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Expression and analysis of the complement resistant trait Iss, from *E. coli* strain χ1378 isolated from poultry colibacillosis in Iran

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Summary

Avian pathogenic *E. coli* (APEC) is responsible for economic losses in all poultry farms. Certain virulence factors have been proposed as a means of controlling APEC infections, including some proteins to be used for vaccination. In the study we report here, one of the major virulence factors, the *iss* (increased serum survival) gene, from *E. coli* strain χ1378, isolated from poultry colibacillosis in Iran, was cloned to construct a prokaryotic expression vector, in order to analyse the Iss protein. The *iss* gene was successfully cloned into the pGEX-3X vector. The construct was transformed into *E. coli* BL21 to express the Iss protein under induction. The Iss protein was expressed as a glutathione-S-transferase (GST) fusion protein. GST::Iss protein was sequenced by MS/MS MALDI-TOF techniques to confirm its amino acid sequence. BLAST analysis of the Iss protein showed high similarity with previously submitted sequences. Overall, it seems that the Iss protein from strain χ1378 could be used as a good antigen to vaccinate against poultry colibacillosis. GST::Iss protein is currently being used as recombinant protein in SPF chicken models with the goal of evaluating the immune response for APEC control. In conclusion, we constructed a prokaryotic expression vector of the *iss* gene, and express and sequence the Iss protein from *E. coli* strain χ1378 isolated from systemic colibacillosis.

Key words: Iss protein, Avian pathogenic *E. coli*, Expression, Vaccination

Introduction

Escherichia coli is present in the normal microflora of the intestinal tract of chickens, however certain strains of extraintestinal pathogenic *E. coli*, known as avian pathogenic *E. coli* (APEC) are capable of causing colibacillosis in birds (Delicato *et al*., 2003; Rodriguez-Siek *et al*., 2005). Different forms of colibacillosis are responsible for significant economic losses to the poultry industry around the world. Infection with APEC generally occurs in 4–9-week-old broiler chickens and in laying hens at the peak of egg production around week 30 (Barnes *et al*., 2008). The most severe manifestation of avian colibacillosis is septicemia, which is characterized by air sacculitis, pericarditis, perihepatitis, salpingitis and swollen head syndrome (Delicato *et al*., 2003).

Several virulence factors for APEC have been introduced including adhesins, toxins, iron acquisition systems, serum resistance proteins, and capsule as well as lipopolysaccharide complexes (Dho-Moulin and Fairbrother, 1999; La Ragione and Woodward, 2002). However, the pathogenesis and virulence mechanisms underlying APEC have not yet been fully elucidated (Foley *et al*., 2000; La Ragione and Woodward, 2002). In recent years, genome-wide analyses have led to a better understanding of the molecular mechanisms of pathogenicity (Li *et al*., 2005). Many of the genes that are characteristic of the APEC

pathotype are linked to large ColV plasmids (Waters and Crosa, 1991).

ColV plasmids have long been associated with *E. coli* virulence in general and with APEC in particular (Waters and Crosa, 1991). One of the traits that has been localized to APEC ColV/BM plasmids is the *iss* (increased serum survival) gene (Binns *et al*., 1979; Nolan *et al*., 2003; Johnson *et al*., 2006). In addition, many *E. coli* strains have the *iss* gene alleles within their chromosomes (Johnson *et al*., 2008). The *iss* gene encodes an outer membrane protein (OMP) that plays a role in serum resistance, protecting against the actions of the complement, and the presence of this gene in pathogenic avian strains has been shown to be highly significant (Pfaff-McDonough *et al*., 2000; Nolan *et al*., 2003; McPeake *et al*., 2005).

Some authors reported that the *iss* gene is a conserved gene and its protein is located on the surface of *E. coli* to stimulate the protective immune response (Lynne *et al*., 2006, 2007; Derakhshandeh *et al*., 2009). So, it seems that the Iss protein could be used as an antigen for vaccination against APEC infection.

