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# Cloning, expression and sequence diversity of iss gene from avian pathogenic *Escherichia coli* (APEC) isolated in Brazil

## Clonagem, expressão e diversidade na sequência do gene *iss* de *Escherichia coli* patogênica para aves (APEC), isolada no Brasil

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### Abstract

The Iss (Increased serum survival) protein is an important characteristic of resistance to complement system of avian pathogenic *Escherichia coli* (APEC). The objectives of this work were to cloning and verify the sequence diversity of *iss* gene from APEC and characterize the recombinant Iss protein. The *iss* gene of 309 bp was amplified by PCR, cloned and expressed in *E. coli* BL21 (DE3) using the pET SUMO vector. The *iss* gene from APEC9 strain was classified as *iss* type 1 by differentiation of the three *iss* gene allele types. The protein was expressed by induction of IPTG and purified in resin charged with the nickel ion. Antibodies IgY anti rIss reacted with rIss showing a molecular mass of 22 kDa, corresponding 11kDa of Iss protein and 11 kDa SUMO protein.

**Key words:** Avian pathogenic *E. coli* (APEC), gene *iss*, cloning, RIss, serum resistance

### Resumo

A proteína Iss (increased serum survival) é uma importante característica de resistência ao sistema complemento da *Escherichia coli* patogênica para aves (APEC). Os objetivos deste trabalho foram clonar e verificar a diversidade da sequência do gene *iss* de APEC e caracterizar a proteína Iss recombinante. O gene *iss* de 309 bp foi amplificado por PCR, clonado e expresso na *E. coli* BL21 (DE3) utilizando o vetor pET SUMO. O gene *iss* da APEC9 foi classificado como *iss* tipo 1 pela diferenciação entre 3 tipos de *iss* alelos. A proteína Iss foi expressa pela indução com IPTG, purificada em coluna com resina ligada ao íon níquel e utilizada na imunização de galinhas poedeiras. Anticorpos da classe IgY anti rIss reagiram com a proteína rIss, a qual apresentou massa molecular de 22 kDa, correspondendo 11kDa da Iss e 11 kDa da proteína SUMO.

**Palavras-chave:** *Escherichia coli* patogênica para aves (APEC), gene *iss*, clonagem, riss, resistência sérica

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## Introduction

*Escherichia coli* strains designated as avian pathogenic *E. coli* (APEC) possess specific virulence factors and are able to cause avian colibacillosis (VIDOTTO et al., 1990; DHO-MOULIN; FAIRBROTHER, 1999). This disease is a serious problem for the poultry industry, since it causes high economic losses. The most severe manifestation of avian colibacillosis is septicemia, which is characterized by air sacculitis, pericarditis, perihepatitis, and salpingitis (GROSS, 1994).

Several potential virulence genes were identified in APEC, and their virulence-associated bacterial properties include adherence to the respiratory tract, resistance to the immunological defences, multiplication under iron-restricted conditions, and production of cytotoxic effects (DELICATO et al., 2003; DHO-MOULIN, FAIRBROTHER, 1999; VANDEKERCHOVE et al., 2005). The genes more frequently found in pathogenic isolates and not detected in faecal isolates from healthy birds are *tsh*, *iutA*, *iss*, *cvaC*, *papC*, which were found in different associations among several putative pathotypes (DELICATO et al., 2003; LARAGIONE, WOODWARD, 2002; RODRIGUEZ-SIEK et al., 2005a, 2005b).

Recently we cloned and characterized the *tsh* and *iutA* genes from APEC. The anti-Tsh inhibited the hemagglutinating activity of strains APEC13 and BL21/pET101-*tsh*. Anti-IutA IgY was able to inhibit the IutA biological activity, inhibiting the sensitivity to cloacin DF13 of APEC9 (SIMÕES et al., 2006; TOKANO et al., 2008). However, anti-IutA IgY did not inhibit the growth of APEC9 in M9 and did not protect chickens inoculated with APEC, suggesting that APEC possess another iron acquisition mechanism distinct of aerobactin (TOKANO et al., 2008).

Published research has shown that the increased serum survival gene (*iss*) is strongly correlated with an APEC isolate's ability to cause disease in poultry (RODRIGUEZ-SIEK et al., 2005; LYNNE et al.,

2007) and has been localized to large plasmids that typify the APEC pathotype (GIDDINGS et al., 2002; JOHNSON; JOHNSON, NOLAN, 2006). Serum resistance in *E. coli* has been related to several structural factors including a K1 antigenic capsule (LEYING et al., 1990) and certain outer membrane proteins, including TraT, Iss, and OmpA (BINNS, MAYDEN; LEVINE, 1982; CHUBA; PALCHAUDHURI; LEON, 1986; WEISER, GOTSCHLICH, 1991).

