Genetic similarity between APEC and *Escherichia coli* strains isolated from *Guaruba guarouba* in a survey on healthy captive psittacine birds

Similaridade genética entre APEC e cepas de *Escherichia coli* isoladas de *Guaruba guarouba* em um estudo com psitacídeos hígidos de cativeiro.

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Abstract

The role of psittacine birds as a reservoir of avian pathogenic *Escherichia coli* (APEC) is not known but would be helpful in understanding the human – animal interface, since the enteric microbiota of these birds consists of Gram positive bacteria. The aim of this study was to identify the presence of APEC in feces of clinically healthy *Guaruba guarouba*. To do this, we isolated and analyzed *E. coli* from cloacal fecal samples taken from 87 psittacine birds from six zoologic parks, three commercial breeders and one conservation breeder. Of the 87 birds examined, 46 (52.87%) presented *E. coli* in feces. The presence of the following eight virulence genes was determined by the polymerase chain reaction (PCR): *irp2*, *iucD*, *iss*, *vat*, *cvi/cva*, *tsh*, *astA*, and *papC*, and 29 (63.04%) of 46 *E. coli* isolates tested were positive at least one of the eight genes studied. The frequency of virulence genes observed in isolates of *E. coli* were 32.6% (15/46) *irp*2, 26% (12/46) *iuc*D, 19.5% *iss* (9/46), 17.4% *vat* (8/46), 17.4% *cvi/cva* (8/46), 8.7% *tsh* (4/46), 4.4% *ast*A (2/46) and 0% *pap*C (0/46). The isolates were grouped in 13 genotypic profiles according to virulence gene combinations, but only 2 isolates were classified as APEC, with the pattern *iuc, iss, cvi/cva, irp* + and *iuc, iss, cvi/cva, irp, tsh, vat* +. This study reveals the presence of APEC in clinically healthy captive *G. guarouba*, suggesting that these psittacine birds may act as reservoir for pathogenic microorganisms. Epidemiological studies are needed to determine the relevance of this species as a reservoir and the implications for conservation of endangered species *G. guarouba*.

Keywords: *Escherichia coli. APE.* Virulence factors. Psittacine birds. G*uaruba guarouba.*

Resumo

O papel dos psitacídeos como reservatório de *Escherichia coli* patogênicas para aves (APEC) não é conhecido, mas será útil para a compreensão da interface humano-animal, uma vez que a microbiota entérica destas aves é composta por bactérias Gram-positivas. O objetivo deste estudo foi identificar a presença de APEC em fezes de *Guaruba guarouba* clinicamente saudáveis. Para isso, foram isoladas e analisadas *E. coli* presentes em fezes cloacais coletadas de 87 psitacídeos, alojados em seis zoológicos, três criatórios comerciais e um criatório conservacionista. Das 87 aves examinadas, 46 (52,87%) apresentaram *E. coli* nas fezes. A presença de oito genes de virulência foi determinada pela reação em cadeia pela polimerase (PCR): *irp2*, *iucD*, *iss*, *vat*, *cvi/cva*, *tsh*, *astA*, e *papC*, e 29 (63,04%) dos 46 isolados foram positivos para pelo menos um dos oito genes estudados. A frequência dos genes de virulência observada nos isolados de *E. coli* foi 32.6% (15/46) *irp*2, 26% (12/46) *iuc*D, 19.5% *iss* (9/46), 17.4% *vat* (8/46), 17.4% *cvi/cva* (8/46), 8.7% *tsh* (4/46), 4.4% *ast*A (2/46) e 0% *pap*C (0/46). Os isolados foram agrupados em 13 perfis genotípicos de acordo com combinações de genes de virulência, mas apenas duas amostras foram classificadas como APEC, com o perfil *iuc, iss, cvi/cva, irp* + e *iuc, iss, cvi/cva, irp, tsh, iuc, iss,* +. Este estudo revela a presença de APEC em aves de cativeiro (*G. guarouba*) clinicamente saudáveis, sugerindo que estes psitacídeos possam atuar como reservatórios de micro-organismos patogênicos. Estudos epidemiológicos são necessários para determinar a relevância desta espécie como reservatório e as implicações para a conservação da espécie ameaçada *G. guarouba*.