In the present study we aimed to construct a prokaryotic expression vector of the *iss* gene, and to express and sequence the Iss protein from *E. coli* strain χ1378 isolated from systemic colibacillosis in Iran (Nayeri Fasaei *et al*., 2009).

Materials and Methods

Bacterial strains and plasmids

Strain *E. coli* χ1378 was isolated from a chicken with systemic colibacillosis in Iran. *Escherichia coli* DH5α (Fermentas, Vilnius, Lithuania) and *E. coli* BL21 (Fermentas) were used as host strains for vector transformation. The plasmid pTZ57R/T (Fermentas) was used to clone the amplified fragment carrying *iss*. The expression vector pGEX-3X (GE-Healthcare, UK), which is designed for the production of proteins fused to glutathione S-transferase (GST), was used in the fusion experiments. Luria-Bertani (LB) broth and agar (Difco, Detroit, USA) were used to grow the bacterial strains. Ampicillin (100 mg l^{-1}) was added when required.

Cloning into pTZ57R/T vector

The coding sequence of the *iss* gene was amplified to clone it into pTZ57R/T using the primers *iss*-Exp-F 5`- AGGGGATCCTG ATGCAGGATAATAAGATGAAAA- `3 and *iss*-Exp-R 5`-CGCCGGAATTCGCAG ATGAGCTCCCCATATC -3`. The PCR was carried out on a total volume of 25 µl containing 3 µl of template DNA and each of the primers at 30 µmol l^{-1}. The amplification program used was 94°C for 4 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and 72°C for 5 min. The amplimer was visualized by electrophoresis in 1% agarose gels in Tris borate EDTA buffer and stained with ethidium bromide. The amplified coding sequence of the *iss* gene was purified from the gel using the QIAquick Gel Extraction Kit (Qiagen Inc., USA) following the manufacturer's instructions and cloned into pTZ57R/T as previously described (Derakhshandeh *et al*., 2009), generating the construct pTZ57R/T::*iss*. pTZ57R/T::*iss* was used to transform electrocompetent cells prepared as described below. The transformed cells were cultured on LB agar with ampicillin (100 mg l^{-1}). Transformants were confirmed by PCR with *iss* primers as described earlier. Plasmid DNA was extracted from the transformed cells using the Qiagen Plasmid midi purification kit (Qiagen Inc.), as recommended by the manufacturer. The presence of the *iss* insert was further confirmed using M13 primers (F: 5`-GTAAAACGACGGCCAGT-3`, R: 5`-CAGGAAACAGCTATGAC- 3`), *iss* primers and by sequencing. The construct containing the coding sequence was digested with restriction enzymes *Eco*RI and *Bam*HI (Fermentas) as described in the manufacturer's protocol. The digest was purified from the agarose gel using a QIAquick Gel extraction kit (Qiagen Inc.).

Construction of the pGEX-3X expression vector

GST and Iss were fused to generate the fusion protein GST-Iss by inserting the *iss* gene fragment into the multiple cloning site of one of the pGEX-3X vectors. Expression

