The expression of plasmid mediated afimbrial adhesin genes in an avian septicemic *Escherichia coli* strain

Eliana Guedes Stehling[1,*]**, Tatiana Amabile Campos**[1]**, Marcelo Brocchi**[1]**, Vasco Ariston de Carvalho Azevedo**[2]**, Wanderley Dias da Silveira**[1]

[1]*Department of Microbiology and Immunology, Institute of Biology, CP 6109, Campinas State University, Campinas, CEP: 13081-862, SP, Brazil*
[2]*Department of Cellular Biology, Biosciences Institute, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*

An *Escherichia coli* strain (SEPT13) isolated from the liver of a hen presenting clinical signs of septicaemia had a LD_{50} of 4.0×10^5 CFU/ml in one-day-old chickens, expressed Ia, Ib, E1, E3, K and B colicins and aerobactin. The strain was ampicillin and streptomycin resistant, and found to have *fim*A, *csg*A and *tsh* DNA related sequences; it could adhere to and invade HEp-2 and tracheal epithelial cells, expressed fimbriae (observed by electron microscopy), and had five plasmids of 2.7, 4.7, 43, 56, and 88 MDa. Transposon mutagenesis of strain SEPT13, with transposon TnphoA, resulted in a mutant strain named ST16 that had a LD_{50} of 1.2×10^{12} CFU/ml. All other biological characteristics of strain ST16 were the same as those detected for strain SEPT13 except for the migration of an 88 MDa plasmid to the 93 MDa position indicating the insertion of the transposon into the 88 MDa plasmid. The 93 MDa plasmid of strain ST16 was transferred, by electroporation assay, to non-pathogenic receptor strains (*E. coli* strains K12 MS101 and HB101), resulting in transformant strains A and B, respectively. These strains exhibited adhesion properties to *in vitro* cultivated HEp-2 cells but did not have the capacity for invasion. The adherence occurred despite the absence of fimbriae; this finding suggests that the 88 MDa plasmid has afimbrial adhesin genes.

Keywords: adhesion, avian, *Escherichia coli*, plasmids

Introduction

Escherichia coli is frequently found as a normal inhabitant of the intestinal tract of humans and animals.

---
*Corresponding author
Tel: +55-19-3521-6269; Fax: +55-19-3521-6276
E-mail: eliana.stehling@gmail.com

However, some strains, capable of causing disease, are pathogenic clones in healthy hosts [23]. Avian pathogenic *E. coli* strains (APEC) are most commonly associated with extraintestinal infections, mainly in the respiratory tract or systemic infections; a variety of diseases can result, which are responsible for severe economic losses in the avian industry [11,17,18].

The pathogenesis and the role of virulence present in APEC strains have not been fully elucidated to date. However, considerable progress has been made recently to establish the mechanisms of pathogenesis [11]. Flagella, toxins and cytotoxins, serum resistance, colicin production, iron sequestering systems, temperature-sensitive hemagglutinin and expression of adhesins, are considered to be the fundamental virulence associated factors for the full expression of APEC pathogenecity [5,9,10,12,36]. Expression of adhesins was first detected by the observation that a virulent and fimbriated strain was less easily cleared from the trachea of turkeys than a non-virulent and less-fimbriated strain [1]. The principal adhesins described for APEC strains are type 1, type P, curli fimbriae and temperature-sensitive hemagglutinin (Tsh). Type 1 and type P fimbriae are encoded by the *fim* and *pap* gene clusters, respectively, that are located on the *E. coli* chromosome [28]. Curli fimbriae have been associated with bacterial adherence to laminin and fibronectin [26] and with chicken red blood cell agglutination, but their involvement in pathogenesis is still unclear and remains to be clarified [27]. The *tsh* gene, which encodes a Tsh, was first identified by Provence and Curtis III [30] and was shown to be associated with APEC but not with *E. coli* isolated from the feces of healthy chickens; [22] this suggested that hemagglutinin could be associated with APEC pathogenesis.

There is strong evidenc that adhesion properties are associated with APEC pathogenicity. The purpose of this stud was to determine the association of pathogenicity and adhesion characteristics expressed by an avian septicaemic

E. coli strain (SEPT13) and to correlate these characteristics with the presence of the 88 MDa plasmid found in this strain. In addition, we compared these results with previous reports on strain SEPT13. Furthermore, once the genetic location of the adhesin operon is determined it could be cloned and expression of the adhesion protein could be studied to improve our understanding of the role of adhesion in Brazilian chicken flocks.

Material and Methods

Bacterial strains and growth media

Escherichia coli strain SEPT 13 was isolated from the liver of a chicken with clinical signs of septicaemia. The *E. coli* strains K12 MS101 (nalidixic acid resistant) and HB101 (streptomycin resistant) are non-pathogenic strains that were used as recipient strains for transformation experiments using the electroporation technique. *E. coli* strain LG 1522 [6] was used as an indicator strain for aerobactin production. *E. coli* strains R80 (all colicins), R81 (col I), R82 (col Ia), R83 (col Ib), R675 (col E1), R676 (col E3), R914 (col ROW-K), R915 (col V), and R996 (col B) were used as indicator strains for specific colicins. They were a gift from Dr. E. C. Souza, at the Federal University of Minas Gerais at Belo Horizonte, MG. *E. coli* V517 is a strain that harbors plasmids of different sizes (32, 5.12, 3.48, 3.03, 2.24, 1.69, 1.51, and 1.25 MDa); [20] they were used as molecular standards in the agarose gel electrophoresis. Plasmid pRT733 [43] containing transposon TnphoA was used for the mutagenesis experiments. LB and LA media [34] were used for routine bacterial growth. All strains were stored in LB medium containing 15% glycerol at -70°C to avoid the loss of plasmids.

