhea. Clinical studies with a larger number of birds are needed to understanding the infection in wild birds.

Among the virulence factors of extraintestinal strains, stands out the presence of the *pap* gene that encoded P fimbriae, and was found in 11 (45.83%) of 24 isolates (Table 2). P pili are a mannose resistant adhesin, associated with human urinary tract infections (cystitis and pyelonephritis) (Blanco et al. 1997c).

The prevalence of P fimbriae in human uropathogenic *E. coli* (UPEC) is high. Among avian pathogenic *E. coli* (APEC) the prevalence of P fimbriae varied between 18 to 30% (Janben et al. 2001, Delicato et al. 2003). The role of P fimbriae in the pathogenesis of avian colibacillosis is still controversial, but the expression of P fimbriae has been associated with colonization of internal organs (Pourbakhsh et al. 1997).

Curli expression promotes bacterial adherence to the laminin and fibronectin; plasminogen activation; and chicken erythrocyte agglutination, but the role of curli in bacterial adherence is polemic, although deletions in the *crl* genes do not inhibit hemagglutination (Provence & Curtis 1992). In this study the structural gene *crl* was present in all isolates (100%), but *csg*A was detected only in five (20.83%). The *csg*A gene is essential for the expression of the major subunit protein of the fibre (CsgA subunit protein).

Another adhesin also detected in APEC, is the tsh protein (temperature sensitive hemagglutinin). This protein possesses a high homology with IgA proteases from *Neisseria gonorrhoeae* and *Haemophilus influenzae* and has been regarded as a virulence marker of APEC (Stathopoulos et al. 1999), contributing to airsacs colonization. The prevalence of the *tsh* gene may vary largely among APEC isolates. Percentiles of 49.7%, 85.3% and 39.5% of *tsh* positive strains were obtained respectively by Dozois et al. (2000); Janben et al. (2001) and Delicato et al. (2003).

In spite of the growth on the iron-deficient medium observed for all isolates in biological assay, only three *E. coli* isolates (12.5%) were positive for the *iuc* gene that encoded aerobactin

(siderophore with high affinity iron uptake system). This result suggests the existence of other iron acquisition systems.

The virulence of APEC has been attributed to the resistance to action of complement and bactericidal effects of serum. This resistance can be conferred by many cellular components like the capsular antigen, lipopolysaccharide (LPS) and the outer membrane proteins Iss and TratT (Monroy et al. 2005). In this study only 7 (29.16%) *iss+* isolates were detected.

The mere presence of virulence genes does not mean that the strain is pathogenic, since some of these genes can be found in asymptomatic carriers (Saidenberg 2008, Knöbl & Menão 2010). According to Ngeleka et al. (2002) the virulence of the *E. coli* strains in the extra intestinal infections depends on the combination of virulence factors giving special attention to *tsh/ iuc* and *tsh/ pap/ iuc* - genotypes considered highly virulent. These genotypes were observed in isolates 15 (G9) and 18 (G10), respectively (Tables 2 and 3). Both were isolated from organs after necropsy.

Saidenberg (2008) compared the frequency of virulence genes between strains isolated from sick and healthy groups. The higher frequency in symptomatic psittacine birds was founded for eight virulence genes (*iss, tsh, sfa, pap, iuc, cnf, pap* and *hly*). The *iss* gene was detected in 51.7% in the symptomatic group and 23.2% in asymptomatic ones, while the *tsh* was presented in 8.6% and 1%, respectively.

In conclusion, our results support that a wide diversity of serogroups and pathotypes among avian *E. coli* exists. *E. coli* isolated from psittacine birds present the same virulence factors as avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC) and Enteropathogenic *E. coli* (EPEC) pathotypes. The detection of virulence factors of *E. coli* strains isolated from psittacine birds by molecular techniques may help to clarify the bacterial pathogenesis. Future studies with new approaches for virulence determinants are needed to appoint the clinical importance of these strains by psittaciformes, to establish the epidemiology of colibacillosis among wild birds and to determine the homology of psittacine birds *E. coli* with pathotypes APEC, UPEC and EPEC.

Acknowledgements.- This study was supported by grants from Xunta de Galicia (Grants PGIDIT04RAG261014PR and PGIDIT05BTF26101PR) and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant 2005/57500-9).