is controlled by the *tac* promoter, which is induced by the lactose analog isopropyl b-D thiogalactoside (IPTG). All pGEX vectors are also engineered with an internal *lac*Iq gene. The *lac*Iq gene product is a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG, thus maintaining tight control over expression of the insert. The circular pGEX-3X plasmid was digested with restriction enzymes *Eco*RI and *Bam*HI (Fermentas) and the linearized plasmid was purified from agarose gel using a Gel extraction kit (Fermentas). The insert DNA isolated from vector pTZ57R/T::*iss* was ligated into pGEX-3X to generate construct pGEX-3X::*iss* according to the manufacturer's protocol. Briefly, the ligation mixture included 3 µl of vector pGEX-3X, 9 µl of insert DNA and 1 µl of T4 ligase. The mixture was incubated at 10°C for 4 h. 10 µl of the ligation mixture was directly used for chemical transformation to competent *E. coli* DH5α following the manufacturer's instructions. The transformed cells were cultured on LB agar with ampicillin (100 mg l^{-1}). Transformants were confirmed by PCR with *iss* primers as described above. The plasmid was extracted from the transformed cells, and the *iss* insert was further confirmed by PCR using pGEX-5 and pGEX-3 primers (pGEX-5: 5`- GGGCTGG CAAGCCACGTTTGGTG-3`, pGEX-3: 5`- CGGGAGCTGCATGTGTCAGAGG-3`) and *iss* primers. In addition, plasmid DNA was digested with restriction enzymes *Eco*RI and *Bam*HI (Fermentas) as described in the manufacturer's protocol, to determine the insert identity and orientation. The *iss* gene was also confirmed by sequencing. pGEX-3X::*iss* constructs were sequenced using ABI PRISMcc BigDye™ Terminator Cycle Sequencing Kits (Applied Biosystems, USA).

Electroporation and expression

Electrocompetent cells were prepared from 50 ml of culture in LB medium with ampicillin and concentrated by centrifugation at 3,000 g for 5 min. They were then washed in 4 ml of ice-cold double-distilled water. After five washing steps, the cells were suspended in 100 µl of ice-cold double-distilled water. The cells were mixed with a corresponding amount of construct (pGEX-3X with insert, approximately 0.5 ng) in an ice-cold microcentrifuge tube and transferred to a 0.2-cm Electroporation cuvette (Bio-Rad, Inc., USA). The cells were electroporated by an Electro cell manipulatorR 600 BTX (Genetronics, USA) at 2.5 kV with 25 F and 200_Ω resistance. After electroporation, 1 ml of LB medium was added to the cuvette. The cells were transferred to a 17-by-100-mm polypropylene tube and incubated in LB medium for 1 to 2 h at 37°C, without shaking. The cells were concentrated ×10 from a 1-ml culture before plating on selective media. The pGEX-3X vector containing the *iss* sequence was electrotransformed into the protease deficient *E. coli* BL21 strain. Also, the same protocol was followed for pGEX-3X without the *iss* gene as a control for GST expression. The transformed cells (containing both pGEX-3X and pGEX-3X::*iss*) were grown overnight on LB plates containing ampicillin (100 mg l^{-1}). Individual colonies were transferred to 10 ml LB broth containing ampicillin (100 mg l^{-1}), and held at 37°C with shaking until an optical density (OD) of between 0.6 and 1.0 was obtained at 600 nm. IPTG was added to the culture to a final concentration of 0.1 mM. The culture was incubated for an additional 3 h. The cells were pelleted by centrifugation, the supernatant was discarded, and the cells were suspended in 100 µl of 10 mM Tris-HCL, pH = 6.7, per absorbance unit at 600 nm. An equal amount of loading buffer (0.125 M Tris-HCL, pH = 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.02% bromophenol blue) was added to the sample, and the mixture was boiled for 6 min prior to being subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis and western blotting

After boiling, the samples were loaded into wells of 12% SDS-polyacrylamide gels for separation of the proteins in a mini-protean II (Bio-Rad) electrophoresis system following the method of Laemmli (Laemmli, 1970). The samples were electrophoresed at 100 V for 1.5 h. The gels were stained with a solution of 0.025% Coomassie blue R-250,