Determination of antibiotic resistance levels

The resistance of antimicrobial drugs (ampicillin, kanamycin, streptomycin, tetracycline, and chloramphenicol) was determined as described by Chulasari and Suthienkul [8]. Concentrations of 5, 10, 25, 50, 100, 250, and 500 μg/ml were used to determine the resistance level for each antibiotic. The maximum concentration of an antibiotic that still had bacterial growth was considered the minimal inhibitory concentration for that antibiotic.

Pathogenicity assay

Pathogenicity assays were performed as described by Fantinatti *et al.* [14]. Briefly, a 1.0 ml suspension (LB medium, 37°C, 14-18 h; washed twice with and resuspended in 0.85% sterilized saline solution) of the strain to be tested was diluted ten-fold (10^{-1} to 10^{-11}) and 0.5 ml of each dilution was injected subcutaneously into the neck region of groups of six one-day-old-male chickens. These groups were observed throughout a 7-day period. The LD_{50} was calculated by the method of Reed and Muench [32] for each strain. All of the experiments were conducted with germ-free white leghorn chickens. Each group of animals was separated into cages that were cleaned daily and fed *ad libido* with sterile water and food.

Colicin production

Colicin was produced as described by Azevedo and da Costa [3]. Briefly, the strains were cultured overnight in LB medium at 37°C and a drop was plated onto LA agar. After the overnight incubation at 37°C all bacterial growth was destroyed by chloroform fumes and then overlaid with 3.0 ml of soft LA medium containing a colicin-indicator strain. The capacity for colicin production (Ia, Ib, E1, E3, K, and B) was determined by the presence of a clear halo around the destroyed bacterial colonies after an overnight incubation period.

Aerobactin production

Aerobactin production was assayed by the method of Carbonetti and Williams [6] using *E. coli* LG 1522 as the indicator strain. For this purpose, symmetric holes were made in the LA medium containing 200 μM α-α-dipyridyl and then filled with the supernatant of the bacterial growth (iron-free LB medium, 37°C, overnight) of each strain to be tested. Once the medium had absorbed all of the liquid, strain LG 1522 was inoculated onto its surface and the Petri dish incubated at 37°C. Growth of LG 1522 colonies, over 72 h, around a given hole, indicated the capacity of that strain to produce aerobactin.

Adhesion and invasion capacities of strains into HEp-2 cells

The capacity for adhesion and invasion of all strains into HEp-2 cells was studied as described by Scaletsky *et al.* [35] and Vidotto *et al.* [44], with slight modifications. Briefly, cultures of these cells were grown in 24-well tissue culture microplates (BD Falcon, USA) where sterile round cover slips (13 mm in diameter) were placed prior to the inoculation with the cells. The growth medium for each microplate well consisted of 0.9 ml of Eagle's minimal essential medium (MEM) with 10% fetal calf serum, 1% D-mannose, and 1% antibiotics solution (penicillin 100,000 U and streptomycin 100 μ/ml). The microplates were incubated in 10% CO_2 atmosphere at 37°C until a semi-confluent monolayer was formed. Afterwards, the monolayers were washed 3 times with sterile phosphate buffered saline (PBS) 0.05 M, pH 7.2. Then, 0.1 ml aliquots of the bacterial culture (37°C -18 h, in LB medium) containing 2×10^7 colony forming units (CFU) were added to the wells. After 3 h of incubation at 37°C, the monolayers were washed 10 times with PBS buffer, fixed with methanol for 10 min, stained with the May-Grunwald and Giemsa stains, and observed under bright field microscopy ($\times$1,000).

Adhesion of strains to tracheal epithelial cells

Adhesion to tracheal epithelial cells was evaluated as described by Dho and Lafont [9] and Pourbakhsh *et al.* [29] using 18-day avian SPF (specific pathogen free) embryonated eggs. Briefly, the trachea was aseptically removed from 18 day avian embryos, rinsed in PBS (pH 7.4), and cut in 5 mm sections. Adherence studies were performed in the 96-well-round-bottom microtiter plates, as described below: two trachea rings and 25 µl of Eagle medium with 5% calf serum were placed into each well. A suspension of each bacterium (10^9 cells/ml) previously grown on LB (37^oC - 18 h) was incubated with the tracheal rings at 37^oC for 30 min, after which they were washed with PBS and incubated for 4 h (37^oC). The tracheal sections were rinsed with PBS-formalin. The tracheal rings were dehydrated, xylol treated and blocked with paraffin. Five µm thick sections were cut using a microtome, mounted on glass slides, hydrated and stained with Giemsa. The adherence assay was performed in the presence and in the absence of 1% D-mannose.