REFERENCES

Baldini M.M., Kaper J.B., Levine M.M., Candy D.C.A. & Moon H.W. 1983. Plasmid-mediated adhesion of enteropathogenic *Escherichia coli*. J. Pediatr. Gastroenterol. Nutr. 2:534-538.

Bangert R.L., Cho B.R., Widders P.R., Strauber E.H. & Ward A.C.S. 1988. A survey of aerobic bacteria and fungi in the healthy psittacine birds. Avian Dis. 32:46-52.

Baudry B., Savarino S.J., Vial P., Kaper J.P. & Levine M.M.A. 1990. A sensitive and specific DNMA probe to identify enteroaggregative *E. coli*, a recently discovered diarrheal pathogen. J. Infect. Dis. 161:1249-1251.

Blanco J.E., Blanco M., Mora A. & Blanco J. 1997a. Production of toxins (enterotoxins, verotoxins, and necrotoxins) and colicins by *Escherichia coli* strains isolated from septicemic and healthy chickens: Relationship with in vivo pathogenicity. J. Clin. Microbiol. 35:2953-2957.

Blanco M., Blanco J.E., Gonzalez E.A., Mora A., Jansen W., Gomes T.A.T., Zerbini F., Yano T., Pestana de Castro A.F. & Blanco G. 1997b. Genes coding for enterotoxins and verotoxins in porcine *Escherichia coli* strains belonging to different O:K:H serotypes: relationship with toxic phenotypes. J. Clin. Microbiol. 35:2958-2963.

Blanco J.E., Blanco M., Mora A., Jansen W.H., Garcia V., Vazquez M.L. & Blanco J. 1998. Serotypes of *Escherichia coli* isolated from septicaemic chickens in Galicia (Northwest Spain).Vet. Microbiol. 61:229-235.

Boom R., Sol C.J.A., Salimans M.M.M., Jansen C.L., Wertheim-Van Dillen P.M.E. & Van Der Noordaa J. 1990. Rapid and Simple Method for purification of Nucleic Acids. J. Clin. Microbiol. 28:495-503.

Delicato E.R., Brito B.G., Gaziri L.C.J. & Vidotto M.C. 2003. Virulence associated genes in *Escherichia coli* isolates from poultry with colibacilosis. Vet. Microbiol. 94:97-103.

Dorrestein G.M., Buitelaar M.N., Van der Hage M.H. & Zwart P. 1985. Evaluation of a bacteriological and mycological examination of psittacine birds. Avian Dis. 29:951-962.

Dozois M.C., Dho-Moulin M., Brée A., Fairbrother J.M., Desaultels C. & Curtiss III R. 2000. Relationship between the Ths autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the *tsh* genetic region. Infect. Immun. 68:4145-4154.

Dziva F. & Stevens M.P. 2008. Colibacilosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol. 37:355-366.

Ewers C., Janben T., Kiebling S., Philipp H.C., Wieler L. 2004. Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. Vet. Microbiol. 104:91-101.

Flammer K. & Drewes L.A. 1988. Species-related differences in the incidence of Gram-negative bacteria isolated from the cloaca of clinically normal psittacine birds. Avian Dis. 32:79-83.

Graham C.L. & Graham D.L. 1978. Occurrence of *Escherichia coli* in feces of psittacine birds. Avian Dis. 22:717-720.

Guinée P.A., Jansen W.H., Wadström T. & Sellwood R. 1981. *Escherichia coli* associated with neonatal diarrhea in piglets and calves. Curr. Top. Vet. Anim. Sci. 13:126-162.

Hoefer H.L. 1997. Diseases of the gastrointestinal tract, p.419-453. In: Altman R.B., Clubb S.L., Dorestein G.M. & Quesenbery K. (Eds), Avian Medicine and Surgery. Saunders Company, Philadelphia.

Horne S.M., Pfaff-Mcdonough S.J., Giddings C.W. & Nolan L.K. 2000. Cloning and sequencing of the *iss* gene from a virulent avian *Escherichia coli*. Avian Dis. 44:179-184.

Janben T., Schwarz C., Preikschat P., Voss M., Philipp H.C. & Wieler L.H. 2001. Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. Int. J. Med. Microbiol.. 291:371-378.