40% methanol, and 7% acetic acid. The bands in the gel corresponding to the proteins were presumptively identified by comparison rainbow protein molecular marker (GE Healthcare, UK). To perform Western blotting, protein samples were electrophoretically separated as described above, but not stained. The proteins in the 12% SDS-polyacrylamide gels were transferred to nitrocellulose membranes (GE Healthcare, UK) using transblot semi-dry transfer cell (Bio-Rad) for 60 min at 75 V. Unoccupied sites on the nitrocellulose membranes were blocked by overnight incubation at 4°C with agitation in Tris-buffered saline (TBS: 0.15 M NaC1, 10 mM Tris/HCl, pH = 7.5) containing 3% bovine serum albumin (BSA) (Sigma, USA). The next day, the membranes were washed three times with TBS plus 0.02% Tween 20. Next, 40 ml of Goat Anti-GST antibody (GE Healthcare) diluted following the manufacturer's instructions (1/1000) was added to the membrane and the membrane was incubated for 2 h at room temperature by rocking. After incubation, the membrane was washed three times with TBS-Tween 20 before the secondary antibody was added. 40 ml of diluted secondary antibody, Anti-Goat IgG Alkaline Phosphatase Conjugate (Sigma), was added to the membrane and incubated for 2 h at room temperature with agitation. The membrane was again washed with TBS-Tween 20, and the blots were developed by incubation at room temperature in a solution of NBT/BCIP (4-nitro blue tetrazolium chloride /5-Bromo-4-Chloro-3-Indolyl-Phosphate) (Roche, Germany). The reaction was allowed to develop for approximately 5 min and was stopped by washing the membrane in distilled water.

Peptide and proteomic analysis

The proteins of the *E. coli* BL21 containing the fusion protein were separated by SDS-PAGE. The gel fragment corresponding to the 34 kDa GST-Iss fusion protein was excised from the main gel. The GST-Iss fusion protein was purified from the gel fragment by electroelution in an Electro Eluter Model 422 system (Bio-Rad) using 25 mM Tris, 192 mM glycine, 0.1% SDS buffer (pH = 8.3). The purified protein was analysed again by electrophoresis and Western blot to confirm the presence of the protein in the fragment excised and the validity of our previous data. The GST-Iss fusion protein was analysed from the fragment excised from the polyacrylamide gel. Purified proteins were in-gel digested with trypsin (sequencing grade modified; Promega, USA) in an automatic Investigator ProGest robot from Genomic Solutions. Briefly, the excised gel spots were washed sequentially with ammonium bicarbonate buffer and acetonitrile. The proteins were then reduced and alkylated by treatment with 10 mM DTT solution for 30 min at 56°C followed by treatment with a 55 mM solution of iodine acetamide. After sequential washings with buffer and acetonitrile, the proteins were digested overnight, at 37°C with 0.27 nmol of trypsin. Tryptic peptides were extracted from the gel matrix with 10% formic acid and acetonitrile; the extracts were pooled and dried in a vacuum centrifuge. Tryptic peptides were analysed by MALDI-TOF/TOF (4700 proteomics analyser, Applied Biosystems). Extracted digests were resolved in 5 µl of 0.1% trifluoroacetic acid. Typically a 0.5-µl aliquot was mixed with the same volume of a matrix solution, 5 mg/ml of α-ciano-4-hydroxycinnamic acid (CHCA) (Sigma) in 50% acetonitrile/0.1% trifluoroacetic acid. MS data were acquired and a maximum of 7 prominent peaks were selected to be characterized further by MS/MS analysis. The resulting spectrum was used to search for matching proteins in the NCBInr database with the MASCOT search program (www.matrixscience.com).

Results

The coding sequence of the *iss* gene from *E. coli* strain χ1378 was amplified by PCR. The amplimer obtained (359 bp) was cloned into a pTZ57R/T cloning vector, and the presence of the insert in the vector was confirmed by PCR (Fig. 1A) and sequencing. Then the *iss* gene was excised from the pTZ57R/T::iss construct and cloned into the pGEX-3X expression vector to create a plasmid for protein expression. The *iss* gene was ligated in the pGEX-3X vector under the control of the Ptac

promoter. The location of the insert in the second construct was confirmed by PCR (Figs. 1B and C) and sequencing.

