Cloning, sequencing, expression, and characterization of the *tsh* gene from an avian pathogenic *Escherichia coli* strain

Clonagem, sequenciamento, expressão e caracterização do gene *tsh* de uma cepa de *Escherichia coli* patogênica de aves

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Abstract

The temperature-sensitive hemagglutinin (Tsh) belongs to a family of high-molecular-weight serine protease autotransporters of *Enterobacteriaceae* (SPATEs), which can cleave different substrates. We isolated and characterised the *tsh* gene from an avian pathogenic *Escherichia coli* (APEC) strain, APEC13 serotype O2:H9, which was cloned in pET101. The 4.2 kb region of cloned DNA coded one protein of approximately 140 kDa (r-Tsh). The recombinant plasmid pET101-*tsh* conferred to *E. coli* BL21 strain (*tsh*) the hemagglutination-positive phenotype against chicken erythrocytes. The r-Tsh was purified by Ni-NTA column and used to produce antibody anti-Tsh. A 1.6 kb fragment of the *tsh* sequence was also amplified and cloned in pCR4, and a partial sequence showed high homology with other sequence analysed. The anti-Tsh reacted with the protein r-Tsh and native Tsh of APEC13, as demonstrated by Western blot, showing that r-Tsh has conserved epitopes and that its antigenicity was preserved. The anti-Tsh also inhibited the hemagglutinating activity of strains APEC13 and BL21/pET101-*tsh*.

Key words: Avian *Escherichia coli*, temperature-sensitive hemagglutinin (Tsh), adherence, virulence factor

Resumo

A hemaglutinina temperatura sensível (Tsh) pertence à família das serino-proteases autotransporte de *Enterobacteriacea* (SPATE), as quais são capazes de clivar diferentes substratos. Nós isolamos e caracterizamos o gene de *Escherichia coli* patogênica aviária (APEC) amostra APEC 13, sorotipo O2:H9, clonado em pET101. A região de 4.2 kb do DNA clonado codifificou uma proteína de aproximadamente 140 kDa (r-Tsh). O plasmídio recombinante pET101-*tsh* conferiu um fenótipo de hemaglutinação positivo para a linhagem BL21 (*tsh*$^-$) para eritrócitos de galinha. A proteína r-Tsh foi purificada em coluna de níquel e utilizada na produção de anticorpos anti-Tsh. Um fragmento de 1.6 kb foi amplificado e subclonado em pCR4, e a seqüência parcial mostrou alta homologia com outras seqüências analisadas. O anti-Tsh reagiu com as proteínas r-Tsh e Tsh nativa da amostra APEC13, como demonstrado pela técnica de Western blot, mostrando que a r-Tsh tem epitopos conservados e que sua antigenicidade foi preservada. O anti-Tsh também inibiu a atividade hemaglutinante das amostras APEC13 e BL21/pET 101-*tsh*.

Palavras-chave: *Escherichia coli* aviária, hemaglutinina temperatura sensível (Tsh), aderência, fator de virulência

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Recebido para publicação 06/02/06 Aprovado em 12/02/06

Introduction

Escherichia coli belongs to normal microbiota of the intestinal and upper respiratory tracts of many avian species, but strains of avian pathogenic *Escherichia coli* (APEC) expresses virulence factors that enable them to cause infections that develop into airsacculitis, pericarditis, perihepatitis and septicemia. This is the most frequent form of avian colibacillosis, and it causes important world-wide economic losses (GROSS, 1994). Several putative virulence factors were detected in APEC, such as aerobactin production, serum resistance, Tsh hemagglutinin, hemolysins, and many types of adhesins and fimbriae that mediate attachment of bacteria to cells of respiratory system (DHO-MOULIN; FAIRBROTHER, 1999). The importance of each virulence factor for the development of infection by APEC is still unclear, however, partly because many virulent strains lack one or several of those factors.

The temperature-sensitive hemagglutinin (Tsh) expressed by APEC confers the phenotype mannose-resistant hemagglutination (MRHA) of chicken erythrocytes to bacteria grown at 26°C on low-osmolarity solid medium (PROVENCE; CURTISS, 1992; PROVENCE; CURTISS, 1994). The *tsh* gene was detected in APEC, but not in *E. coli* isolated from the faeces of healthy chickens, which suggests that it possibly has a role in the pathogenicity of APEC (MAURER et al., 1998; DOZOIS et al., 2000). The deduced sequence of 4.4 kb of Tsh presented homology to the serine-type immunoglobulin A (IgA) proteases of *Neisseria gonorrhoeae* and *Haemophilus influenzae* (PROVENCE; CURTISS, 1994), and as Tsh is secreted similarly to type IgA serine-proteases, it was classified into the subfamily of autotransporter proteins denominated "SPATE" ("serine protease autotransporters of *Enterobacteriaceae*") (HENDERSON; NAVARRO – GARCIA; NATARO, 1998; STATHOPOULOS; PROVENCE; CURTISS, 1999; DOZOIS et al., 2000).