The protein Iss product of *iss*, is thought to occur as a 10 to 11 kD lipoprotein in the bacterial outer membrane (HORNE et al., 2000) and presents 90% similarity with the Bor protein, a lipoprotein of the cell envelope of *E. coli* lambda lysogens (LYNNE et al., 2007).

Recently, an alignment of all of *iss* and *bor* sequences revealed three genetically distinct alleles of *iss*, which have been designated *iss* types 1 to 3 and can discriminate the *E. coli* pathotypes (JOHNSON; WANNEMUEHLER; NOLAN, 2008).

The objective of this study was cloning, sequencing and expression of the *iss* gene from APEC isolated in Brazil, and in silico comparative sequence analysis of *iss* from our Brazilian APEC vs. other *iss* sequences in GenBank.

## Materials and Methods

### Bacterial strain

The avian pathogenic *Escherichia coli* strain APEC9 used in this study was recovered from the trachea of a colisepticemic chicken in the Parana State-Brazil (VIDOTTO et al., 1990). This strain shows resistance to tetracycline, ampicillin and serum complement. It produces colicin V, and has an iron uptake system mediated by aerobactin and carries one large plasmid of approximately 120 kilobases (Kb) (VIDOTTO et al., 1990). The APEC9 strain presented serotype O2:H9:K1 (MOURA; IRINO; VIDOTTO, 2001) and was pathogenic to 1-day-old chickens by means of pathogenicity test,

presenting LD<sub>50</sub> of 1 x 10<sup>5</sup> cells/ ml. The *iutA*, *tsh*, *iss*, *papC*, *papG* and *cvaC* genes were detected in the *E. coli* APEC9 strain (DELICATO et al., 2002; DELICATO et al., 2003).

#### *Cloning and sequence analysis of the iss gene*

The entire *iss* open reading frame was amplified from APEC9 genomic DNA by PCR. A pair of primers was constructed according to sequences in GenBank, *iss* forward 5' – ATGATGCAGGATAATAAGATGAAAAAATG – 3' and *iss* reverse- 5'-CTATTGTGAGCAATATACCCGG – 3'. PCR was carried out in a total volume of 50 µl containing 50 ng of DNA template, 1 µl each of the primers at 20 pmol, and 200 µM of each deoxynucleoside triphosphate, 2U Taq DNA polymerase (Invitrogen Life Technologies, São Paulo, Brazil). The PCR conditions were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, annealing at 50°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 7 min in a thermal cycler (mastercycler personal eppendorf®). The amplified DNA was visualized in 1.0% agarose gels stained with ethidium bromide.

PCR product was quantified and 50 ng were used as insert in the pET SUMO® vector (5.5 Kb) (Invitrogen, Carlsbad, CA, USA). Chemically competent *E. coli* host strain Mach cells (Invitrogen) were then transformed with 3 µl of the cloning reaction. The transformants colonies were selected on plates containing 30 µg of kanamicin, and the recombinants plasmids were extracted by alkaline lysis (SAMBROOK; FRITSCH; MANIATIS, 1990). The presence of *iss* genes were confirmed by PCR and subsequent restriction enzyme digestion of recombinant plasmids with EcoRI and SmaI. The correct position of *iss* gene was confirmed by sequencing with primers SUMO forward (5'-AGATTCTTGTACGACGGTATTAG-3') T7 reverse (5'-TAGTTATTGCTGAGCGGTGG-3')

and *iss* primers, utilizing commercial kit BigDye Terminator (Applied Biosystems, CA, USA).

The obtained sequences were analyzed by BLAST through the NCBI website (<http://www.ncbi.nlm.nih.gov/>) to verify the sequence identity. DNA and amino acid sequence analyses were carried out by the software “CAP3 Contig Assembly Program” and “Clustal W (1.81) Multiple Sequence Alignments” and “Six Frame Translation of Sequence” (LARKIN et al., 2007).