Palavras-chave: *Escherichia coli. APEC.* Fatores de virulência. Pistacíde. G*uaruba guarouba*.

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Introduction

The *Guaruba guarouba* (Golden conure, also called Ararajuba or Queen of Bavaria conure) is a medium-sized (34 cm) neotropical psittacine bird, characterized by bright yellow plumage with green remiges in outer wings[1]. This bird is endemic in the Amazonian biome (Brazil) but is now listed on the International Union for Conservation of Nature (IUCN) Red list as an endangered species, with extinction risk due to deforestation, capture and illegal wildlife trade[2].

The wild population is estimated as 1,200 individuals distributed over approximately 100,000 km^2, while the captive population in Brazil is about 600 birds. Studies that contribute to a better understanding the ecology of the species and sanitary health status, therefore, are important for conservation of wild and captive psittacine birds[3].

The mortality of captive parrots and macaws is often due infection by *Escherichia coli* or other members of the gram-negative Enterobacteriaceae family[4]. Such bacteria are considered abnormal inhabitants of psittacine birds, so they are viewed by some as pathogenic and opportunistic microorganisms in this context[5,6,7]. However, there is controversy about the meaning of the infection in clinically healthy birds, such as whether they are a reservoir of potentially pathogenic bacterial species for other birds and mammals[7,8,9].

Avian pathogenic *Escherichia coli* (APEC) is an etiological agent of colibacillosis, affecting production birds and causing economic losses in the poultry industry. Pathogenic *E. coli* from contamination with broiler feces can enter the respiratory tract and colonize the air sacs. Aerossacculitis is followed by extraintestinal infections including pneumonia, pericarditis, perihepatitis and death after septicemia[10]. The psittacine birds diseases is correlated with immunossupression, poor hygiene and daily care in captivity[6]. The most frequent clinical disorders include pneumonia, sinusitis, and enteric disease, being common the sepsis[4].

Recently, the interest in this agent has increased due to the zoonotic potential of some strains with genetic similarities to human extraintestinal pathogenic *E. coli* (ExPEC). Broilers are now considered a potential reservoir for pathogenic strains of *E. coli*, but the presence of this agent in wild birds is still unknown[11].

The APEC strains exhibit considerable genomic diversity and have a wide range of virulence factors, including adhesins, iron uptake systems, capsule proteins and toxins, which are often encoded in plasmids or on pathogenicity islands. The APEC pathotype shares virulence associated traits and is represented mainly by O1, O2 and O78 serogroups[12]. Ewers et al. (2005) classifies APEC as any strain with four of the eight genes: P fimbriae (*pap*), aerobactin (*iuc*), iron repressible protein (*irp*), temperature-sensitive haemagglutinin (*tsh*), vacuolating autotransportes protein (*vat*), enteroaggregative toxin (*ast*), increased serum survival protein (*iss*) and colicin plasmid operon genes (*cva/cvi*)[13].

The aim of this study was investigate the virulence genes present in *E. coli* isolated from feces of captive *G. guarouba*. In the present study, we analyzed cloacal fecal samples of clinically healthy birds to determine if these psittacine act as a reservoir of APEC.

Materials and methods

Bacterial strains

The study was conducted with *Escherichia coli* isolated from 87 psittacine birds (*Guaruba guarouba*), housed in ten institutions in São Paulo state, Brazil, including six zoologic parks, three commercial breeders and one conservation breeder. The project was approved by the Ethics Committee of São Paulo University (1595/2009) and authorized for scientific purposes (SISBIO: 19066-1).

