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Rapid Detection of *Salmonella dublin* by PCR Amplification of the *SopE* Gene and its Cloning

[1]M.H. Mirmomeni, [1]S. Kiani and [2]S. Sisakhtnezhad
[1]Cell and Molecular Unit, Department of Biology, Razi University, Kermanshah, Iran
[2]Medical Biology Research Center, Kermanshah University of Medical Science, Kermanshah, Iran

Abstract: This study is directed towards the method of amplifying and cloning the *SopE* gene, that encodes *Salmonella* outer protein E. Strains used in this study were *S. dublin* collected from Kermanshah province. Genomic DNA was extracted by the general boiling method. Using the specific primers, a part of *SopE* gene was multiplied. The PCR product was inserted into the cloning vector (pTZ57R/T). Furthermore, *E. coli DH5α* bacteria were transformed to amplify the recombinant plasmid. Recombinant clones were identified by blue/white selection. Recombinant plasmids were purified by alkaline lysis procedure. Moreover, identity of the *SopE*/pTZ57R/T product was confirmed by restriction enzyme digestion assay and sequencing. Finally, the cloned gene was compared with that published by the NCBI Genbank (L78932). The results showed that the obtained sequence differed in four nucleotides which resulted in two amino acid differences. The cloned *SopE* was submitted to the NCBI Genbank (EU399750).

Key words: *Salmonella dublin*, *SopE* gene, PCR, Cloning, bacterial survival, cattle

INTRODUCTION

Salmonella is a worldwide pathogen and can be found in a large number of dairy farms and in many species of animals including mammals, birds, insects, reptiles and humans. *Salmonella dublin* (*Salmonella enterica* subsp. enterica serovar *dublin*) is a non-typhoidal *Salmonella* serovar which is most frequently recovered from cattle, to which it is regarded as strongly host adapted. It can be transmitted to humans via meat and dairy products. The human infections are severe and may be fatal, especially in AIDS and other immunocompromised persons (Kerouanton *et al*., 1996; Selander *et al*., 1992; Rotger and Casadesus, 1999; Mcdonough *et al*., 1999).

S. dublin receives much attention in the cattle industry for several reasons. It is a food-borne zoonotic bacterium that causes severe invasive infections in humans usually after consumption of milk products that have not been pasteurized properly or of insufficiently cooked meat.

In cattle, *S. dublin* causes economic losses from disease and death among calves and young animals, as well as abortion and reproductive disorders among adult cattle, extra labour and increased veterinary expenses (Nielsen *et al*., 2004; Santos *et al*., 2003). Salmonellosis due to *S. dublin* infection in cattle results in high morbidity and mortality in calves and significant morbidity in adult cattle (Rice *et al*., 1997).

Invasion and survival in mammalian cells by *Salmonella enterica* is mediated by bacterial proteins that are delivered to the host cell cytoplasm by type III secretion system. Type III secretion systems are highly conserved in a variety of gram-negative pathogenic bacteria. The main function of these systems is translocation of the effector proteins into the target eukaryotic cells. These effector proteins are Sop_s, AvrA and SptP. The Sop_s are *Salmonella* outer proteins which are encoded by *Sop* genes. Several polymorphisms of *Sop* genes have been identified (*Sop* A-E). One such translocated effector protein is *SopE* that contributes to the expression of *Salmonella* invasion by stimulating membrane ruffling (Jones *et al*., 1998; Rahman *et al*., 2004; Bakshi *et al*., 2000; Hapfelmeier *et al*., 2003; Wooda *et al*., 2004; Waterman and Holden, 2003). Although the genes encoding the type III secretion system are fairly well conserved between species, the secreted injected proteins are for the most part unique to each bacterial species (Schesser *et al*., 2000).

Because of *Salmonella* outer proteins, genes can play an important role in salmonella pathogenesis and its protein products can induce immune response in the animals, they can also be employed as an effective candidate to detection and intervention strategies in infected animals (Barrow, 2007).

Corresponding Author: M.H. Mirmomeni, Cell and Molecular Unit, Department of Biology, Razi University, Kermanshah, Iran

The aim of this investigation is to detection of *Salmonella dublin* from spacemen which were collected from Kermanshah, using the PCR assay for *SopE* gene and its cloning.