Plasmid DNA extraction and agarose gel electrophoresis

Plasmid DNA was extracted as described by Sambrook *et al.* [34] and suspended in sterilized deonized water and stored frozen until use. The plasmid DNA to be used in the electroporation experiments was cleaned using the Wizard DNA Clean-up columns (Promega, USA). Plasmid DNA electrophoresis and ethidium bromide staining of the gels were carried out as described by Sambrook *et al.* [34].

Electroporation experiments

The electroparation assays were performed as described by Dower et al. [13] with minor modifications. For this, the recipient strains were grown in LB medium (50 ml, 37^oC, 150 rpm) until an absorbance of 0.5, at a wavelength above 500 nm. Then, they were extensively washed with iced 10% (10 ml) glycerol and resuspended in 100 µl of the washing solution. Next, 60 µl of the suspension was electroporated (2,500 V; 800 ohms of resistance; 25 µF of capacitance in 15.3 sec) with 20 µl of the plasmid DNA suspension in a Gene Pulser II (Bio-Rad, USA). Transformant strains were selected on the LA medium containing specific antibiotic markers for the recipient strains and the electroporated plasmid DNA.

Transposon mutagenesis

Transposon mutagenesis (TnphoA) was accomplished as described by Taylor *et al.* [43] using plasmid pRT733. Mutants were obtained on LA medium containing 40 µg/ml of 5-bromo-4-chloro-3-indolyl phosphate and selective antibiotics. Blue, kanamycin resistant colonies were analyzed by agarose gel electrophoresis to establish the plasmid DNA profiles. All strains that presented with an increased plasmid size, as observed by agarose gel electrophoresis, were tested for the LD_{50} using a method described previously.

TnphoA molecular probe and hybridization with plasmid DNA

A 3,450 bp DNA fragment of transposon TnphoA was cut from the plasmid pRT733 using the restriction enzyme *Bst* EII, and then purified from the agarose gel using the dialysis method as described by Sambrook *et al.* [34]. This fragment was labeled using the Alk-Phos kit (Amersham Pharmacia, Sweden), and then hybridized with plasmidial DNA (88 MDa mutagenized plasmid) fragments that were obtained after treatment with the restriction enzymes *Eco* RI, *Eco* RV and *Bst* EII; the fragments were separated by agarose gel electrophoresis as described by Sambrook *et al.* [34].

Electronic microscopy studies

The Electronic Microscopy was carried out as described by Sperandio and Silveira [39]. For this purpose, the bacterial strain was grown in LB medium at 37^oC, overnight. After centrifugation ($13,000 \times g$; 30 sec), the pellet was resuspended in 200 µl of milli-Q water and 10 µl of this growth was mixed and fixed with 1% phosphotungstic acid for 30 sec. This bacterial suspension was added onto a 400 mesh grid coated with Formvar; the grids were dried in a carbon-evaporator and observed using a transmission electronic microscope (LEO 906; LEO Elektronenmikroskopie, Germany).

Detection of pathogenicity related sequences by PCR

A total of 20 ng of genomic bacterial DNA was extracted as described by Ausubel *et al.* [2] and resuspended in TE buffer plus 10 mg/ml RNAse and used for PCR. The primers used for the amplification of the pathogenicity related sequences and the PCR conditions were the same as those described by the authors cited in Table 1. All amplification reactions were performed in a Mastercycle termocycle (Eppendorf, Germany). The PCR products were analyzed by electrophoresis in a 1.0% submersed agarose gel stained with ethidium bromide and visualized under UV light as described by Sambrook *et al.* [34].

Results

Table 2 shows the biological characteristics of the wild type SEPT 13 strain and its derivative strains. SEPT 13 is an APEC (wrinkled) strain that was isolated from a chicken with clinical signs of septicaemia. It expresses colicins Ia, Ib, E1, E3, K and B; it produces aerobactin and is resistant to ampicillin, tetracycline and streptomycin. In addition, it harbors five different plasmids 2.7, 4.7, 43, 56, and 88

Table 1. Genes and primers evaluated in strain SEPT13 and derivative strains

| Strains	Gene	Primers (5' – 3')	Fragment (bp)	References
APEC	*fim*A	GTTGATCAAACCGTTCAG AATAACGCGCCTGGAACG	331	[21]
APEC	*tsh*	GGGAATGACCTGAATGCTGG CCGCTCATCAGTCAGTACCAC	420	[22]
UPEC	*pap*A	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGATGCAATA	328	[19]
APEC	*csg*A	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC	200	[22]
UPEC	*afa*	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCG	710	[4]
UPEC	*sfa*	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410	[4]
EPEC	*eae*	ACGTTGCAGCATGGGTAACTC GATCGGCAACAGTTTCACCTG	815	[16]
EHEC	*lpf*A$_{O157/O141}$	CTGCGCATTGCCGTAAC ATTTACAGGCGAGATCGTG	412	[41]
EAEC	*fyu*A	GCCACGGGAAGCGATTTA CGCAGTAGGCACGATGTTGTA	787	[37]
EAEC	*irp*-2	AAGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	287	[37]
Shigella flexneri	*sit*A	CGCTGAAAGCAGTAGTTATC TTTTGACGACAGGGACCAG	608	[33]
EHEC	*tox*B	ATACCTACCTGCTCTGGATTGA TTCTTACCTGATCTGATGCAGC	1305	[42]
EIEC	*ial*	GTGGATGGTATGGTGAGG GGAGGCCAACAATTATTTCC	320	[23]
EHEC	*efa*	GAGACTGCCAGAGAAAG GGTATTGTTGCATGTTCAG	479	[24]
Yersinia enterocolitica	*inv*	CTGTGGGGAGAGTGGGGAAGTTTGG GAACTGCTTGAATCCCTGAAAACCG	570	[31]
EHEC	*chu*A	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279	[7]
E. coli K12	*yja*A	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211	[7]
EHEC	*Tsp*E4.C2	GAGTAATGTCCGGGGCATTCA CGCGCCAACAAAGTATTACG	152	[7]
E. coli K12	*fli*C	ATCGCACAAGTCATTAATACCCAAC CTAACCCTGCAGC AGAGACA	variable	[15]