Crude protein preparation of transformed *E. coli* BL21 containing plasmid construct (pGEX::*iss*) expressed a 34 kDa protein (Fig. 2A). The construct and BL21 containing pGEX-3X without insert overexpressed a 26-kDa protein (Fig. 2A). These sizes correspond to the predicted protein that should be expressed from these plasmids after induction of IPTG; since GST has 26 kDa, Iss should be 8 kDa. Overall, our fusion protein should be 34 kDa. Western blot analysis confirmed and identified the two proteins with the predicted sizes of 34 and 26 kDa by detection with Goat Anti-GST antibody (Fig. 2B).

In order to detect and determinate correct expression, a strip of the SDS-PAGE slab gel corresponding to the site of migration of the 34-kDa GST-Iss fusion proteins and the 26-kDa GST protein was carefully cut out and electrophoretically eluted. The partially purified proteins were then concentrated and processed by SDS-PAGE, showing a clear band of the expected size (Fig. 3). The presence of the GST and the GST-Iss fusion protein was confirmed by Western blot (Fig. 3).

The purified protein GST-Iss was sequenced (Fig. 4). The GST fragment was

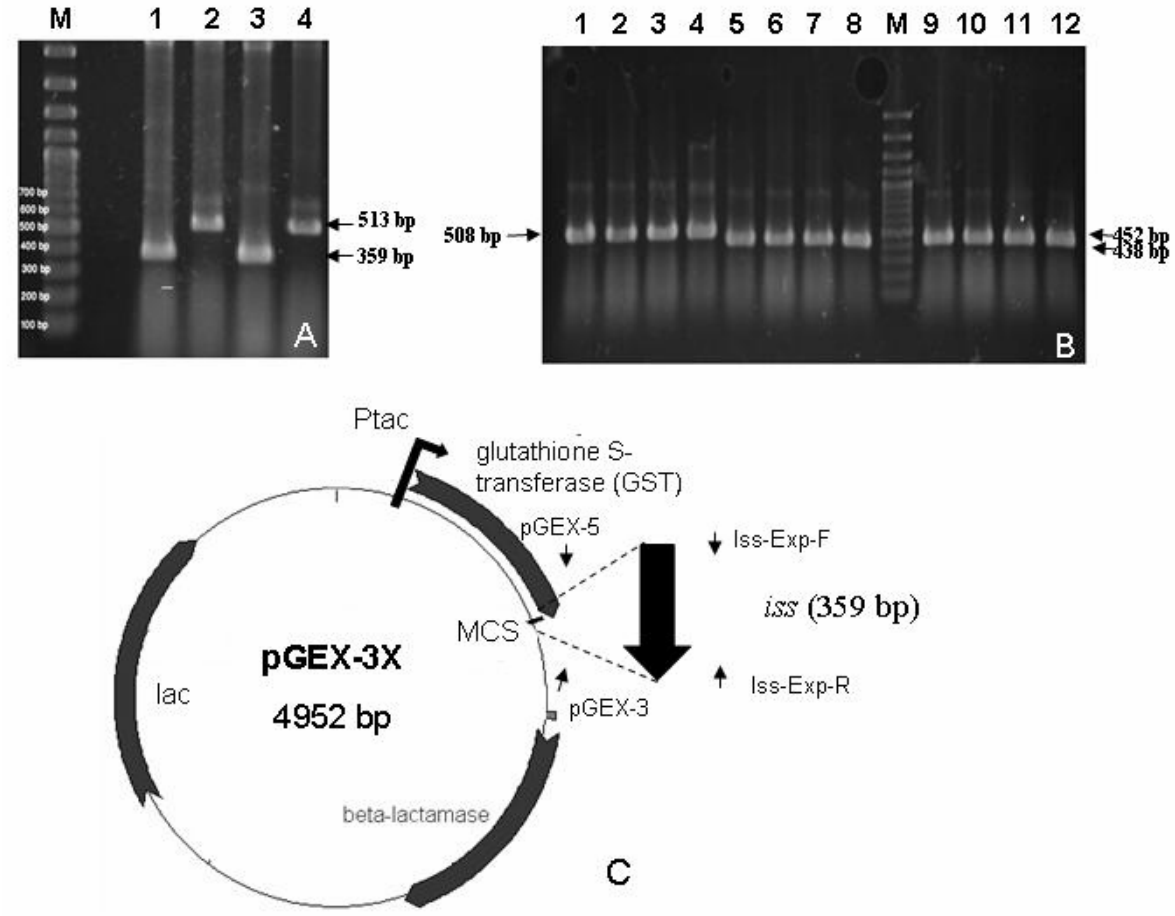

Fig. 1: Cloning of the *iss* gene in pTZ57R/T and pGEX-3X expression vectors. (A) Cloning of the *iss* gene in plasmid pTZ57R/T. Verification of the insert in the plasmid was performed with *iss*-Exp F and R primers (Lanes 1 and 3) (359 bp) and universal M13 primers (Lanes 2 and 4) (513 bp): Marker: 100 bp. (B) Cloning of the *iss* gene in the expression plasmid pGEX-3X. Primer pairs used for detection of the construct were *iss*-Exp F and R primers and pGEX-F and R primers. Lanes 1-4: Amplimer obtained with primers pGEX-F/pGEX-R; Lanes 5-8: Amplimer obtained with primers pGEX-R/*iss*-Exp F; Lanes 9-12: Amplimer obtained with primers pGEX-F/*iss*-Exp F. (C) Schematic map of the generation of the pGEX-3X::*iss* indicating the precise position in which the *iss* gene was incorporated, the size of the fragment and the primers used to amplify the gene and to confirm gene insertion

