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Rapid Detection of *Salmonella enteritidis* by PCR Amplification of The *SefA* Gene and It's Cloning

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Abstract: The emergence of *Salmonella enteritidis* as an important food-borne pathogenesis in humans, demands the development of novel detection and intervention strategies. It is generally accepted that fimbriae are an important factor in bacterial survival and persistence in the host. This study is directed towards the method of amplifying and cloning the *SefA* gene, which encode *Salmonella enteritidis* fimbrial protein. Strains used for these studies were *S. enteritidis* (E3), which were collected from Kermanshah region. Chromosomal DNA was extracted by boiling method and PCR reaction was performed and single band of 511 bp amplified by SefA-F and SefA-R primers. The resulting PCR product was inserted into the cloning vector (pTZ57R/T). In order to amplify the recombinant plasmid, *E. coli* DH5α bacteria were transformed with SefA-pTZ57R/T. Recombinant clones were identified by blue/white selection and purified recombinant plasmids were indicated by an alkaline lysis procedure. Identity of the SefA-pTZ57R/T product was confirmed by RFLP and sequencing. Nucleotide and protein alignment with BLAST software showed that the sequence of the *SefA* gene derived from *S. enteritidis* (E3), which was cloned in the pTZ57R/T vector, was 99% identical to that of the Genbank (L11008). The sequence of the *SefA* gene from *S. enteritidis* (E3) differed only in two nucleotides and one amino acid. The cloned *SefA* gene from *S. enteritidis* (E3) was submitted to the NCBI Genbank (EF553334).

Key words: *Salmonella enteritidis*, PCR, cloning, bacterial survival

INTRODUCTION

Microbial diseases constitute a major cause of death in many parts of the world. *Salmonella* are considered major health problem worldwide. Salmonella has been identified as an important food and water-borne pathogen (Laconcha *et al*., 1998; Wilks *et al*., 2000). *S. enteritidis* emerged as an important pathogen in poultry and human (Kisiela *et al*., 2003). Human *S. enteritidis* infections showed a dramatic increase since 1980s, particularly in developing countries and has became the most commonly isolated serotype in many countries (Sakai and Chalermchaikit, 1996; Rabsch *et al*., 2001; Wilks *et al*., 2000). Recently it has been identified that poultry, consumption of raw eggs, poultry meat, processed products, fast food and international food trade between countries are the major sources of infection and transmission of *S. enteritidis* in humans (Nayak *et al*., 2004; Wilks *et al*., 2000; Landeras *et al*., 1998; Gibson, 2000).

Fimbriae, also called pili, are surface appendage and filamentous structures which are primarily composed of low molecular weight protein (14 kDa), *Salmonella enterica* subsp. *Enterica serovar enteritidis* elaborates morphologically distinct fimbriae designated SeF21, Sef17, Sef14 and probably an additional kinds of LPF and PEF (Rajashekara *et al*., 2000). It is generally accepted that fimbriae are an important factor in bacterial survival and persistence in the host. Fimbriae play an important role in adhesion of bacteria to different cell surfaces. It's known that adhesion of *S. enteritidis* to cell surface is an essential stage in colonization and pathogenesis of salmonellosis (Rajashekara *et al*., 2000; Ogunniyi *et al*., 1997; Darwin and Miller, 1999; Kisiela *et al*., 2003).

It has been shown that the *S. enteritidis* fimbriae Sef14 are composed of major fimbrial protein called SefA, which are coded by *SefA* gene (Clouthier *et al*., 1993; Collinson *et al*., 1996; Muller *et al*., 1991). Because of their structure and localization, fimbriae are excellent targets for the immunological system of the host and highly immunogenic (Kisiela *et al*., 2003; Kuczkovski *et al*., 2004). *S. enteritidis* fimbriae antigen are useful as an antigen for immunoassay diagnosis of *S. enteritidis* infection or evidence and treatment of infection (Kisiela *et al*., 2003; Kuczkovski *et al*., 2004; Ochoa-Reparaz *et al*., 2004).