MATERIALS AND METHODS

Preparation of bacterial isolate: The cattle fecal samples were cultured into Selenit broth and Rappaport Vassiliadis broth and incubated at 37 and 42°C for 24 h, respectively. Then, these broth cultures were cultured in *Salmonella-shigella* selective medium and incubated at 37°C for 24 h and *Salmonella* suspected colonies were isolated. Furthermore, biochemical tests were performed. In this study, the control isolate, RITCO 1817, was provided by Razi institute (Karaj-Iran).

Preparation of genomic and plasmid DNA: Genomic DNA was extracted by the general boiling method. In this method one Bacteria colon from S.S agar medium was cultured in 10 mL Leuria Bertani (LB) broth, overnight at 37°C. Followed by centrifugation at 4000 rpm for bacterial precipitation. The bacterial cell was resuspended in a micro centrifuge tube with 300 μL of DNase-RNase free distilled water by vortexing. For extraction chromosomal DNA, the sample was heated in boiling water for 20 min and centrifuged for 5 min at 10000 rpm. Supernatant was collected and analyzed by 1% agarose gel electrophoresis (Sambrook *et al*., 2001). Plasmid DNA was isolated by an alkaline lysis procedure (Sihavy *et al*., 1984).

PCR: The primers including 5'-ACACACTTTCACCGAGGAAGCG-3' (forward primer) and 5'-GGATGCCTTCTGATGTTGACTGG-3' (reverse primer) were used for PCR amplification of *SopE* gene, designed by Rahman *et al*. (2004). The PCR mixture (50 μL) contained 5 μL 10x PCR buffer, 6 μL forward primer, 5 μL reverse primer, 1 μL dNTP, 1.5 μL $MgCl_2$, 0.5 μL Taq polymerase and 2 μL DNA template. The PCR-amplification was performed with temperature program consisting of initial denaturation (94°C, 3 min) and 30 cycles of denaturation (94°C, 1 min), primer annealing (55°C, 1 min) and primer extension (72°C, 2 min). For final extension, this was followed by incubation at 72°C for 10 min. A volume of 5 μL of the PCR product was analyzed by electrophoresis in 1% agarose gel.

Cloning bacteria strain, plasmid, media and growth conditions: *E. coli DH5α* was used as the host for cloning experiments with pTZ57R/T vector (Fig. 1). Stock culture was maintained at -70°C in 20% (v/v) glycerol. All culture was grown in LB broth at 37°C.

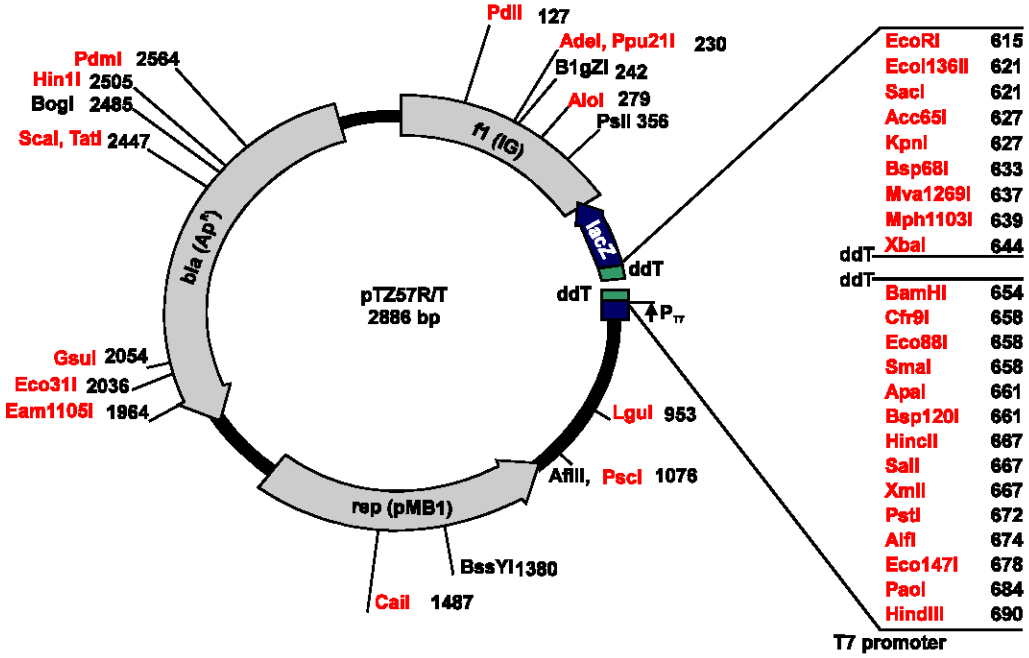

Fig. 1: pTZ57R/T cloning vector and its restriction map. rep (pMB1): A replicon (rep) from the pMBI plasmid is responsible for the replication. bla (AP^R): β-lactamase gene conferring resistance to ampicillin. f1 (IG): Synthesis of a single-stranded DNA (http://www.fermentas.com/catalog/kits/kitinstaclone.htm)