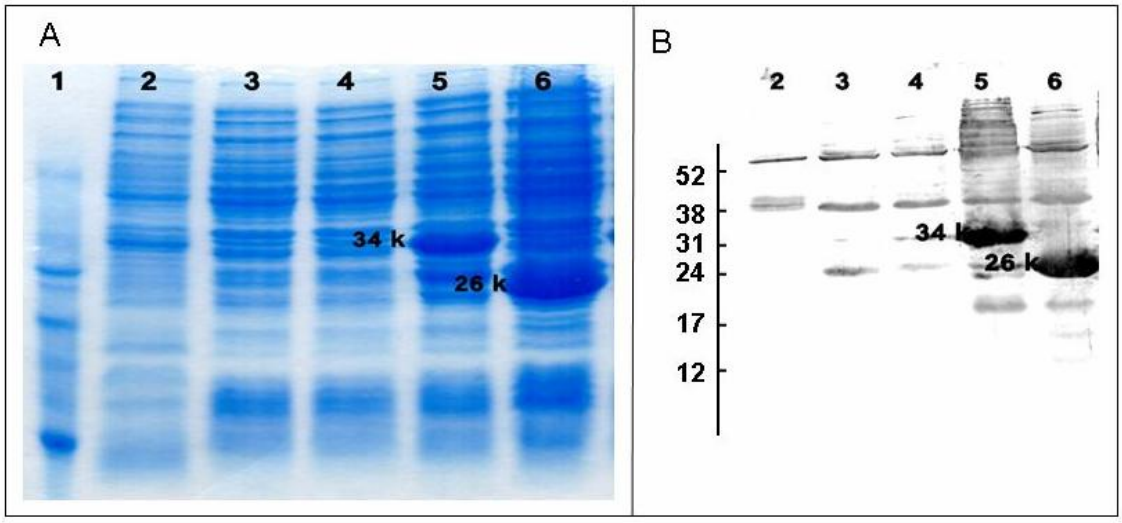

Fig. 2: (A) Coomassie brilliant blue-stained SDS-polyacrylamide gel of SDS-PAGE of crude protein preparation of *E. coli* BL21 containing pGEX::*iss.* Lane 1: LMW-SDS marker (defined in materials and methods); Lane 2: *E. coli* strain χ1378; Lane 3: *E. coli* BL21 containing pGEX-3X::*iss* before induction; Lane 4: *E. coli* BL21 conatining pGEX-3X before induction; Lane 5: *E. coli* BL21 containing pGEX-3X::*iss* induced with 0.1 M IPTG (34 kDa); Lane 6: *E. coli* BL21 conatining pGEX-3X induced with 0.1 M IPTG (26 kDa). (B) Western blot (Goat Anti-GST antibody) of crude protein preparation of *E. coli* BL21 containing the expression vector with and without the *iss* gene and Goat Anti-GST antibody. Lane 1: rainbow molecular weight marker; Lane 2: *E. coli* strain χ1378; Lane 3: *E. coli* BL21 conatining pGEX-3X before induction Lane 4: *E. coli* BL21 containing pGEX::*iss* before induction; Lanes 5 and 7: *E. coli* BL21 containing pGEX::*iss* induced with 0.1 M IPTG (34 kDa); Lanes 6 and 8: *E. coli* BL21 containing pGEX induced with 0.1 M IPTG (26 kDa)

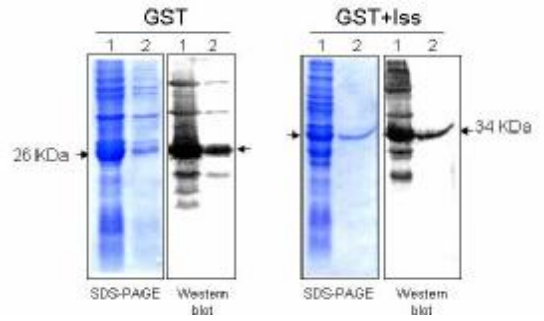

Fig. 3: Coomassie brilliant blue-stained SDS-polyacrylamide gel of SDS-PAGE and Western blot (with Goat Anti-GST antibody) of purified GST protein (26 kDa) and purified fusion protein GST-Iss (34 kDa). Lane 1: crude protein preparation expressed from the BL21 cultures containing pGEX or pGEX::*iss* induced with 0.1 M IPTG; Lane 2: GST and GST-Iss proteins purified after cutting a strip of the SDS-PAGE slab gel from the zone to which the 26 kDa and 34 kDa fragments migrate and eluting the strips electro-phoretically