#### *Expression of genes on E. coli strain and purification of recombinants Iss proteins.*

The recombinant plasmids were utilized in transformation reactions with *E. coli* BL21 Star (DE3) One Shot (Invitrogen Life Technologies, Sao Paulo, Brazil). The BL21/pET SUMO-*iss* strain was grown to an optical density of OD<sub>600 nm</sub> 0.5, and IPTG (Invitrogen Life Technologies, São Paulo, Brazil) was added to 1mM and aliquots were removed at different times to choose the best time for expression. The cells were collected by centrifugation and treated with the buffer containing 6 M guanidine-HCl and sonicated on the ice with three 5-second pulses at the high intensity. The lysate was then centrifuged at 3000 x g for 15 min and the supernatant was transferred to Ni-NTA resin (Qiagen, Sao Paulo, Brazil); previously washed with the Denaturing Binding Buffer (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, pH 8.0). The supernatant and resin were incubated for 1h on a rotation wheel. Then the resin was washed once with the Denaturing Binding Buffer at pH 8.0, twice with the buffer at pH 6.3, and three times with the buffer at pH 5.9. The protein was eluted using 8 M urea buffer at pH 4.0. The protein concentration was measured using the Bradford method (1976) and analyzed on SDS-PAGE 15%.

### *Production of IgY and hyperimmune serum in hens*

The procedures used were approved by the Ethics Committee for Experiments in Animals (CEEAA, nº 39/07), Universidade Estadual de Londrina. Two Shaver breed chickens were utilized for the production of IgY anti-rIss. Each animal received three inoculations of 100µg (LARSSON; CARLANDER; WILHELMSSON, 1998) of protein at intervals of 14 days by the intramuscular via. During the first inoculation the complete Freund adjuvant (Sigma Immuno Chemicals, São Paulo, Brazil) was used, while the incomplete Freund adjuvant was administered in all other inoculations. The eggs were collected daily, beginning one week before immunization until two weeks after the last inoculation. The IgY extraction was realized as described Akita and Nakai (1993). The IgY purifity was then centrifuged, the obtained sediment resuspended in sterile water dialysed and maintained at - 20 °C until usage.

Extractions of post-immunization IgY were adsorbed with *E. coli* BL21 Star for use in Western blot.

### *SDS PAGE and Western blot*

The expression and the localization of recombinant Iss was demonstrated by SDS-PAGE and Western blot. Purified recombinant proteins were loaded in a 15% SDS-PAGE gel. The gel were either stained with Comassie Brillant blue or transfer to a nitrocellulose membrane (Amersham International, Amersham, UK) for Western blotting analysis (TOWBIN; GORDON, 1984). The membrane was blocked with 5% skim milk for 1 h at room temperature, washed in PBS-T (PBS + 0.1% Tween 20), and incubated for 1h with a 1:500

dilution of anti-rIss. The membrane were then incubated with chicken anti-IgY (1:10,000) stained with peroxidase (Sigma). The membrane was washed and the rIss were detected by means of the enhanced chemiluminescence (ECL) Western Blotting System (Amersham International, Amersham, UK). Protein molecular mass markers were used as standards.

## **Results**

The insertion of the *iss* gene of APEC9 into the plasmids pETSUMO/*iss* was confirmed. The recombinant plasmid pETSUMO/*iss*, which was cleaved with the enzyme EcoRI, presented molecular mass of 5.8 Kb, 5.5 Kb of the vector and 0.3 Kb of the *iss* gene. The *iss* gene was amplified using the pETSUMO/*iss* plasmids, and the sequence of the *iss* gene showed that it was inserted at the correct positions into the vector.

The complete *iss* sequence from APEC9 was deposited in GenBank (Accession Nº FJ824853). The Figure 1 shows the alignment of the *iss* sequence from APEC9 with the sequence of three genetically distinct alleles of *iss*, designated *iss* types 1 to 3 and with the *bor* sequence, demonstrating that APEC9 has the *iss* gene type 1. There was 100% of similarity among the *iss* sequence from APEC9 with those of others APEC strains isolated of different countries and different serotypes; from USA O1:K1 (DQ381420), O2 (AF042279, AY545598), O78:K80 (AF449498), O103 (CP001232); from Australia O non-typeable:H28 (EU330199); from Iran O78 (FJ416147) and from China O1 (DQ295188), O2 (DQ295187), O78 (DQ309288, DQ309289, DQ309290, O109 (DQ309281, DQ309291), O119 (DQ309292). Also, this sequence type 1 was found in chicken fecal strain O5 from China (DQ299279, DQ29980, DQ299399, DQ299400).