Maturation of Tsh produces two proteins, a 106kDa extracellular protein (Tsh_s), and a 33 kDa outer membrane protein (Tsh_b). The 106 kDa protein contains the serine-protease motif, which is also found in secreted IgA proteases, but it did not cleave human IgA, or chicken IgA (STATHOPOULOS; PROVENCE; CURTISS, 1999), although Tsh did cleave bovine submaxillary gland mucin and coagulation factor V (DUTTA et al., 2002). Tsh_s exerts proteolytic activity against casein (KOSTAKIOTI; STATHOPOULOS, 2004).

APEC isolated in Brazil often carry the *tsh* gene (DELICATO et al., 2002), whereas non-pathogenic strains do not carry it, which strengthens the contention that it is an important virulence factor and a possible target for vaccine development. In this study we cloned and expressed the *tsh* gene from a Brazilian isolate of APEC.

Material and Methods

Strains

APEC 13 strain, serotype O2: H9 (MOURA; IRINO; VIDOTTO, 2001), was isolated from colibacillosis lesions. The *E. coli* BL 21 Star™ (DE3) strain (Invitrogen) was used as receptor of recombinant plasmid, which allows high-level expression of T7-regulated genes. APEC strains were grown in agar colonization factor antigen (CFA).

Cloning of tsh gene and DNA sequence analysis

DNA from APEC 13 strain was released from whole organisms by boiling and used to amplify a region that contains the *tsh* gene by polymerase chain reaction (PCR). A pair of primers was constructed according to the GeneBank sequence (Accession number **L27423**), *tsh*1 (5'-CACCATGAACAGAATTTATTCTC-3') and *tsh*2 (5'-GAATGAATAACGAATATTAGC-3'). The

forward PCR primer contains the sequence CACC, at the 5' end of the primer, which base pair with the overhang sequence, GTGG in pET 101/D-TOPO® vector (Invitrogen, Carlsbad, CA, USA).

PCR was carried out in a total volume of 25 ul containing 5 ul of template DNA, each of the primers at 20 pmol, the four deoxynucleoside triphosphates (each at 200mM), *pfx* platinum buffer, 50 mM of magnesium sulphate and 2.5 U of *pfx* platinum DNA polymerase. PCR conditions were as follows: denaturation at 94 for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min in a Thermal Cycler (Gene Amp PCR System 9700/Perkin Elmer). The amplified DNA was visualised in 1.0% agarose gels stained with ethidium bromide. The 100-pb ladder (Promega, Madison, WI) was used as standard for determining molecular mass of PCR products.

The amplification product was purified with kit "CONCERT – System of extraction from gel" (GIBCO BRL), and 20ng were used to insert into the pET 101/D-TOPO® vector. The host strain TOP10 chemically competent *E. coli* cells (Invitrogen) were then transformed with 3 µl of the cloning reaction and incubated on ice for 30 min. After heat-shocking the cells for 30 seconds at 42°C, 250 µl of SOC medium were added and the preparation was incubated at 37°C for 30min. Then, 200 µl of transformation were spread on selective plates containing 100 ¼g of ampicilin and incubated at 37°C overnight to obtain colonies denominated positive clones. These clones were grown in LB containing ampicilin for extraction of the plasmid by alkaline lysis (Mini Prep) (SAMBROOK). Presence of *tsh* inserts in these plasmids was confirmed by restriction digests with *Xma*I and PCR as described above.

Cloning of 1.6 kb initial sequence of *tsh* gene was realised for sequencing. Genomic DNA from APEC13 was used for amplification of DNA by PCR with primers 5'-TGAACAGAATTTATTCTCTTCGC-3' and 5'-AGTCCAGCGTGATAGTGG-3' as described previously (DOZOIS et al., 2000). The fragment amplified of the *tsh* gene was cloned in plasmid pCR4-TOPO (TA cloning Kit for sequencing; Invitrogen, San Diego, Calif.), resulting in plasmid pCR4-tsh. *E. coli* TOPO10 was used as the host strain. Plasmid isolation and further subcloning procedures were performed as described by Sambrook, Fritsch e Maniast (1989)

DNA and amino acids sequence analysis was done with the sequence analysis software package by Genetics Computer "CAP3 Contig Assembly Program", "Clustal W (1.81) Multiple Sequence Alignments"and "Six Frame Translation of Sequence".