identified and corresponded to a 209-Aa fragment and the Iss corresponded to a 102-Aa fragment. The amino acid sequence of χ1378-Iss fitted with the prediction obtained from translation of the DNA to the protein.

BLAST analysis of χ1378-Iss with protein sequences in the genetic bank sequences was performed. From 10 sequences of the Iss protein submitted, two protein sequences of poultry origin showed total identity with our protein (100%), while five sequences showed few differences between 97 and 99% (Fig. 4). Among others, *iss* sequences with accetion number P19592 (mammalian origin) showed at least identity (86%). Iss sequences from *E. albertii* and *E. coli* O157:H7 showed 97 and 98 percent identity, respectively (Fig. 4).

Discussion

In recent years, awareness of the importance of poultry colibacillosis has increased. It causes significant morbidity and mortality as well as multimillion-dollar annual losses to the poultry industry (Horne *et al*., 2000; Rodriguez-Siek *et al*., 2005). Emphasis has been placed on the detection, diagnosis and management of colibacillosis. However, due to the fact that the exact

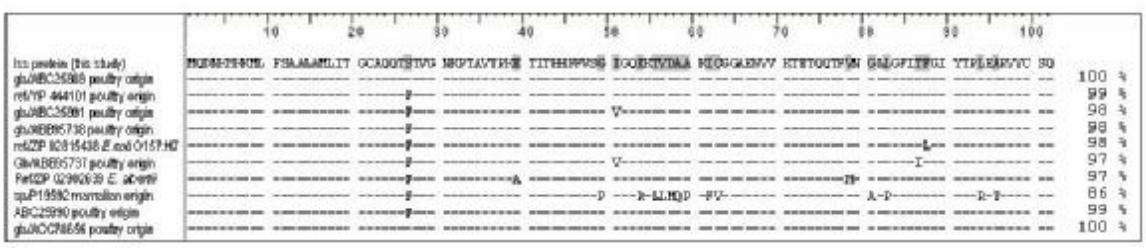

Fig. 4: Multiple alignments of the Iss amino acid sequence from strain χ1378 and other sequences deposited in the gene bank database. The differences in the sequence of each strain are specified by letters indicating corresponding amino acid residues and in grey in the Iss sequence from our study; positions containing amino acid residues which are the same in the Iss sequence of the databank as the gene in this study are indicated by dashes. Percent of identity is indicated on the right-hand side