MATERIALS AND METHODS

Bacterial strains, plasmid, media and growth conditions: *S. enteritidis* E3 was provided by S. Ghazaey

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(Mirmomeni *et al*., 2007). *E. coli* DH5α was used as the host for the cloning experiments with pTZ57R/T vector. Stock culture were maintained at -70°C in 20% (v/v) glycerol. All cultures were routinely grown in ss-agar at 37°C. Cells for DNA extraction were grown in Leuria broth/LB at 37°C.

Preparation of genomic and plasmid DNA: Chromosomal DNA was isolated by boiling method. In this method one bacteria colon from ss-agar medium was cultured in 10 mL LB broth overnight at 37°C. Cultured *S. enteritidis* in the LB broth was centrifuged at 4000 rpm for 5 min for bacterial precipitation. Then precipitated *S. enteritidis* was suspended in a micro centrifuge tube with a capacity of 1.5 mL with 300 µL of DNAse-RNAse-free distilled water by vortexing. Micro centrifuge tube was heated in boiling water for 15 min and then the suspension was centrifuged for 5 min at 10000 rpm. The supernatant was extracted carefully and made ready for electrophoresis. Plasmid DNA was isolated by an alkaline lysis procedure (Silhavy *et al*., 1984).

PCR: The oligonucleotid primers used for the specific amplification of the *SefA* gene, SefA-F (5′-GCGCCATGGGTAAATCAGCA TCTGC-3′) and SefA-R (5′-CCCTCTAGAAGGTTTGATACTG CT-3′) were designed by Kisiela *et al*. (2003). A volume of 4 µL of the DNA template solution was added to 48 µL of reaction mixture containing ddH_2O, 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP, 45 P mol μL^{-1} each primer, 5 U μL^{-1} Taq polymerase. Amplification was carried out in UK thermocycler (Model FTGRAD2D (with temperature programme consisting of initial denaturation (3 min at 94°C), 30 amplification cycles (1 min at 94°C, 1 min at 52°C, 1.5 min at 72°C) and the final extension (10 min at 72°C). A volume of 5 µL of the PCR product was analyzed by electrophoresis in 1% agarose gel (Sambrook *et al*., 2001).

Restrictive endonuclease digestion: For confirming the PCR product, 2 µL of 10X buffer, 2 µL of DDT (1%), 2 µL of BSA (1%) and 0.25 µL of AvaII (20 $\mu l\ L^{-1}$) were added in a restrictive endonuclease system. The total volume was brought up to 20 µL with PCR products. Then it was incubated at 37°C for 1 h and checked in a 1% agarose gel electrophoresis at 40 V and developed with UVI Doc. For confirming the SefA-pTZ57R/T construct, the same method was used with PstI restriction enzyme.

Cloning of *SefA* gene: The *SefA* gene were obtained by PCR from chromosomal DNA isolated from *S. enteritidis* E3. The resulting PCR product inserted in the corresponding site of cloning vector, pTZ57R/T with Fermentase Ins T/A clone™ PCR product Cloning Kit (# K1213).

Transformation: In order to insert recombinant vector (SefA/pTZ57R/T) in to the component cells (*E. coli* DH5α) Transform Aid™ Bacterial Transformation System were used (Fermentase Ins T/A clone™ PCR product Cloning Kit). Transformed *E. coli* DH5α were grown at 37°C in LB-Ampocillin agar plates containing 50 $\mu g\ mL^{-1}$ Ampicillin, 0.1 M IPTG (Isopropyl-thiogalactopyranoside) and 20 mg mL^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Sequencing: Sequencing was performed by Korean Microgene Co. Ltd., with automation dideoxy chain determination method.

