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SOME ADHESINS OF AVIAN PATHOGENIC *ESCHERICHIA COLI* (APEC) ISOLATED FROM SEPTICEMIC POULTRY IN BRAZIL

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ABSTRACT

Three hundred and fifty strains of *E. coli* isolated from septicemic poultry from seven states of Brazil were examined for presence of nine adhesion-encoding genes, hemagglutination and adherence to chicken tracheal cells (*in vitro*). Analysis of the strains by colony hybridization tests demonstrated that 93.7% of the isolates were *fim* +, 17% *pap*+ and 5.7% were *sfa*+. The mannose sensitive fimbriae occur with similar frequency in APEC isolated from all Brazilians states, while significant differences among *pap* and *sfa* genes distributions were observed. The results showed that 0.85% and 0.28% of APEC were positive for genes that encoded enteroaggregative adhesins and EPEC adherence factor, respectively. None of APEC was positive for DA, afa, Bfp and Eae probes. The adherence to chicken tracheal cells showed 96% positive strains, while hemagglutination assays showed 26.5% of the isolates were mannose sensitive and 21.7% were mannose resistant.

Key words: APEC, *Escherichia coli,* adhesins, colibacillosis, poultry

INTRODUCTION

Avian Pathogenic *Escherichia coli* (APEC) are associated with extra intestinal infections and development of septicemia in broilers. Colibacilosis is an opportunistic disease, responsible for severe economic losses for the poultry industry due to a lowered production, increased mortality rate, carcass condemnation and cost of treatment (3,5).

Extra intestinal *E. coli* strains encode many adhesins that promote the attachment of the bacteria to cell receptors. These virulence factors are very important for the host infection and to development of septicemia (10,24,29,31-34,39,40). Type 1 fimbriae have been involved with the initial stages of the upper respiratory colonization, whereas the P fimbriae are involved in colonization of the internal organs (31).

Type 1 fimbriae are found in many different species of *Enterobacteriaceae* and are characterized by their ability to mediate agglutination of guinea pig erythrocytes in the absence of α-D-mannose, but not in its presence. The role of type 1 fimbriae in colibacillosis has been associated with mucus adherence and trachea and intestinal tract colonization (8,11,13,28,31,35).

P fimbriae are mannose resistant hemagglutinating fimbriae present in *E. coli* strains causing urinary tract infections in humans and also may be expressed by some *E. coli* of avian origin. They are associated with internal organs colonization, septicemia and lethality in one-day-old chicks (12,31).

Epidemiological studies with APEC have shown the presence of other selected genes for fimbrial and afimbrial adhesins of human origin. The role of these adhesins in pathogenesis of colibacillosis has not been elucidated, but poultry may act as a reservoir for human pathogenic *E. coli* (12,19,20,24,30,36,40).

The purpose of this survey was to investigate the distribution of adhesion-encoding genes among avian

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pathogenic *E. coli* (APEC) isolated from septicaemic poultry in seven Brazilians states.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A total of 350 *E. coli* strains were isolated from different poultry farms between 1994 and 2004 in seven states of Brazil - Ceará, Pernambuco, Minas Gerais, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul.

The strains were isolated from the liver of broilers with colisepticemia. Standard bacteriological methods were employed for isolation and identification of the organism (3). All strains were stored at -80ºC in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) containing 15% glycerol, added after incubation.

Colony Hybridization

The colony hybridization assays were performed as described by Maas (27). The test strains were examined using specific DNA probes labeled with [α-^{32}P]-dATP by Nick translation. The DNA probe donors, recombinant plasmids, fragments size and relevant literature are given in Table 1. Detection of *pap, afa*, and *sfa* genes involved oligonucleotide fragments obtained by polymerase chain reaction (Table 2). Positive and negative controls were included in all hybridization assays.

Hemagglutination tests

The presence of fimbriae was detected by the ability of strains to agglutinate erythrocytes from guinea pig, human, chicken, cattle and sheep in the presence or absence of 2% D-Mannose (14).

Tracheal ring cell preparation and adherence

To assess *E. coli* isolates for adherence, the bacteria were grown on colonization factor antigen agar (CFA) at 37ºC for 18-24 h and then suspended in 50 mM PBS. Ten-day-old Specific-Pathogen-Free chicks were killed humanely and the tracheas removed aseptically. Tracheal sections were cut in 4 mm length, rinsed three times in Krebs Ringer Tris saline buffer with 0.05 M Tris-HCl (pH 7.4) (18).