MDa (Fig. 1, Lane 2). This strain demonstrated a D-mannose resistant diffuse adhesion to HEp-2 cells cultivated *in vitro* (Fig. 2A), an adherence to tracheal epithelial cells (Fig. 3A) and was able to invade HEp-2 cells (Table 2). Fimbriae expression was detected when this strain was studied under an electron microscope (Fig. 4A). In the one-day-old chicken assay the LD$_{50}$ of strain, SEPT 13 was determined to be 4.0×10^5 CFU/ml (Table 2). The PCR experiments demonstrated, in this strain only, the presence of *fimA*, *csgA* and *tsh* genes, and was negative for all the other genes as noted in Table 1 (data not shown).

Mutagenesis of the SEPT 13 strain with transposon TnphoA (Km^r, alcaline phosphatase gene) resulted in 12 mutant strains. Agarose gel electrophoresis of these strains demonstrated that the transposon TnphoA had been inserted into the 88 MDa plasmid that was increased in size (93 MDa), in three of these transformant strains. These strains where evaluated by the LD$_{50}$ pathogenicity assay; one of them was found to have a decrease in pathogenicity (LD$_{50}$ of 1.2×10^{12} CFU/ml) (Table 2). This mutant strain was termed strain ST16. In addition to the decreased pathogenicity, all other biological characteristics were present in the mutant ST16 (Table 2). To characterize the biological characteristics of the 93 MDa plasmid, a total
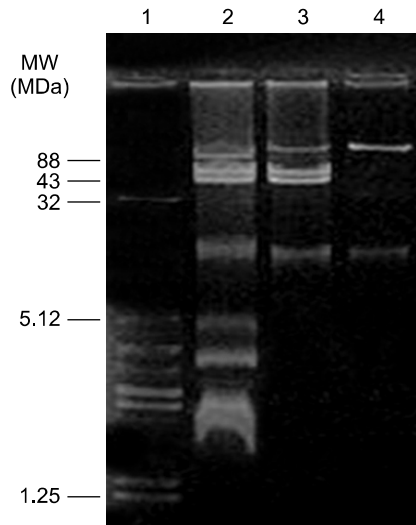
Fig. 1. Agarose gel electrophoresis (0.7%) of plasmid DNA from the SEPT13 strain, its derivative transformant strains and the reference plasmids Lane 1: Strain V517 (32 MDa), Lane 2: Strain SEPT13, Lane 3: Strain ST16, Lane 4: Recipient strain MS101 harboring the 93 MDa plasmid (Strain transformant A).
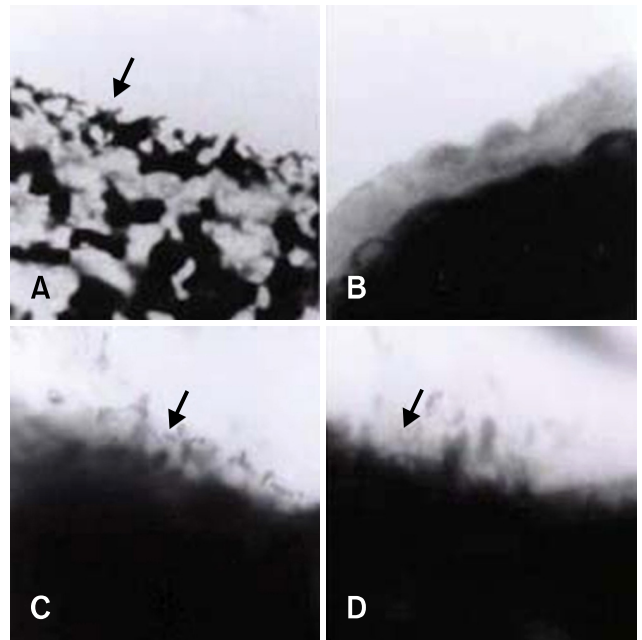
Fig. 3. Adhesion of strains SEPT 13 and its derivative transformant strains to tracheal epithelial cells. (A) Strain SEPT 13; (B) Recipient strain MS101; (C) Strain ST16; (D) Recipient strain MS101 harboring the 93 MDa plasmid (Strain transformant A). Arrowheads identify bacterial cells adherent to the tracheal epithelial cells. ×1,000.
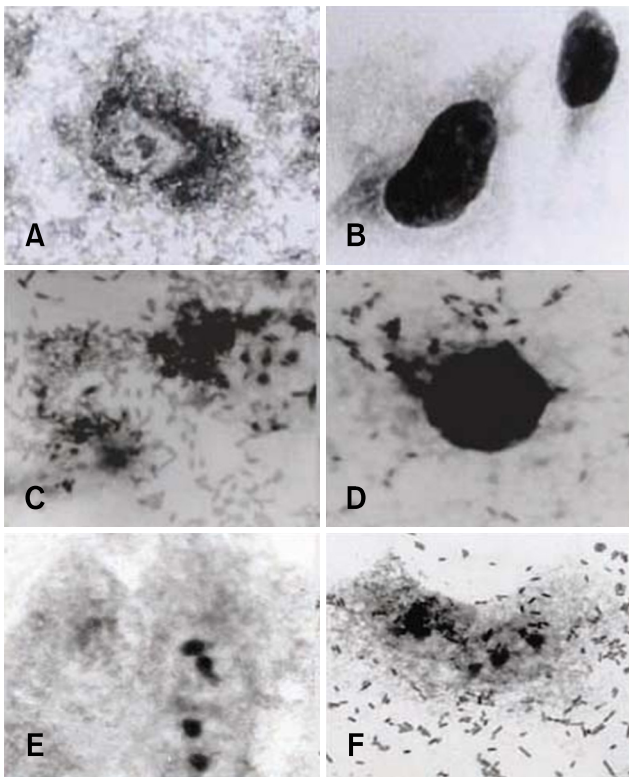
Fig. 2. Adhesion of strain SEPT13 and its derivative transformant strains to Hep-2 cells. (A) Strain SEPT 13; (B) Strain MS101 (C) Strain ST16; (D) Strain transformant A; (E) Strain HB101; (f) Strain transformant B. ×1,000.
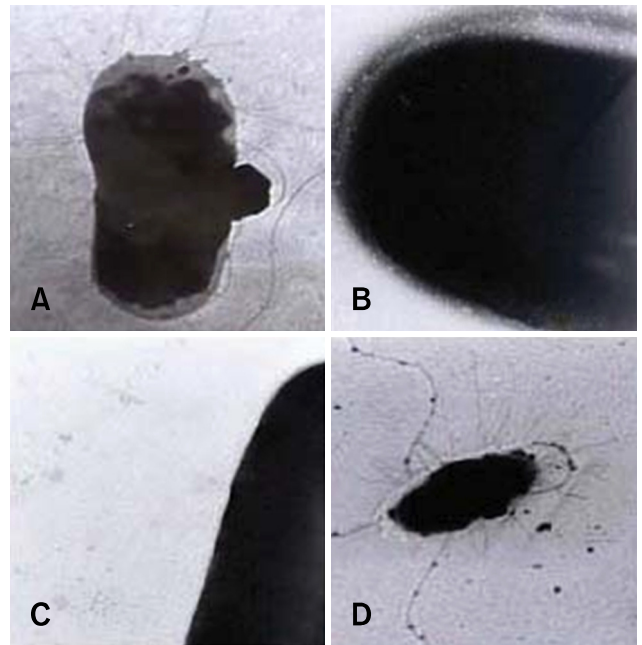
Fig. 4. Electron microscopy studies of fimbria expression by the *E. coli* strains. (A) Strain SEPT 13, ×32,000; (B) Recipient strain HB101, ×80,000; (C) Recipient strain HB101 harboring the 93MDa plasmid (Strain transformant B), ×40,000; (D) Strain MS101, ×18,000.