IssAPEC9	ATGCAGGATAATAAGATGAAAAAAATGTTATTTTCTGCCGCTCTGGCAATGCTTATTAC	
	AGGATGTGCTCAACAAACGTTTACTGTTGGAAACAAACCGA.....	100
Iss type1	.....	100
Iss type2	.....G.....G.....	100
Iss type3	.....G.....G.....T.....	100
Bor	.....C.C.CG.A.T.G....CC.....G.A.....CA.....A.....	085
IssAPEC9	CAGCAGTAACACCAAAGGAAACCATCACTCATCATTTCTTCGTTTCGGGAATTGGACA	
	AGAGAAAACTGTTGATGCAGCCAAAATTTGTGGCGGTGCAGA .....	200
Iss type1	.....	200
Iss type2	.....C..C.....G.....C.....	200
Iss type3	.....C.....G.GA....C.....A.....T.....	200
Bor	.....G.....C.....T.....G.GA....C.....T.....C.....	185
Iss APEC9		
IssAPEC9	AAATGTTGTTAAAACAGAAACTCAGCAAACATTCGTAAATGGATTGCTCGGTTTTATC	
	ACTTTTGGCATCTATACTCCGCTGGAAGCCCGGGTATATTGC.....	300
Iss type1	.....	300
Iss type2	.....C.....T...A...T.....G.T.G.....	300
Iss type3	.....C.....T...A...T.....G.T.G.....	300
Bor	.....C.....T...A...T.....G.T.G.....	285
Iss APEC9 TCACAATAG		309
Iss type1	.....	309
Iss type2	.....A.....	309
Iss type3	.....A.....	309
Bor....A.....	.....A.....	294

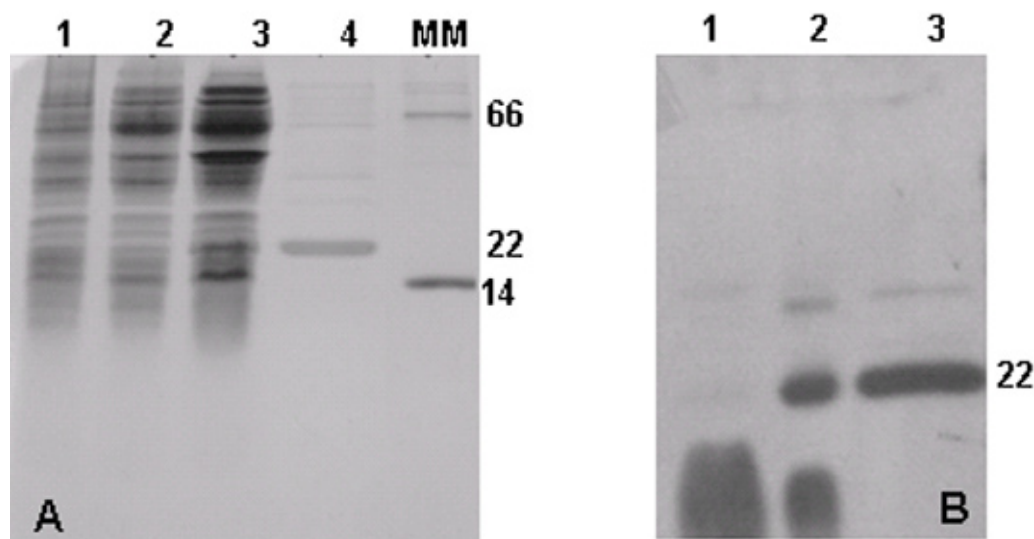
**Figure 1.** Alingment of *iss* gene of APEC 9 strain from Parana State-Brazil and *iss* type 1, 2, 3, and *bor* genes sequences, indicating the nucleotides that are different from APEC 9 and type 1 sequences.

The Fig. 2A shows the rIss protein, an expected band around 22 kDa, corresponding 11 kDa of Iss and 11 kDa of SUMO protein, which was more evident after 4 h of induction with IPTG (lane 3). This band was observed in the insoluble fraction or inclusion bodies and was absent in the negative control. rIss was solubilized and purified with the Ni-NTA purification system under denaturing

conditions. The results obtained from SDS-PAGE showed that rIss was successfully expressed in BL21 (DE3) and purified by means of Ni-NTA resin (Fig.2 A, lane 4), although the SUMO-Iss was not cleaved with SUMO protease at different conditions as manufacturer's recommendations.

The expression of rIss protein and its reactivity with the hen hyper-immune serum by Western blotting are shown in Figure 2 B.