Adherence studies were performed in 96-well round-bottom microtiter plates. Each well received three tracheas and MEM without calf serum. Bacterial strains were incubated with tracheal rings at 37ºC for 30 min, after which they were washed with 50 mM PBS (pH 7.4) and incubated for an additional 4 h. Then, the tracheal rings were fixed with buffered formalin, processed and stained with Giemsa for examination by light microscopy. *E. coli* K 12 strain C600 was used as a negative control (18).

Statistical analysis

All data were analyzed by means of the software EpiInfo - Centers for Disease Control and Prevention, Atlanta, GA, USA (6). Fisher´s exact test and the χ^2 test were used for univariate analysis of the significance associations. Differences were considered statistically significant if P d≤0.05.

RESULTS

The results of the DNA hybridization tests, hemagglutination assays and tracheal adherence tests are summarized in Table 3.

Type 1 probe showed that the relevant sequence was present in 328 (93.7%) isolates. These fimbriae occurred with similar frequency in strains from the seven Brazilians states.

Sixty one (17.4%) isolates were *pap*+ and twenty (5.7%) were *sfa*+. Significant differences were observed in the distribution of mannose-resistant fimbriae among several Brazilians states. The *pap*+ gene frequency varied from 18 and 26% in the majority of the states, while Ceará and Minas Gerais presented only 6 and 10% of *pap*+ APEC, respectively. The *sfa* genes were detected with a higher frequency in isolates from Paraná (30%) than in isolates from São Paulo (4%), Minas Gerais (4%) and Rio Grande do Sul (2%), and were absent from strains isolated from other states.

None of *E. coli* isolates carried the genes encoding for afimbrial adhesin (*afa*), diffuse adhesion (DA) and attaching and effacing lesion (*eae*).

The phenotypic assays showed that 93 (26.5%) strains presented mannose sensitive hemagglutination and 76 (21.7%) mannose resistant hemagglutination (Table 3). HAMS profiles varied among 12% and 46% in Brazilian states and were considered very lower than results obtained in colony hybridization for type 1 fimbriae (88% to 98%). Results of HAMR varied greatly among different Brazilian states (Table 3) and didn't correlate with results of P and S DNA positive probe.

In relation to adherence tests, 336 (96%) strains adhered on tracheal cells in absence of D-mannose. There were no significant differences among the results obtained in several Brazilian states ($p<0.05$).

DISCUSSION

Epidemiological and pathogenesis researches on the *E. coli* are concentrated on adhesion investigations, because fimbriae are good candidates for vaccine against APEC (23,36,37,38). The present study shows that the presence of mannose-resistant adhesins varies among strains isolated from different Brazilian states, while the mannose sensitive adhesins presents a uniformly distribution (Table 3).

This study also confirms previous observations that type 1 fimbriae are frequently detected in APEC (7,20,29,40). A total of 93.7% *E. coli* isolates were found to hybridize with *fim* DNA probe, but only 26.5% exhibited mannose-sensitive hemagglutination pattern (MSHA). The results of MSHA were lower than colony hybridization for type 1 fimbriae in the seven

Table 1. DNA probes for adhesins detection in avian pathogenic *Escherichia coli* (APEC).

Virulence factor	DNA probe	Recombinant Plasmid	Restriction enzime	Fragment size	References
Type 1 pili	*fim*B-H	pIB254	*Hin*dIII, *Sal*I	9.6 kb	(22)
Difuse adherence	*daa*C	pSLM852	*Pst*I	350pb	(5)
Enteroaggregative adherence	AA	pCVD432	*Xba*I, *Sma*I	~1.0Kb	(4)
Bundle forming pilus	*bfp*	pMSD207	*Eco*RI	852 pb	(17)
EPEC adherence factor	EAF	pJPN16	*Bam*HI, *Sal*I	1.0Kb	(2)
E. coli attaching and effacing	*eae*A	pCVD434	*Sal*I, *Kpn*I	1.0Kb	(21)

Table 2. Oligonucleotide sequences for adhesins detection in avian pathogenic *Escherichia coli* (APEC).