Expression of tsh gene on E. coli strain and purification of recombinant his-tagged Tsh

E. coli BL 21 was transformed with the recombinant plasmid pET 101- *tsh.* The BL21/ pET 101- *tsh* strain was grown to an optical density of $OD_{600\,nm}$ = 0.5 to 0.8 (mid log), and IPTG (isopropyl-1-²- D- thiogalactopyranoside) was then added and grown at 37 °C with shaking. Aliquots were sampled at different times to choose the best time for expression.

The induced culture for 3 h at 37 °C with shaking was centrifuged at 5000 rpm for 5 min. in a Sorvall SS-34 rotor. To the pellet, buffer containing 6 M guanidine-HCl, 20 mM $NaPO_4$, pH 7.8 and 500 mM NaCl was added, and the cells were slowly rocked for 5-10 min at room temperature to assure thorough cell lysis, and the cell lysate was sonicated on ice with three 5-seconds pulses at high intensity. The lysate was then centrifuged at 3.000 x g for 15 min to pellet the cellular debris. The supernatant was transferred to ProBond™ resin (Invitrogen) that had previously been washed with Denaturing Binding Buffer (8 M urea, 20 mM $NaPO_4$, 500 mM NaCl, pH 7.8). The supernatant and resin were incubated for 1h on a rotation wheel. After centrifugation at 2000 rpm (Sorvall SS-34 rotor) the resin was washed twice with denaturing binding buffer, twice with denaturing wash buffer (8 M urea, 20 mM $NaPO_4$, 500 mM NaCl, pH 6.0), and with 8

M urea buffer at pH 5.3. The protein was eluted using 8 M urea buffer at pH 4.0 and concentrated on centrifugal microconcentrators. Protein concentration was measured by Lowry's modified method (MILLER, 1959).

Anti-Tsh antibody production

The purified r-Tsh protein (100mg) was resolved by SDS-PAGE (8% gels), the 140kDa band was removed and intramuscularly inoculated together with Freund's coadjuvant into chickens. Four days after the second boost, the chickens were bled, and the serum was separated, inactivated, and adsorbed on E. coli BL 21 Star™ (DE3). Control serum was obtained from non-immunised chickens.

Analysis of Tsh by SDS PAGE and Western Blot

The uninduced and induced protein lysates and purified protein were suspended in electrophoresis sample buffer (0.025M Tris-HCl, 2% SDS, 15% glycerol, 2.5% 2-mercaptoethanol, pH 6.8), boiled for 5 min, and electrophoresed an SDS-8% polyacrylamide gel (SDS-PAGE). Gels were either stained with Comassie blue or set up for Western blot. Proteins were transferred onto nitrocellulose membranes (Pharmacia Biotech) and the membranes were blocked in blocking buffer (PBS + 0.1% Tween 20 + 5% nonfat dry milk) for 1 h at room temperature under agitation. Membrane was washed in PBS-T (PBS + 0.1% Tween 20) and incubated for 1 h in a 1:5000 dilution of Anti-His(C-term)-HRP (Invitrogen) directed against the hexamer histidine tag and in a 1:40000 dilution of serum anti-Tsh. The membrane was washed once again and recombinant TSH was detected by means of enhanced chemoluminescence (ECL) Western Blotting System (Amersham International, Amersham, United Kindom). Protein molecular weight markers (Rainbow™ coloured, Amersham Life Science) were used as standards.

Hemagglutination assay

Hemagglutination activity was tested by microhemagglutination (PROVENCE; CURTISS, 1992). Bacteria grown on colonisation factor antigen (CFA) agar plates at 26 °C for 48 h were harvested and suspended in 0.85% NaCl. When cells were assayed for hemagglutination activity, the suspension of cells was serially diluted in 0.85% NaCl containing (1%) methyl-±-D-mannopyranoside (Sigma, St. Louis, USA) to inhibit hemagglutination by type1 pili and then was added to each well of a 96-well round-bottom microplate containing a suspension of fresh chicken erythrocytes. The reactions were incubated for 1 h on ice. Wells containing an even sheet of erythrocytes across the well were considered positive, whereas those containing a small erythrocyte pellet at the bottom of the well were considered negative. To test the presence of inhibitory antibodies in the immune serum, APEC 13, BL21/pET101-*tsh* and BL21 strains were incubated with anti-Tsh serum and control serum for 30min on ice, and then tested by the micro-hemagglutination assay.