RESULTS AND DISCUSSION

Chromosomal DNA was extracted from *S. enteritidis* E3. The PCR reaction was performed and single band of 511 bp was amplified by SefA-F/SefA-R primers (Fig. 1). The product was checked with 1% agarose gel electrophoresis. The PCR product was performed by

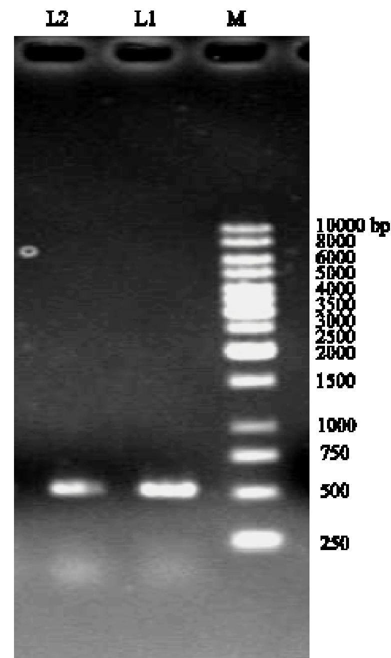

Fig. 1: Specific PCR for amplification of *Salmonella enteritidis SefA* gene with primer pair SefA-F/SefA-R. L1 and L2 referred to control and *Salmonella enteritidis* E3 isolate PCR product, respectively. M = DNA marker (GeneRuler™ 1kb DNA Ladder #SM0313)

```
>gi|310645|gb|L11008.1|STYSEFA  Salmonella enteritidis fimbrial protein (SefA) gene, complete
cds
Length=498

 Score =  963 bits (486),  Expect = 0.0
 Identities = 492/494 (99%), Gaps = 0/494 (0%)
 Strand=Plus/Plus

Query  5    GTAAATCAGCATCTGCAGTAGCAGTTCTTGCTTTAATTGCATGTGGCAGTGCCCACGCAG  64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5    GTAAATCAGCATCTGCAGTAGCAGTTCTTGCTTTAATTGCATGTGGCAGTGCCCACGCAG  64

Query  65   CTGGCTTTGTTGGTAACAAAGCAGAGGTTCAGGCAGCGGTTACTATTGCAGCTCAGAATA  124
            |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  65   CTGGCTTTGTTGGTAACAAAGCAGTGGTTCAGGCAGCGGTTACTATTGCAGCTCAGAATA  124

Query  125  CAACATCAGCCAACTGGAGTCAGGATCCTGGCTTTACAGGGCCTGCTGTTGCTGCTGGTC  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125  CAACATCAGCCAACTGGAGTCAGGATCCTGGCTTTACAGGGCCTGCTGTTGCTGCTGGTC  184

Query  185  AGAAAGTTGGTACTCTCAGCATTACTGCTACTGGTCCACATAACTCAGTATCTATTGCAG  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  AGAAAGTTGGTACTCTCAGCATTACTGCTACTGGTCCACATAACTCAGTATCTATTGCAG  244

Query  245  GTAAAGGGGCTTCGGTATCTGGTGGTGTAGCCACTGTCCCGTTCGTTGATGGACAAGGAC  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245  GTAAAGGGGCTTCGGTATCTGGTGGTGTAGCCACTGTCCCGTTCGTTGATGGACAAGGAC  304

Query  305  AGCCTGTTTTCCGTGGGCGTATTCAGGGAGCCAATATTAATGACCAAGCAAATACTGGAA  364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  AGCCTGTTTTCCGTGGGCGTATTCAGGGAGCCAATATTAATGACCAAGCAAATACTGGAA  364

Query  365  TTGACGGGCTTGCAGGTTGGCGGGTTGCCAGCTCTCAAGAAACGCTAAATGTCCCTGTCA  424
            |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  365  TTGACGGGCTTGCAGGTTGGCGAGTTGCCAGCTCTCAAGAAACGCTAAATGTCCCTGTCA  424

Query  425  CAACCTTTGGTAAATCGACCCTGCCAGCAGGTACTTTCACTGCGACCTTCTACGTTCAGC  484
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425  CAACCTTTGGTAAATCGACCCTGCCAGCAGGTACTTTCACTGCGACCTTCTACGTTCAGC  484

Query  485  AGTATCAAAACTAA  498
            ||||||||||||||
Sbjct  485  AGTATCAAAACTAA  498
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Fig. 2a: Nucleotide alignment of *Salmonella enteritidis* E3 *SefA* gene with standard isolate (L11008). This alignment show that *Salmonella enteritidis* E3 carried out two nucleotide mutation in the positions of 89 and 387

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>gi|462119|sp|P12061|FM SALEN  Fimbrial protein precursor
 gi|310646|gb|AAA27219.1|  fimbrial protein
Length=165

 Score =  264 bits (674),  Expect = 2e-69
 Identities = 151/152 (99%), Positives = 151/152 (99%), Gaps = 0/152 (0%)
 Frame = +1

Query  40   IACGSAHAAGFVGNKAEVQAAVTIAAQNTTSANWSQDPGFTGPAVAAGQKVGTLSITATG  219
            IACGSAHAAGFVGNKA VQAAVTIAAQNTTSANWSQDPGFTGPAVAAGQKVGTLSITATG
Sbjct  14   IACGSAHAAGFVGNKAVVQAAVTIAAQNTTSANWSQDPGFTGPAVAAGQKVGTLSITATG  73

Query  220  PHNSVSIAGKGASVSQQVATVPFVDGQGQPVFRGRIQGANINDQANTGIDGLAGWRVASS  399
            PHNSVSIAGKGASVSGGVATVPFVDGQGQPVFRGRIQGANINDQANTGIDGLAGWRVASS
Sbjct  74   PHNSVSIAGKGASVSGGVATVPFVDGQGQPVFRGRIQGANINDQANTGIDGLAGWRVASS  133

Query  400  QETLNVPVTTFGKSTLPAGTFTATFYVQQYQN  495
            QETLNVPVTTFGKSTLPAGTFTATFYVQQYQN
Sbjct  134  QETLNVPVTTFGKSTLPAGTFTATFYVQQYQN  165
```