Table 2. Biological characteristics of the SEPT13 strain and its derivative transformants

Strains	LD 50% (CFU/ml)	Colicins	Aerobactin	Antibiotic Resistance*	Adhesion (Hep-2 cells)	Invasion (Hep-2 cells)	Adhesion (trachea cells)	Plasmids (Mda)	PCR[†]
13-Sep	4.0×10^5	Ia, Ib, E1, E3, K, B	+	Ap; Tc; Sm	DA	+	DA	2.7; 4.7; 43; 56; 88	*fimA, csgA, tsh*
ST16	$1,2 \times 10^{12}$	Ia, Ib, E1, E3, K, B	+	Ap, Tc, Sm, Km	DA	+	DA	2.7; 4.7; 43; 56; 93	*fimA, csgA, tsh*
A	$>10^{11}$	-	-	Km, NA	DA	-	DA	93	-
MS101	$>10^{11}$	-	-	NA	-	-	-	-	-
HB101	$>10^{11}$	-	-	Sm	-	-	ND	-	-
B	$>10^{11}$	-	-	Km; Sm	+	-	ND	93	-

*Ap: ampicillin, Sm: streptomicin, Tc: tetracycline, Km: kanamycine, NA: nalidixic acid; DA: diffuse adhesion, ND: not determined; [†]PCR detection of *fimA, csgA, papA* and *tsh* genes.

plasmidial DNA preparation of strain ST16 was electroporated into strains MS101 (non-pathogenic, nalidixic acid resistant) and HB101 (a non-fimbriated, non-pathogenic, streptomycin resistant). Although of different genetic backgrounds, both of the recipient strains are adhesion and invasion negative to HEp-2 cells. The transformant strains containing only the 93 MDa plasmid (corresponding to the 88MDa plasmid carrying the transposon TnphoA), as determined by agarose gel electrophoresis, were selected in the LB plates with Km, resulting in the transformant strains A (Fig. 1) and B (data not shown), derived from strains MS101 and HB101, respectively. Hybridization experiments using a 3,450 bp *Bst* EII fragment of transposon TnphoA as a molecular probe confirmed the insertion of TnphoA into the 93 MDa plasmid (data not shown).