Results

Cloning of the tsh gene and its DNA sequence

Of the transformant colonies obtained, five were analysed by PCR by means of primers for the *tsh* gene, and all colonies showed the amplicon of approximately 4.2 kb, as expected from the insert. A fragment of 9.9 kb was released from the extracts of the recombinant plasmid pET101-*tsh* from a positive clone, after cleavage with the restriction enzyme *Xma*I that cleaves the vector pET 101; this fragment corresponds to 5.7 kb of the vector plus 4.2 kb of the insert.

A 1.6 kb fragment at the 5'region of the *tsh* gene was amplified and cloned in the TOPO/ pCR4 vector. The clone *E. coli* TOP10/pCR-*tsh* contains the amplicon that corresponds to the initial sequence of

tsh gene. The nucleotide sequence of this amplicon was determined and deposited in the GenBank date base with accession number AY280856. Analysis reveals that this region is homologous to the previously characterized *tsh* gene. The deduced amino acid sequence encoded by this region shows homology to serine-protease autotransporters and has high similarity to Tsh described sequence (Figure1).

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APEC13          MNRIYSLRYSAVARGFIAVSEFARKCVHKSVRRLCFPVLLLIPVLFSAGSLAGTVNNELG
tsh             MNRIYSLRYSAVARGFIAVSEFARKCVHKSVRRLCFPVLLLIPVLFSAGSLAGTVNNELG

APEC13          YQLFRDFAENKGMFRPGATNIAIYNKQGEFVGTLDKAAMPDFSAVDSEIGVATLINPQYI
tsh             YQLFRDFAENKGMFRPGATNIAIYNKQGEFVGTLDKAAMPDFSAVDSEIGVATLINPQYI

APEC13          ASVKHNGGYTNVSFGDGENRYNIVDRNNAPSLDFHAPRLDKLVTEVAPTAVTAQWAVAGA
tsh             ASVKHNGGYTNVSFGDGENRYNIVDRNNAPSLDFHAPRLDKLVTEVAPTAVTAQGAVAGA
                                                                         *

APEC13          YLGKERYPVFYRLGSGTQYIKDSNGQLTKMGGAYSWLTGGTVGSLSSYQNGEMISTSSGL
tsh             YLDKERYPVFYRLGSGTQYIKDSNGQLTQMGGAYSWLTGGTVGSLSSYQNGEMISTSSGL
                  *                         *

APEC13          VFDYKLNGAMPIYGEAGDSGSPLFAFDTVQNKWVLVGVLTAGNGAGGRGNNWAVIPLDFI
tsh             VFDYKLNGAMPIYGEAGDSGSPLFAFDTVQNKWVLVGVLTAGNGAGGRGNNWAVIPLDFI

APEC13          GQKFNEDNDAPVTFRTSEGGALEWSFNSSTGAGALTQGTTTYAMHGQQGNDLNAGKNLIF
tsh             GQKFNEDNDAPVTFRTSEGGALEWSFNSSTGAGALTQGTTTYAMHGQQGNDLNAGKNLIF

APEC13          QGQNGQINLKDSVSQGAGSLTFRDNYTVTTSNGSTWTGAGIVVDNGVSVNWQVNGVKGDN
tsh             QGQNGQINLKDSVSQGAGSLTFRDNYTVTTSNGSTWTGAGIVVDNGVSVNWQVNGVKGDN

APEC13          LHKIGEGTLTVQGTGINEGGLKVGDGKVVLNQQADNKGQVQAFSSVNIASGRPTVVLTDE
tsh             LHKIGEGTLTVQGTGINEGGLKVGDGKVVLNQQADNKGQVQAFSSVNIASGRPTVVLTDE

APEC13          RQVNPDTVSWGYRGGTLDVNGNSLTFHQLKAADYGAVLANNVDKRATITLD---------
tsh             RQVNPDTVSWGYRGGTLDVNGNSLTFHQLKAADYGAVLANNVDKRATITLDYALRADKVA
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Figure 1. Comparison of the deduced amino acid sequence of Tsh with that of the Tsh from APEC13 (AY280856). '*' indicates positions which have a amino acid did not conserved.