Fig. 2b: Translation alignment of *Salmonella enteritidis* E3 SefA protein with standard isolate (L11008). This alignment show that *Salmonella enteritidis* E3 carried out on amino acid mutation in the position of 56

AvaII restriction enzyme and showed 217 and 281 bp fragments. The results indicated that *SefA* gene was amplified.

The PCR product was inserted in the corresponding site of cloning vector (pTZ57R/T) using Fermentase Ins T/A clone™ PCR product Cloning Kit.

In order to amplify the recombinant plasmids, *E. coli* DH5α bacteria was transformed with SefA/pTZ57R/T, via Transform Aid™ Bacterial Transformation system. Recombinant clones were identified by blue/white selection, since the vector is *LacZ* genetically marked. The correct insertion (SefA/pTZ57R/T product) was confirmed by restriction endonuclease analysis. The results showed that SefA/pTZ57R/T were correctly transformed in the *E. coli* DH5α.

Identification of the SefA/pTZ57R/T product was confirmed by sequencing. The sequence was compared with that published by the Genbank (L11008) which indicated that two point mutations exist in 89 and 387 nucleotide positions. Nucleotide and protein alignment with BLAST software showed that the sequence of the *SefA* gene derived from *S. enteritidis* E3, which was cloned in the pTZ57R/T vector, was 99% identical to that in the gene bank (L11008). The sequence of the *SefA* gene from *S. enteritidis* E3 differed only in two nucleotides (Fig. 2a) and this resulted in one amino acid difference (Fig. 2b). Thus, this method is simple and rapid and results obtained in less than 20 h proved to be highly specific and sensitive. The results of cloning and sequencing showed that the specimen from Kermanshah area not very different with other areas which are addressed in Genbank and these point mutation do not have a major effect on diagnostic of this bacteria.

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