Strains A and B were unable to produce colicin or aerobactin, were invasion negative for HEp-2 cells (data not shown) but had mannose resistant adhesion to this cell type (Figs. 2D and F, respectively). In this assay, the wild type strains MS101 (Fig. 2B) and HB101 (Fig. 2E) were non-adherent. On the other hand, and as previously pointed out, SEPT 13 and the isogenic mutant strain ST 16 presented with a diffuse adherence pattern (Figs. 2A and C, respectively). The PCR assay was unable to amplify any of the genes that were previously detected in the SEPT 13 strain (*tsh, csgA,* and *fimA*) using the genomes of transformants A and B as templates. In addition, strains A and B, as well as mutant ST16, had a LD_{50} of more than 10^{11} CFU/ml (Table 2) when evaluated by the one-day-old chicken assay.

The adherence of strains, onto the tracheal epithelial cells, was also assayed (Fig. 3). As expected, the MS101 strain was non-adherent (Fig. 3B). On the other hand, SEPT 13, ST16 and transformant A were adherent to the tracheal epithelial cells (Figs. 3A, B and D, respectively). Transformant B was not tested in this assay.

Electron microscopy studies were performed with strains SEPT13, ST16, transformant A, B, MS101, and HB101. With the exception of transformant B and HB101, all other strains, including the receptor strain MS101, exhibited fimbriae on their surface, as noted in Figure 4. Despite the absence of fimbrial structures on the surface of transformant B (Fig. 4C), this strain was able to adhere to HEp-2 cells (Fig. 2F), in contrast to the results exhibited by the HB101 recipient strain (Fig. 2E).

Discussion

The aim of this study was to correlate the presence of a high-molecular weight plasmid (88 MDa) with virulence and the biological traits of strain SEPT 13. For this, strain SEPT 13 was transposon-mutagenised resulting in a less virulent strain (strain ST 16).

In a previous study performed by Stehling *et al.* [40], a 43 MDa plasmid present in SEPT 13 was transferred to a recipient strain that resulted in a transformant called transformant E that expressed fimbriae and harbored the gene *tsh*. This gene was proposed as a candidate responsible for the adhesion and invasion characteristics of strain SEPT 13. They demonstrated that this plasmid (43 MDa) was not associated with the major factors responsible for pathogenicity in strain SEP 13 as observed in the one-day-old chicken assay. This is because the transfer of the plasmid to the recipient strains did not increase virulence.

The results of the mutagenesis experiments herein accomplished suggest that the 88 MDa plasmid might be responsible, at least in part, for the pathogenicity observed in strain SEPT 13. This is because strain ST16 was less virulent than SEPT 13 and had the insertion of the transposon in this plasmid as indicated by the plasmid profile and hybridization experiments. Previous studies

[25,38,45] have also indicated that high-molecular weight plasmids could have genes involved in the pathogenicity of avian *E. coli* strains.

Transformant A exhibited fimbriae expression and adhesion to HEp-2 and chicken embryo tracheal cells, but was unable to invade the HEp-2 cells. The fimbriae expressed by this transformant cell, might have been expressed by the MS101 strain; therefore, we transferred the plasmid to a non-fimbriated strain (HB101). As a result, no fimbriae were expressed by the new transformant strain (transformant B). However, the transformant strains (A and B) had adhesion characteristics identified in the HEp-2 cells. This indicates that these strains were expressing adhesin that was not expressed by strains MS101 and HB101. In addition, transformant A was able to adhere to chicken embryo tracheal epithelial cells, unlike strain MS101. These results suggest that afimbrial adhesins were encoded by genes present in the 88 MDa plasmid, and likely responsible for the adhesion characteristics of the transformant strains. In addition, the insertion of transposon TnphoA in the 88 MDa plasmid did not knock out the adhesion genes. Although strain SEP13 harbors *fimA*, *tsh* and *csgA* genes, as detected by PCR, they were not responsible for the observed adhesion in the transformed strains since they were not transferred to these strains.

Our results support those of Stehling *et al.* [40]; in that the SEPT13 strain was found to have, a 43 MDa plasmid, with genes responsible for the expression of fimbrial adhesins that are responsible for the adhesion and invasion properties observed in this strain. In addition, this strain appears to have afimbrial adhesin genes located in the 88 MDa plasmid responsible for the adhesion properties herein studied. These observations suggest that the SEPT 13 strain has more than one adhesin type responsible for all adhesion properties; as demonstrated by adhesion genes expressed in different plasmids. The attenuation of virulence exhibited by the mutant ST16 in the one-day-old chicken assay is remarkable. Considering that the invasion capacity is mediated by the 43 MDa plasmid, we speculate that the 88 MDa plasmid may carry genes related to serum resistance or *in vivo* replication, abilities that almost invariably are exhibited by bacteria that cause systemic infections. Therefore, insertion mutagenesis of the 88 MDa plasmid, mediated by the TnphoA transposon, probably impaired the function of the genes essential for mediation or regulation of the expression of such characteristics. However, further studies are needed to characterize these genes.