**Figure 2.** Expression of the rIss protein by clone *E. coli*/pETSUMO-*iss* and immunological response of this protein in chickens. (A) SDS-PAGE 15% stained with Coomassie brilliant blue. Lane 1- *E. coli* BL21; lane 2 – BL21/pETSUMO-*iss* non induced; lane 3 – BL21/pETSUMO-*iss* induced with IPTG 1mM; lane 4 – eluted rIss; lane 5 – Mass Molecular. The band of approximately 22 kDa correspond to recombinant Iss protein. (B) Western blot with anti-rIss IgY and anti-chicken IgG conjugated with peroxidase. Lane 1 – *E. coli* BL21; lane 2 – clone BL21/pETSUMO-*iss* induced with IPTG 1mM; lane 3 – eluted rIss.

## Discussion

APEC has been widely studied for its role as agent extraintestinal infections, which causes high losses in the poultry industry (MINHARRO et al., 2001; ASSIS et al., 2003; BARNES, GROSS, 1997). An important gene responsible by serum resistance presented by APEC is the *iss* gene (BINNS; MAYDEN; LEVINE, 1979; LYNNE et al., 2007).

Recently, the differentiation of the three *iss* gene allele types in *E. coli* provides an additional tool for discriminating among *E. coli* pathotypes (JOHNSON; WANNEMUEHLER; NOLAN, 2008). The plasmid-borne *iss* allele (designated type 1) was highly prevalent among APEC (78%) and neonatal meningitis-associated *E. coli* isolates (66%) but not among uropathogenic *E. coli* isolates. The types 2 and 3 was highest among necrotoxicogenic and human ExPEC strains, respectively (JOHNSON; WANNEMUEHLER; NOLAN, 2008). The different *iss* types appear to have evolved from a *bor*-containing phage precursor, with several key events leading to the current *iss* alleles present

on different prophage elements and conjugative plasmids (JOHNSON; WANNEMUEHLER; NOLAN, 2008).

In this work, we sequenced the *iss* sequence from APEC9 (O2:H9:K1) and the alignment this sequence with those described in the GeneBank showed that it belong to *iss* type 1 (Fig.1). This sequence has 100% of similarities with those of different serotypes APEC strains from of USA, Australia, Iran and China. In China the sequence type 1 was found in *E. coli* O5 strain isolated from chicken fecal, however we found *E. coli* O5 as APEC in Brazil (MOURA; IRINO; VIDOTTO, 2001).

The *iss* type 1, as well as others genes associated with the virulence of APEC, is commonly plasmid-linked (JOHNSON et al., 2002; JOHNSON; JOHNSON; NOLAN, 2006; RODRIGUEZ-SIEK et al., 2005a, 2005b, Skyberg et al., 2006) and the APEC plasmids may serve as reservoirs of resistance or virulence genes for human ExPEC. Thus, APEC could be a possible source of UPEC causing UTIs or other diseases in human beings

(EWERS et al., 2007; JOHNSON et al., 2007; RAMCHANDANI et al., 2005; RODRIGUEZ-SIEK et al., 2005a). Several plasmid-linked APEC-derived sequences (*tsh*, *cvaB*, *traR*, *traC* and *sopB*) were predominantly present in APEC, as compared to UPEC (KARIYAWASAM; SCACCIANOCE; NOLAN, 2007), however the *iss* gene of UPEC is commonly found in the chromosome and it is of the *iss* type 2 (JOHNSON; WANNEMUEHLER; NOLAN, 2008). In our analysis, the *iss* type 1 was found in only a plasmid of UPEC (AY205565).

Iss and Bor proteins are exposed on *E. coli* outer membrane where they may be recognized by the host's immune system (LYNNE et al., 2007). In this work, the *iss* gene was successfully cloned into pETSUMO and the recombinant protein SUMO-Iss was purified in Ni-NTA resin with denaturing conditions (Fig.2). Although SUMO, expressed by pETSUMO vector, increases the solubility of recombinant proteins, the results showed that rIss is insoluble. The SUMO-Iss was not cleaved with SUMO protease and the antibodies elicited by SUMO-Iss were directed against SUMO and not Iss. This response can be due the size of SUMO-Iss that is two times the size of Iss. Others authors also cloned the *iss* gene, but they used the pGEX-6P-3 vector, and the purification of protein was by affinity chromatography with Glutathione-Sepharose (FOLEY et al., 2000; LYNNE; FOLEY; NOLAN, 2006). However, chicken immunize with GST-Iss were able to produce antibody against Iss (LYNNE; FOLEY; NOLAN, 2006).

In conclusion, the *iss* gene from APEC 9 strain is the genotype 1 that is highly prevalent among APEC, and the antibodies IgY anti recombinant Iss could be used in diagnostic protocols.

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