Jerse A.E., Jun Y., Tall B.D. & Kaper J.B. 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl Acad. Sci. USA 87:7839-7843.

Johnson J.T., Kariyawasam S., Wannemuehler Y., Mangiamele P., Johnson S.J., Doetkott C., Skyberg J.A., Lynne A.M., Johnson J.R., Nolan L. 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. J. Bacteriol. 189:3228-3226.

Knöbl T., Baccaro M.R., Moreno A.M., Vieira M.A., Ferreira C.S. & Ferreiara A.J. 2001. Virulence properties of *Escherichia coli* isolated from ostriches with respiratory disease. Vet. Microbiol. 2283:71-80.

Knöbl T., Godoy S.N., Matushima E.R., Guimarães M.B. & Ferreiara A.J. 2008. Caracterização molecular dos fatores de virulência de estirpes de *Escherichia coli* isoladas de papagaios com colibacilose aviária. Braz. J. Vet. Res. Anim. Sci. 45:54-60.

Knöbl T. & Menão M. 2010. *Escherichia coli* enteropatogênica (EPEC) isoladas de psitacídeos. FIEP Bulletin 80:839-841.

Maas R. 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid 10:296-298.

Marietto-Gonçalves G.A., Almeida S.M., Lima E.T. & Andreatti Filho R.L. 2010. Detecção de *Escherichia coli* e *Salmonella* spp. Em microbiota intestinal

de Psittaciformes em fase de reabilitação e soltura. Braz. J. Vet. Res. Anim. Sci., 47:185-189.

Marietto-Gonçalves G.A., Lima E.T., Sequeira J.L. & Andreatti Filho R.L. 2007. Colisepticemia em papagaio verdadeiro (*Amazona aestiva*). Revta Braz. Saúde Prod. Anim. 8:56-60.

Mattes B.R., Consiglio S.A.A., Almeida B.Z., Guido M.C., Orsi R.B., Silva R.M., Costa A., Ferreira A.J.P. & Knöbl T. 2005. Influência da biossegurança na colonização intestinal por *Escherichia coli* em psitacídeos. Arqs Inst. Biológico, São Paulo, 72:13-16.

Maurer J.J., Brown T.P., Steffens W.L. & Thayer S.G. 1998. The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin Tsh among avian *Escherichia coli*. Avian Dis. 42:106-118.

Menão M.C., Ferreira C.S.A., Castro A.G.M., Knöbl T. & Ferreira A.J.P. 2002. Sorogrupos de *Escherichia coli* isolados de frangos de corte com doença respiratória crônica. Arqs Inst. Biológico, São Paulo, 65:15-17.

Monroy M.A., Knöbl T., Bottino J.A., Ferreira C.S. & Ferreira A.J.P. 2005. Virulence characteristics of *Escherichia coli* isolates obtained from broilers breeders with salpingitis. Comp. Immunol. Microbiol. Infect. Dis. 28:1-15.

Nakazato G., Campos T.A., Stehling E.G., Brocchi M. & Da Silveira W.D. 2009. Virulence factors of avian pathogenic *Escherichia coli* (APEC). Pesq. Vet. Bras. 29:479-486.

Nardi A.R., Salvatori M.R., Coswig L.T., Gatti M.S., Leite D.S., Valadares G.F., Neto M.G., Shocken-Iturrino R.P., Blanco J.E. & Yano T. 2005. Type 2 heat-labile enterotoxin (LTII) producing *Escherichia coli* isolated from ostriches with diarrhea. Vet. Microbiol. 105:245-249.

Ngeleka M., Brereton L., Brown G. & Fairbrother J.M. 2002. Pathotypes of avian *Escherichia coli* as related to tsh-, pap-, pil-, and iuc-DNA sequences, and antibiotic sensitivity of isolates from internal tissues and the cloacae of broilers. Avian Dis. 46:143-152.

Olsivik O. & Strockbine N.A. 1993. p.271-276. In: Persing D.H., Smith T.F., Tenover F.C., T.J. White T.J. (Eds), Diagnostic Molecular Microbiology: Principles and applications. American Society for Microbiology, Washington, DC.