In conclusion, the results of this study show that a 88 MDa plasmid has genes responsible for adhesion in avian pathogenic *E. coli* to *in vitro* cultured cells and to tracheal epithelial cells. These adhesion characteristics are likely mediated by a non-fimbriated adhesin. In addition, this plasmid probably carries genes or operons essential for the pathogenicity observed in the one-day-old chicken assay, which requires additional study. These results, together with those obtained in a previous work conducted by our research group [40] indicate that the pathogenesis of APEC is very complex and further investigations are necessary to improve our understanding of it. In addition, the recognition that these strains express more than one adhesin suggest that molecular cloning of these compounds may help improve our understanding of the pathogenicity of avian *Escherichia coli*.

Acknowledgments

References

1. **Arp LH, Jensen AE.** Piliation, hemagglutination, motility, and generation time of *Escherichia coli* that are virulent or avirulent for turkeys. Avian Dis 1980, **24**, 153-161.
2. **Ausubel FM, Brente R, Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K.** Cur Prot Mol Biol Green Publishing Associates, Brooklyn, NY, 1988.
3. **Azevedo JL, da Costa SOP.** Exercícios Práticos de Genética. pp. 171-174, EDVSP, São Paulo, 1973.
4. **Blanco M, Blanco JE, Alonso MP, Mora A, Balsalobre C, Muñoa F, Juárez A, Blanco J.** Detection of *pap*, *sfa* and *afa* adhesin-encoding operons in uropathogenic *Escherichia coli* strains: relationship with expression of adhesins and production of toxins. Res Microb 1997, **148**, 745-755.
5. **Campos TA, Stehling EG, Ferreira A, Castro AFP, Brocchi M, Silveira WD.** Adhesion properties, fimbrial expression and PCR detection of adhesin-related genes of avian *Escherichia coli* strains. Vet Microbiol 2005, **106**, 275-285.
6. **Carbonetti NH, Williams PH.** A cluster of five genes specifying the aerobactin iron uptake system of plasmid ColV-K30. Infect Immun 1984, **46**, 7-12.
7. **Clermont O, Bonacorsi S, Bingen E.** Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 2000, **66**, 4555-4558.
8. **Chulasiri M, Suthienkul O.** Antimicrobial resistance of *Escherichia coli* isolated from chickens. Vet Microbiol 1989, **21**, 189-194.
9. **Dho M, Lafont JP.** *Escherichia coli* colonization of the trachea in poultry: comparison of virulent and avirulent strains in gnotoxenic chickens. Avian Dis 1982, **26**, 787-797.
10. **Dho M, Lafont JP.** Adhesive properties and iron uptake ability in *Escherichia coli* lethal and lethal for chicks. Avian Dis 1984, **28**, 1016-1025.
11. **Dho-Moulin M, Fairbrother JM.** Avian pathogenic *Escherichia coli* (APEC). Vet Res 1999, **30**, 299-316.
12. **Dozois CM, Daigle F, Curtiss R III.** Identification of

pathogen-specific and conserved genes expressed *in vivo* by an avian pathogenic *Escherichia coli* strain. Proc Natl Acad Sci U S A 2003, **100**, 247-252.

13. **Dower WJ, Miller JF, Ragsdale CW.** High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res 1988, **16**, 6127-6145.

14. **Fantinatti F, Silveira WD, Castro AFP.** Characteristics associated with pathogenicity of avian septicemic *Escherichia coli* strains. Vet Microbiol 1984, **41**, 74-86.

15. **Fields PI, Blom K, Hughes HJ, Helsel LO, Feng P, Swaminathan B.** Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-restriction fragment length polymorphism test for identification of *E. coli* O157:H7 and O157:NM. J Clin Microbiol 1997, **35**, 1066-1070.

16. **Gannon VP, Rashed M, King RK, Thomas EJ.** Detection and characterization of the eae gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. J Ciln Microbiol 1993, **31**, 1268-1274.

17. **Gross WB.** Diseases due to *Escherichia coli* in poultry. In: Gyles CL (eds.). *Escherichia coli* in Domestic Animals and Humans. 1st ed. pp. 237-259, CAB International, Wallingford, 1994.

18. **La Ragione RM, Woodward MJ.** Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Res Vet Sci 2002, **73**, 27-35.

19. **Le Bouguenec C, Archambaud M, Labigne A.** Rapid and specific detection of the *pap*, *afa*, and *sfa* adhesin-encoding operons in uropathogenic *Escherichia coli* strains by polymerase chain reaction. J Clin Microbiol 1992, **30**, 1189-1193.

20. **Macrina FL, Kopecko DJ, Jones KR, Ayers DJ, McCowen SM.** A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid 1978, **1**, 417-420.

21. **Marc D, Dho-Moulin M.** Analysis of the fim cluster of an avian O2 strain of *Escherichia coli*: serogroup-specific sites within *fimA* and nucleotide sequence of fimI. J Med Microbiol 1996, **44**, 444-452.