Virulence factor (Gene)	Prototype Strains	Oligonucleotide primer pairs (5´→3´)	Amplicom (pb)	References
pap	J96	GAC GGC TGT ACT GCA GGG TGT GGCG ATA TCC TTT CTG CAG GGA TGC AAT A	328	(26)
afa	KS52	CAT CAA GCT GTT TGT TCG TCC GCCG GCT GGG CAG CAA ACT GAT AAC TCT C	750	(26)
sfa	HB101 (pANN801-13)	CGG AGG AGT AAT TAC AAA CCT GGCA CTC CGG AGA ACT GGG TGC ATC TTA C	410	(26)

Table 3. Colony hibridization, HA test and tracheal ring cells adherence of APEC isolated from poultry in some Brazilian states.

States	DNA probs					Hemagglutination assay		Tracheal adherence
	F1	P	S	AA	EAF	MS	MR	
Rio Grande do Sul	49/50 (98%)	13/50 (26%)	1/50 (2%)	–	–	6/50 (12%)	15/50 (30%)	42/50 (84%)
Paraná	47/50 (94%)	9/50 (18%)	15/50 (30%)	2/50 (4%)	–	23/50 (46%)	3/50 (6%)	50/50 (100%)
Santa Catarina	44/50 (88%)	11/50 (22%)	–	–	1/50 (2%)	9/50 (18%)	13/50 (26%)	50/50 (100%)
São Paulo	48/50 (96%)	10/50 (20%)	2/50 (4%)	–	–	(28%) (28%)	(14%) (14%)	(92%) (92%)
Minas Gerais	47/50 (94%)	5/50 (10%)	2/50 (4%)	–	–	10/50 (20%)	12/50 (24%)	50/50 (100%)
Pernambuco	47/50 (94%)	10/50 (20%)	–	–	–	17/50 (34%)	11/50 (22%)	48/50 (96%)
Ceará	46/50 (92%)	3/50 (6%)	–	1/50 (2%)	–	14/50 (28%)	15/50 (30%)	50/50 (100%)
Total	328/350 (93.7%)	61/350 (17.4%)	20/350 (5.7%)	3/350 (0.85%)	1/350 (0.28%)	93/350 (26.5%)	76/350 (21.7%)	336/350 (96%)

MR – mannose resistant; MS- mannose sensitive.

Brazilian states, suggesting that hemmaglutination assay was a low sensitive phenotypic test.

Dho and Lafont (9) have associated the virulence of avian *E.coli* with its ability to adhere to tracheal epithelial cells. In this study, 96% of *E. coli* isolates presented adherence to tracheal epithelium in absence of D-mannose, and there were no significant differences among the results obtained in several Brazilian states ($p<0.05$), as show the Table 3. The presence of type 1 fimbriae was correlated with tracheal cells adherence, and this phenotypic expression model was more sensitive than hemagglutination assay. However, some isolates that were positive to tracheal cells adherence didn't hybridize with *fim* operon, suggesting a low specificity of this adherence model.

Vandemaele *et al.* (37) analyzed the sequence of *fim*H and *fim*A genes in 24 isolates of APEC and demonstrated that *fim*H is a conserved adhesin, while *fim*A presents a variable sequence, although, the immunization with the binding domain of *fim*H does not protect chickens against avian pathogenic *E. coli* (38).

Pourbakhsh *et al.* (31) demonstrated the involvement of type 1 fimbriae in the colonization of the upper respiratory tract in experimentally inoculated chickens and suggested that P fimbriae may be involved in the colonization of internal organs and in the development of septicaemia. However, the major part of *E.coli* isolated from colibacillosis was negative for fimbriae P, and the mannose resistant adherence cannot always be attributed to P pili (33,40).

Epidemiological studies about APEC present relative diversity among *pap* frequency (7,20,24,29,33,40). For instance, the *pap* gene was detected in 30% of isolateds by Janben *et al.* (20), 23.9% of isolates by Stouder *et al.* (34) and 16% of isolates by Knöbl *et al.* (24).

In Brazil, the epidemiological studies on APEC were concentrated in the Paraná state, with 14% of *E. coli pap*+ isolated by Vidotto *et al.* (40); 18.5% by Delicato *et al.* (7); and 17.4% in this study. Amabile de Campos *et al.* (1) studied 45 APEC obtained from chickens suffering from septicemia, swollen head syndrome and omphalitis, isolated from individuals in different regions of Brazil and detected 11 (22.4%) *pap* positive strains. Our results suggest that the frequency of *pap* gene in Brazil can vary between 6 and 26%. The regional variation in the frequency of P fimbriae may be considered one limitation for protection against colibacilosis by fimbriae vaccine. Other limitation of the P pilus vaccine is the highly polymorphic nature of Pap A main subunit. Vandemaele *et al.* (36) showed that the *pap*GII and *pap*GIII sequences of APEC have high homology with human *pap*G sequences. Moreover to mention the zoonotic consequences, the authors suggested that the conserved character makes it a promising vaccine candidate against APEC.