Expression and purification of recombinant Tsh protein

Tsh protein was produced by transcription/translation system *in vitro* in *E. coli* BL21 transformed with recombinant plasmid pET101-*tsh*. The "his-tagged" Tsh protein was best induced after 3h of incubation with 1 mM of IPTG. The profile of bands on SDS-PAGE shows the presence of induced Tsh (Figure 2A, line 2) by comparison with strain *E. coli* BL21 (Figure 2A, line 1). The recombinant Tsh (r-Tsh) was effectively purified from resin ProBond and presents approximately 140 kDa (Figure 2A, line 3). Western blot with anti-his monoclonal antibody detected the induced Tsh in the cell lysate. The reactivity of recombinant Tsh with the anti-Tsh serum on Western blot is shown on line (Figure 2B, lines 2 and 3).

Tsh hemagglutinating activity

Both the clone expressing Tsh and APEC13 agglutinated chicken erythrocytes at 26ºC, and this hemagglutination was inhibited by the anti-Tsh antibody, whereas the BL21 strain (tsh^-) was non-agglutinating, indicating that pET101-*tsh* contains the structural gene that codifies the hemagglutinin Tsh. The 140kDa purified r-Tsh protein caused agglutination of chicken erythrocytes, which was inhibited by the anti-Tsh antibody.

Discussion

The isolation and partial characterisation of the *tsh* gene from APEC 13, serotype O2:H9 shows that it is highly similar to the *tsh* gene from APEC X7122, serotype O78:H9 characterised by Provence e Curtiss (1994). In agreement with those authors, we verified that *E. coli* BL21 strain transformed with *tsh* gene agglutinates chicken erythrocytes and expresses a protein of approximately 140 kDa. Thus, the *tsh* gene is not only common in the Brazilian isolates that we studied (DELICATO et al, 2002), but it also seems to be homologous to the gene isolated in a very distant region, from which one might plausibly infer that *tsh* is an important virulence factor.

The results obtained from SDS-PAGE showed that r-Tsh was successfully expressed in BL21 (DE3) and purified by means of Ni-NTA. The r-Tsh used to immunize chickens induced an immune response that produced specific anti-Tsh reactive with both recombinant (140 kDa) (Figure 2), and native Tsh, inhibiting the agglutination of chicken erythrocytes caused by APEC13. The data showing that r-Tsh has conserved epitopes and maintained antigenicity.

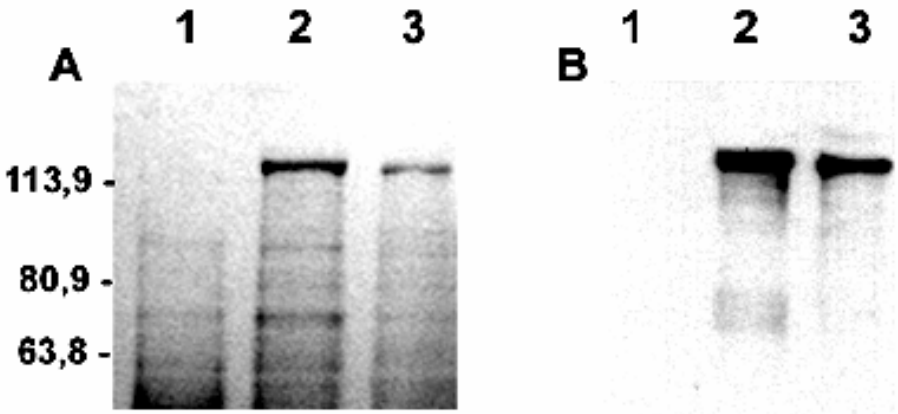

Figure 2. Expression of the 140 kDa protein by clone *E. coli*/pET101-*tsh* and immunological response of this protein in chickens. (A) SDS-PAGE 8% stained with Comassie brilhant blue. (B) Western blot with anti-Tsh antibody and anti-chicken IgG conjugated with peroxidase. Lane 1, *E. coli* BL21; lane 2, clone BL21/pET101-*tsh* induced with IPTG 1mM; lane 3, eluted Tsh. The band of approximately 140 kDa corresponding to Tsh protein.

In conclusion, the *tsh* gene cloned from a Brazilian strain of APEC is homologous to the gene previously characterised, and r-Tsh protein induced the antibodies in chickens, which inhibited its biological activity. Thus, the Tsh protein should therefore be evaluated as a vaccine candidate, since it is highly conserved in APEC strains.

Acknowledgments

This work was supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) and "Conselho de Aperfeiçoamento de Pessoal de Ensino Superior" (CAPES).

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