All birds were considered clinically healthy upon physical examination at the time of collection, and age and sex were variable. The swabs were collected from cloacae and then transported to the laboratory under refrigeration. Standard bacteriological methods were employed for *E. coli* isolation and identification. The swabs were cultured in BHI broth and incubated at 37°C for 24 hours. After enrichment, the samples were plated on MacConkey agar, and incubated at 37°C for 24 hours. Genera identification was carried out by conventional biochemical tests (EPM, Mili and Simmons Citrate – Probac®). All isolates were stored in Lúria Bertani broth, with 15% glycerol at -70 °C.

Polymerase chain Reaction for the detection of virulence factors

DNA was extracted according to the protocol described by Boom et al.[14] Polymerase chain reaction (PCR) was performed in 50 µL reaction mixtures with 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each deoxyribonucleotide (dATP, dCTP, dGTP, dTTP), 50 pmol of each oligonucleotide primer, 1.0 U of Taq DNA polymerase, autoclaved ultra pure water, and 5 µL of DNA template. Primers for specific amplification of the eight APEC virulence factor genes, amplicon size and references are described in figure 1. The amplified products were separated by electrophoresis on a 2% agarose gel and stained with Syber Safe® (Invitrogen). The 100 bp DNA ladder was used as a molecular size marker.

Results

Escherichia coli were isolated from cloacal of 46/87 (52.7%) birds from 10 facilities, and of the isolates, 18 were negative for all virulence genes, and 28 (60.8%) were positive at least one of the eight genes studied, as shown in figure 2.

Results from PCR revealed that 12 (26%) isolates were positive for the aerobactin gene (*iuc*D); 9 (19.5%) for increased serum survival protein (*iss)*; 4 (8.7%) for the temperature-sensitive hemagglutinin (*tsh)*; 8 (17.4%) for the colicin plasmid operon genes (*cvi/cva)*; 15 (32.6%) for the iron repressible protein (*irp*2); 2 (4.4%) for enteroaggregative toxin (*ast*A); and 8 (17.4%) for vacuolating autotransporte protein (*vat)*. None were positive for P fimbriae (*pap*C) (Figure 2).

Genetic profiles based on combinations of virulence genes are listed in figure 3. Only 2 of the 13 strains were classified as APEC, according to the genotype profiles G13 [*iuc, iss, cvi/cva, irp* + (EC.45)] or G12 [*iuc, iss, cvi/cva, tsh, irp, vat* + (EC.11)].

Discussion

Previous studies have shown that *E. coli* isolated from psittacine birds have the same virulence traits of avian pathogenic *E. coli* (APEC) and enteropathogenic *E. coli* (EPEC) pathotypes[7,8,9]. However, the diversity of serogroups and pathotypes from *E. coli* isolated from birds is an obstacle to understanding the pathogenesis of colibacillosis in psittacines. The clinical significance of these microorganisms in the microbiota of healthy parrots is yet to be determined.

Xenoulis et al.[5] molecularly characterized the cloacal microbiota in wild and captive parrots, showing lower bacterial diversity in wild birds (*Amazona* spp. and *Ara ararauna*). *Staphylococcus saprophyticus* was significantly more abundant in wild birds, while *E. coli* was more abundant in captive birds.

The present study is the first report of APEC in captive healthy *G. guarouba*, and the large sampling (n=87) covers about 15% of total Brazilian captive population. The results showed a colonization of 52.7% of the birds, and 60.8% of isolates harbored at least one of the eight virulence genes (Figure 2). The recovery frequency of *E. coli* was higher than in other Brazilian surveys that found 25% colonization in the white-eyed conure (*Aratinga leucophthalmus*) and 18.6% in blue-fronted parrots (*Amazona aestiva*)[8].