22. **Maurer JJ, Brown TP, Steffens WL, Thayer SG.** The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin Tsh among avian *Escherichia coli*. Avian Dis 1998, **42**, 106-118.

23. **Nataro JP, Kaper JB.** Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 1998, **11**, 142-201.

24. **Nicholls L, Grant TH, Robins-Browne RM.** Identification of a novel genetic locus that is required for in vitro adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. Mol Microbiol 2000, **35**, 275-288.

25. **Nolan LK, Wooley RE, Cooper RK.** Transposon mutagenesis used to study the role of complement resistance in the virulence of an avian *Escherichia coli* isolate. Avian Dis 1992, **36**, 398-402.

26. **Olsén A, Jonsson A, Normark S.** Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature 1989, **338**, 652-655.

27. **Olsén A, Arnqvist A, Hammar M, Sukupolvi S, Normark S.** The RpoS sigma factor relieves H-NS-mediated transcriptional repression of csgA, the subunit gene of fibronectin- binding curli in *Escherichia coli*. Mol Microb 1993, **7**, 523-536.

28. **Orndorff PE.** *Escherichia coli* type 1 pili. In: Miller VL, Kaper JB, Portnoy DA, Isberg RR. (eds.). Molecular Genetics of Bacterial Pathogenesis. 1st ed. pp. 91-111. ASM Press, Washington DC, 1994.

29. **Pourbakhsh SA, Boulianne M, Martineau-Doizé B, Dozois CM, Desautels C, Fairbrother JM.** Dynamics of *Escherichia coli* infection in experimentally inoculated chickens. Avian Dis 1997, **41**, 221-233.

30. **Provence DL, Curtiss R III.** Isolation and characterization of a gene involved in hemagglutination by an avian pathogenic *Escherichia coli* strain. Infect Immun 1994, **62**, 1369-1380.

31. **Rasmussen HN, Rasmussen OF, Andersen JK, Olsen JE.** Specific detection of pathogenic *Yersinia enterocolitica* by two-step PCR using hot-start and DMSO. Mol Cell Probes 1994, **8**, 99-108.

32. **Reed LJ, Muench H.** A simple method of estimating fifty per cent endpoints. Am J Epidemiol 1938, **27**, 493-497.

33. **Runyan-Janecky LJ, Reeves SA, Gonzales EG, Payne SM.** Contribution of the Shigella *flexneri* Sit, Iuc, and Feo iron acquisition systems to rion acquisition *in vitro* and in cultured cells. Infect Immun 2003, **71**, 1919-1928.

34. **Sambrook J, Fritsch EF, Maniatis T.** Molecular Cloning: a Laboratory Manual. 2nd ed. p. 1584. Cold Spring Harbor, New York, 1989.

35. **Scaletsky ICA, Silva MLM, Trabulsi LR.** Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. Infect Immun 1984, **45**, 534-536.

36. **Schouler C, Koffmann F, Amory C, Leroy-Sétrin S, Moulin-Schouleur M.** Genomic subtraction for the identification of putative new virulence factors of an avian pathogenic *Escherichia coli* strain of O2 serogroup. Microbiology 2004, **150**, 2973-2984.

37. **Schubert S, Rakin A, Karch H, Carniel E, Heesemann J.** Prevalence of the "high-pathogenicity island" of Yersinia species among *Escherichia coli* strains that are pathogenic to human. Infect Immun 1998, **66**, 480-485.

38. **Sekizaki T, Nonomura I, Imada Y.** Loss of virulence associated with plasmid curing of chicken pathogenic *Escherichia coli*. Nippon Juigaku Zasshi 1989, 51, 659-661.

39. **Sperandio V, Silveira WD.** Comparison between enterotoxigenic *Escherichia coli* strains expressing F42, F41 and K99 colonization factors. Microbiol Immun 1993, **37**, 869- 875.

40. **Stehling EG, Campos TA, Ferreira A, Silveira WD.** Adhesion and invasion characteristics of a Septicaemic avian *Escherichia coli* strain are plasmid mediated. J Appl Res Vet Med 2003, **1**, 27-36.

41. **Szalo IM, Goffaux F, Pirson V, Piérard D, Ball H, Mainil J.** Presence in bovine enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* of genes encoding for putative adhesins of human EHEC strains. Res Microbiol 2002, **153**, 653-658.

42. **Tarr CL, Large TM, Moeller CL, Lacher DW, Tarr PI, Acheson DW, Whittam TS.** Molecular characterization of a serotype O121:H19 clone, a distinct Shiga toxin-producing clone of pathogenic *Escherichia coli*. Infect Immun 2002, **70**,

6853-6859.

43. **Taylor RK, Manoil C, Mekalanos JJ.** Broad-host-range vectors for delivery of TnphoA: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J Bacteriol 1989, **171**, 1870-1878.

44. **Vidotto MC, Muller EE, de Freitas JC, Alfieri AA,** **Guimarães IG, Santos DS.** Virulence factors of avian *Escherichia coli*. Avian Dis 1990, **34**, 531-538.

45. **Wooley RE, Gibbs PS, Dickerson HW, Brown J, Nolan LK.** Analysis of plasmids cloned from a virulent avian *Escherichia coli* and transformed into *Escherichia coli* DH5-alpha. Avian Dis 1996, **40**, 533-539.