Pakpinyo S., Ley D.H., Barnes J.P., Vaillancourt J.P. & Guy J.S. 2002. Prevalence of enteropathogenic *Escherichia coli* in naturally occurring cases of poultry enteritis-mortality syndrome. Avian Dis. 46:360-369.

Parreira V.R. & Gyles C.L. 2003. A novel pathogenicity island intregrated adjacent to the thrWtRNA gene of avian pathogenic *Escherichia coli* encodes a vacuolating autotransporter toxin. Infect. Immun. 71:5087-5096.

Parreira V.R. & Yano T. 1998. Cytotoxin produced by *Escherichia coli* isolated from chickens with swollen head syndrome (SHS). Vet. Microbiol. 62:111-119.

Pollard D.R., Johnson W.M., Lior H., Tyler S.D. & Rozes K.R. 1990. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. J. Clin. Microbiol. 28:540-545.

Pourbakhsh S.A., Dho-Moulin M., Brée A., Desautels C., Doize B.M. & Fairbrother J.M. 1997. Localization of the *in vivo* expression of P and F1 fimbriae in chickens experimentally inoculated with pathogenic *Escherichia coli.* Microbiol. Pathog. 22:331-341.

Provence D.L. & Curtiss R. 1992. Role of *crl* in avian pathogenic *Escherichia coli*: a knockout mutation of *crl* does not affect hemagglutination activity, fibronectin binding, or curli production. Infect. Immun. 60:4460-4467.

Ron E.Z. 2006. Host specificity of septicemic *Escherichia coli*: Human and avian pathogens. Curr. Opin. Microbiol. 9:28-32.

Saidenberg A.B.S. 2008. Detecção dos fatores de virulência de *Escherichia coli* isoladas de psitacídeos com diferentes manifestações clínicas. Dissertação de Mestrado, Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo. 91p.

Salvatori M.R., Yano T., Carvalho H.E., Parreira V.R. & Gyles C.L. 2001. Vacuolating cytotoxin produced by avian pathogenic *Escherichia coli*. Avian Dis. 45:43-51.

Schremmer C., Lohr J.E., Wastlhuber U., Kösters J., Ravelshofer K., Steinrück H. & Wieler L.H. 1999. Enteropathogenic *Escherichia coli* in psittaciformes. Avian Pathol. 28:349-354.

Schultsz C., Pool G.J., Van Ketel R., Wevek D., Speelman P. & Dankert J. 1994. Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. J. Clin. Microbiol. 32:2393-2397.

Stathopoulus C., Provence D.L. & Curtiss R. 1999. Characterization of the avian pathogenic *Escherichia coli* hemagglutinin Tsh, a member of the immunoglobulin A protease-type family of autotransporters. Infect. Immun. 67:772-781.

Styles D.K. & Flammer K. 1991. Congo red binding of *Escherichia coli* isolated from the cloacae of psittacine birds. Avian Dis. 35:46-48.

Tsuji T., Joya J.E., Honda T. & Miwatani T. 1990. A heat-labile enterotoxin (LT) purified from chicken enterotoxigenic *Escherichia coli* is identical to porcine LT. FEMS Microbiol. Lett. 55:329-332.

Tsukamoto T. 1997. PCR method for detection of K1 antigen and serotypes of *Escherichia coli* isolated from extraintestinal infection. Kansenshogaku Zashi 71:125-129.

Vila J., Vargas M., Henderson I.R., Gascon J. & Nataro J.P. 2000. Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhea strains. J. Infect. Dis. 182:1780-1783.

Vidal M., Kruger E., Dúran C., Lagos R., Levine M., Prado V., Toro C. & Vidal R. 2005. Single multiple PCR assay to identify simultaneously the si categories of diarrheagenic *Escherichia coli* associated with enteric infections. J. Clin. Microbiol. 43:5362-5365.

Woodward M.J., Carroll P.J. & Wray C. 1992. Detection of entero and verocytotoxin genes in *Escherichia coli* from diarrhea disease in animals using polymerase chain reaction. Vet. Microbiol. 31:251-261.

Yamamoto S., Terai A., Yuri K., Kurazono H., Takeda Y. & Yoshida O. 1995. Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. FEMS Immunol. Med. Microbiol. 12:85-90.