Figure 1 - Primers used for APEC virulence gene amplification, amplicon size and references

Genes	**Oligonucleotide *primer pairs* (5`-3`)**	***Amplicon* (bp)**	Reference
iuc	TACCGGATTGTCATATGCAGACCGT AATATCTTCCTCCAGTCCGGAGAAG	602	YAMAMOTO et al, 1995 [15]
tsh	GGGAAATGACCTGAATGCTGG CCGCTCATCAGTCAGTACCAC	420	MAURER et al, 1998 [16]
iss	GTGGCGAAAACTAGTAAAACAGC CGCCTCGGGGTGGATAA	760	HORNE et al., 2000 [17]
*pap*C	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAAC	501	JANSEN et al., 2001 [12]
*irp*2	AAGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	281	SCHUBERT et al., 1998 [18]
*ast*A	TGCCATCAACACAGTATATCC TAGGATCCTCAGGTCGCGAGTGACGGC	116	YAMAMOTO e ECHEVERRIA, 1996 [19]
vat	TCCTGGGACATAATGGTCAG GTGTCAGAACGGAATTGTC	981	EWERS et al., 2004 [20]
cvi/ cva	TCCAAGCGGACCCCTTATAG CGCAGCATAGTTCCATGCT	598	EWERS et al., 2007 [21]

These differences can be attributed to many factors such as inter-species variation, hygiene and handling practices, diet, environmental factors and interaction with domestic species and humans[6].

The most frequently observed genes in this study were *iuc*D (26%) and *irp*2 (32.6%), which encode aerobactin and yersiniabactin siderophores, respectively, as well as other iron acquisition systems identified in APEC and human enteroaggregative *E. coli*.[12] The iron uptake systems are much conserved among members of Enterobacteriaceae family and promote capture and transport of iron, allowing bacteria to grow in restricted or limited free-iron conditions[10]. The aerobactin siderophore is encoded by *iuc* operon on the plasmid ColV. The *cvi/cva* genes (Col V plasmids) were also detected in 17.4% of isolates.

Saidenber[22] compared the frequency of virulence genes in strains isolated from sick and healthy psittacine birds. While the genes were found in higher frequency in symptomatic birds, some virulence genes were also present in asymptomatic individuals. The *iss* gene was most frequently identified, detected in 23.2% in asymptomatic groups, while the *tsh* was present only in 1%[22]. Similarly, our results showed a frequency of 19.5% for *iss* and 8.7% for *tsh*.

The *iss (increased serum survival)* gene is located on conjugated high molecular weight plasmids with virulence factors associated with the resistance to complement, bactericidal effects of serum, and antibiotics[21]. TSH protein is a temperature-sensitive hemagglutinin that possesses high homology with IgA proteases from *Neisseria gonorrhoeae* and *Haemophilus influenzae*[16]. Although the role of this protein in the pathogenesis of avian colibacillosis is not fully understood, it has been regarded as a virulence marker of APEC, and the genotype *iuc+, iss+, tsh+* has been correlated with highly pathogenic strains[23]. These genes encode proteins that facilitate the air sac colonization, iron uptake and serum survival in extra-intestinal infections[16,17,21,23].

By grouping virulence genes, 13 genotypic profiles were identified in this study. Two strains, G12 and G13, possessed four or more virulence genes and therefore are considered APEC (Figure 3). G13 had the highly pathogenic phenotype containing *iuc*, *iss*

Figure 2 - Virulence genes of *Escherichia coli* isolated from *Guaruba guarouba*, Brazil - 2012