Stoudeur *et al.* (34) analyzed a collection of 1601 of extra intestinal or intestinal *Escherichia coli* isolated from chickens, turkeys and ducks, in Belgium, France and Spain and observed that 4.2% of strains were S-positive. Knöbl *et al.* (24) showed that the frequency of these fimbriae in Brazil can vary between 4 and 16% among isolates from respiratory disease and omphalitis, respectively. Chicks may become infected due to poor hygiene during handling of eggs in the hatchery. The fecal-oral route can be responsible for the widespread of infection.

Significant differences were appointed in the frequency of *sfa* gene in this investigation. The *sfa* gene was detected in 4% of isolates from São Paulo and Minas Gerais states, while in Paraná state, 30% of APEC were *sfa*+. Vidotto *et al.* (39) examined APEC isolates from Paraná state using colony hybridization and found that 40% were positive for *sfa*DE and 30% for *fac*A genes. Amabile de Campos *et al.* (1) found *sfa* adhesion sequence in 4.16% of septicaemic *E. coli* isolated from chickens in Brazil. The epidemiology of *Escherichia coli sfa*+ isolated from poultry in Brazil needs to be better characterized.

The S fimbriae are able to promote the adherence of *E. coli* to endothelial and epithelial cells in human coroid plexus and cerebral ventriculus. The presence of S fimbriae was rarely detected in APEC and the role of these fimbriae on pathogenesis of colibacillosis had not been elucidated (19,24,34).

Certain adhesins associated with *E. coli* causing intestinal disease in humans are found sporadically in APEC (20,25). To our knowledge, this work is the first report of enteroaggregative *E. coli* isolated from poultry. The importance of these strains for poultry is unclear, but domestic animals may constitute reservoirs of strains that are pathogenic for humans. Janben *et al.* (20), using PCR for identifying virulence-associated genes in 150 APEC, found enteroaggregative heat stable toxin gene (*ast*A) in 17.3% of the field strains.

EAF gene sequences in strains of avian origin are also rare, but other virulence determinants of EPEC, like *eae*, gene have been described by many authors (16,25,30). In this study only one strain presented the EAF sequences, but this APEC was negative for EAE probe.

The *afa* sequences were not found in this investigation and this result is in accordance with results obtained by Delicato *et al.* (7). However, the *afa* sequence was detected in 4.8% of isolates from avian origin by Stordeur *et al.* (34) and 12.5% by Amabile de Campos *et al.* (1).

In conclusion, the results of this study confirm the regional differences of frequency of mannose resistant adhesion genes in APEC isolated from Brazil. A more complete understanding about these fimbriae is necessary to support a vaccine programs.

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RESUMO

Algumas adesinas de *Escherichia coli* aviária (APEC) isoladas de aves com colisepticemia no Brasil

Trezentas e cinqüenta amostras de *E. coli* isoladas de aves com septicemia em sete estados do Brasil foram examinadas para a presença de nove genes codificadores de adesinas, hemaglutinação e aderência em células da traquéia (*in vitro*). A análise das amostras pela hibridização de colônias demonstrou que 93,7% dos isolados eram *fim* +, 17% *pap*+ e 5,7% eram *sfa*+. As fímbrias manose sensíveis apresentaram uma distribuição uniforme em todos os estados do Brasil. No entanto, diferenças significativas na distribuição dos genes *pap* e *sfa* foram observadas. Os resultados mostraram que 0,85% e 0,28% das APEC foram positivas para os genes que codificam as adesinas enteroagregativas e o fator de aderência de EPEC, respectivamente. Nenhuma amostra foi positiva para as sondas DA, afa, Bfp e Eae. A aderência em células de traquéia de aves revelou 96% de amostras positivas, enquanto os testes de hemaglutinação mostraram 26,5% dos isolados mannose sensíveis e 21,7% manose resistentes.

Palavras-chave: APEC, *Escherichia coli*, adesinas, colibacilose, ave

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