mechanism of pathogenesis and the role of virulence factors are both unclear, and also because of the extensive gene diversity in *E. coli* (Foley *et al*., 2000; La Ragione and Woodward, 2002; Mokady *et al*., 2005), it has not been thoroughly controlled and its incidence has increased around the world, especially in Iran. Few management options (such as improved hygiene, vaccination, the use of competitive exclusion products and antimicrobial chemotherapy) are currently available and, further, these approaches are largely ineffective (La Ragione *et al*., 2001, 2004; Peighambari *et al*., 2002; Gomis *et al*., 2003; Johnson *et al*., 2004). So, the development of a novel, effective and safe vaccine has been the subject of much research into the control of poultry colibacillosis. In a previous study we sequenced the *iss* gene from *E. coli* (O78:K80) strain χ1378 isolated from Iranian poultry (Derakhshandeh *et al*., 2009). In this current study, the *iss* gene from *E. coli* strain χ1378 has been successfully expressed by IPTG induction and the encoded protein has been sequenced. This study is the first description of a work of this kind performed in Iran.

One of the traits involved in APEC virulence is Iss, which is reported to play a subtle role in the resistance of APEC to complement serum (Nolan *et al*., 2003). Some studies show that the *iss* gene is a conserved gene and that it occurs more frequently in APEC affected poultry than in healthy chickens in different serotypes and with different origins (Pfaff-McDonough *et al*., 2000; Johnson *et al*., 2006; Derakhshandeh *et al*., 2009). So the Iss protein from strain χ1378 could be used to control APEC in Iranian avian isolates. Our data emphasize that the Iss protein sequence of strain χ1378 has a high homology with other sequences, although it is not identical, and that it could be used for vaccination. Through sequential determination and BLAST analysis, between the Iss protein of *E. coli* strain χ1378 and other sequences that were previously submitted, the Iss sequence is identical to some sequences and extremely similar to others. Former analyses (Derakhshandeh *et al*., 2009) indicated that the Iss protein was very conservative in APEC. As mentioned above, vaccination against colibacillosis is prepared by routine methods such as a killed or attenuated vaccine, mutant vaccine and fimbrial adhesion protein. Due to the considerable diversity in APEC strains, many virulence factors have proved to be ineffective as vaccines when applied in avian communities different from those used to select and prepare the vaccine (Melamed *et al*., 1991; Peighambari *et al*., 2002; Amoako *et al*., 2004; Vandemaele *et al*., 2005). For this reason we attempted to construct a prokaryotic expression vector using the extract of the Iss protein isolated from our strain. This will ensure that, independent of the conservation of this character and their common occurrence among APEC strains (Pfaff-McDonough *et al*., 2000; Johnson *et al*., 2006; Derakhshandeh *et al*., 2009), we will succeed when applying it to our own avian communities. In addition, the use of a recombinant protein is a feasible way to generate new vaccine against avian colibacillosis. Recombinant proteins have a very low risk of infection; they can be produced in large quantities and do not need

an adjuvant. Recombinant proteins have been used for several purposes such as vaccination against fowl cholera (Nattawooti *et al*., 2008).

These results could serve as the basis for further research into the usefulness of this gene and its expression product in the development of a subunit vaccine, DNA vaccine and diagnostic reagent against avian colibacillosis. Currently, GST::Iss protein is being used as a recombinant protein in SPF chicken models with the goal of evaluating the immune response for APEC control.

Acknowledgements

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