Facilites	*E. coli* strains	APEC virulence genes							
		iucD	*iss*	*tsh*	*cvi/ cva*	*irp2*	*astA*	*vat*	*papC*
Zoo 1	**5**	-	+	-	-	-	-	-	-
Zoo 2	**7**	-	+	+	-	-	-	-	-
	11*	+	+	+	+	+	-	+	-
Breeder 1	**13**	-	-	-	-	+	-	+	-
Zoo 3	**21**	-	-	-	-	+	-	-	-
Zoo 4	**26**	-	-	-	-	+	+	-	-
Breeder 2	**28**	-	-	+	-	-	-	-	-
	29	-	-	+	-	-	-	-	-
Breeder 3	**32**	-	-	-	-	+	+	-	-
	35	+	-	-	+	-	-	-	-
	37	-	-	-	-	+	-	-	-
	38	-	-	-	-	+	-	-	-
	40	+	-	-	+	-	-	-	-
	41	-	+	-	-	-	-	-	-
	42	+	+	-	+	-	-	-	-
	43	+	+	-	+	-	-	-	-
	44	+	+	-	+	-	-	-	-
	45*	+	+	-	+	+	-	-	-
	47	+	+	-	+	-	-	-	-
Breeder 4	**65**	+	-	-	-	+	-	+	-
	66	+	-	-	-	-	-	-	-
	68	-	-	-	-	+	-	+	-
	74	+	-	-	-	-	-	+	-
	75	-	-	-	-	+	-	-	-
	76	-	-	-	-	+	-	+	-
	80	-	-	-	-	+	-	-	-
	82	-	-	-	-	+	-	+	-
	86	+	-	-	-	+	-	+	-
	Total	12/46	9/46	4/46	8/46	15/46	2/46	8/46	0/46
	%	26%	19,5%	8,7%	17,4%	32,6%	4,4%	17,4%	0%

* isolates classified as APEC

and *tsh*, as well as *cvi/cva, irp* and *vat*. APEC strains were found in two birds from two different facilities: Zoo2 (G13) and Breeder 3 (G12). Future studies with experimental infection are needed to appoint the clinical significance of these strains in psittaciformes.

The *vat* gene (vacuolating cytotoxin), a serine protease auto transporter protein[20], was present in G13, and also in seven other isolates (17.4%) with profiles G6, G9 and G11. This heat-labile toxin is produced by APEC isolated from broilers with cellulitis, and induces the formation of intracellular vacuoles in cultured cells. Vat plays a key role in the inflammatory process mediated by increased levels of TNF- α nd IL-10[24].

Several authors have described the prevalence of the gene *ast*A in virulent strains of APEC, but its exact involvement in the pathogenesis of avian colibacillosis is not well understood[13,12,20]. The *astA* gene product, enteroaggregative heat-stable toxin EAST1, is mainly related to diarrheagenic enteroaggregative strains[12]. These strains show an aggregative adherence pattern in cultured cells and are associated with persistent

Figure 3 - Genotypic profiles of *Escherichia coli* isolated from *Guaruba guarouba*, Brazil - 2012

Profiles	Combination of genes	No. of strains	Facilities
G 1	*iss*	2	Zoo 1; Breeder 3
G 2	*irp2*	5	Zoo3; Breeder 3; Breeder 4
G 3	*tsh*	2	Breeder 2
G 4	*iucD*	1	Breeder 4
G 5	*iss, tsh*	1	Zoo 2
G 6	*irp2, vat*	4	Breeders 1; Breeder 4
G 7	*irp2, astA*	2	Zoo 4; Breeder 2
G 8	*iucD, cvi/cva*	2	Breeder 3
G 9	*iucD, vat*	1	Breeder 4
G 10	*iucD, iss, cvi/cva*	4	Breeder 3
G 11	*iucD, irp2, vat*	2	Breeder 4
G 12	*iucD, iss, cvi/cva, irp2*	1	Breeder 3
G 13	*iucD, iss, tsh, cvi,cva, irp2, vat*	1	Zoo 2
Total		28	

watery diarrhea in humans. Our results showed only 4.4% of isolates were *astA* positive, all of which fell in the G7 profile (*ast*A+ , *irp* 2+).

The prevalence of the *pap*C gene, which encodes for P fimbriae, may vary largely among APEC isolates, but it is very common in human uropathogenic *E. coli* (UPEC) strains. None of isolates were positive for *pap*C gene, agreeing with Saidenberg et al.[9] in a survey in asymptomatic free-ranging parrots[9].

Conclusion

The present study shows that *Escherichia coli* isolated from asymptomatic *Guaruba guarouba* contains similar virulence factors to those reported for *E. coli* strains from commercial poultry. Clinical healthy birds may act as reservoir for APEC, and further studies are needed for conservation of these captive psittacine birds.

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