Restriction endonuclease digestion: For confirming the PCR product, 1 μL 10x PCR buffer, 4 μL ddH_2O, 3 μL product and 2 μL of *AluI* restriction enzyme were mixed and incubated at 37°C for 1 h and checked in a 1% agarose gel electrophoresis at 40 V and developed with UVIDoc. For confirming the *SopE*/pTZ57R/T construct, the same method was used with *BamHI* and *EcoRI* restriction enzymes.

Cloning of *SopE* gene: The *SopE* gene was obtained by PCR-amplification of *S. dublin* chromosomal DNA. The resulting PCR product was inserted into the corresponding site of cloning vector, pTZ57R/T.

Transformation: In order to insert recombinant vector (*SopE*/pTZ57R/T) into the component cells (*E. coli DH5α*), Transform Aid™ Bacterial Transformation System were used. Moreover, transformed *E. coli DH5α* were grown at 37°C in LB-Ampicilin agar plates containing 50 μg mL^{-1} Ampiciline, 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

Genomic DNA was extracted from *S. dublin*. PCR-amplification for *SopE* gene was successfully used. The PCR reaction was performed and specific band of PCR product was observed at the position corresponding to the expected size of the DNA amplification products about 399 bp for *SopE* gene. The PCR product was checked by agarose gel electrophoresis and stained with ethidium bromide (Fig. 2). Confirmation of PCR product was performed by *AluI* restriction enzyme and two 250 and 149 bp fragments were observed. The results indicated that *SopE* gene was amplified.

The resulting PCR product was inserted into 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 (*SopE*/pTZ57R/T), *E. coli DH5α* was transformed and identified by blue/white selection, since the vector is genetically marked. By using restriction endonuclease enzymes, the correct insertion was confirmed. Confirmation of *SopE*/pTZ57R/T product was performed

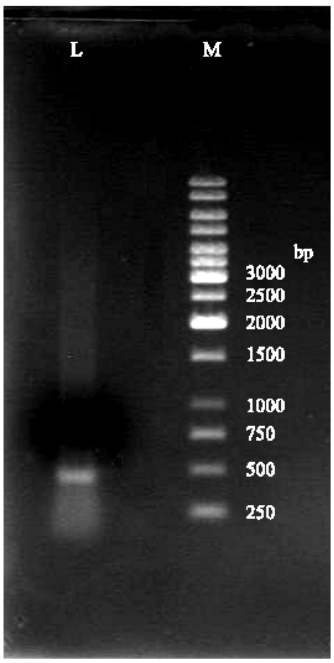

Fig. 2: The PCR product of the *SopE* gene. L refer to *S. dublin* isolate. M indicates the DNA marker (GeneRulerTM 1kb DNA Ladder # SM0313)

by *BamHI* and *EcoRI* restriction enzymes and two 2886 and 399 bp fragments were observed. The results showed that *SopE*/pTZ57R/T was correctly transformed in the *E. coli DH5α*.

The presence of correct insertion (*SopE*/pTZ57R/T) was confirmed by DNA sequencing. Hence, sequence was compared with another control sequence from Genbank (L78932) with BLAST software (Fig. 3). The results showed that four point mutations exist in 70, 246, 299 and 300 nucleotide positions, which proved 99% identical to that published by Genbank (L78932). Also, protein alignment with BLAST software showed that two amino acid differences exist in the obtained *SopE* amino acid sequence. The results showed that Serine and Methionine amino acids in L78932 isolate were replaced with Threonine and Leucine in the present isolate (Fig. 4).

The results obtained in this study showed the rapid and successful use of PCR in the identification of doubtful samples in comparison with traditional microbiological culture methods that may take several days to complete. The cloning and sequencing results showed that the specimen from Kermanshah region are not considerably different with other areas which are addressed in Genbank and these point mutations do not

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>gb|L78932.1|STYSOPE  Salmonella dublin SopE (sopE) gene, complete cds
Length=1011

Score =  715 bits (387),  Expect = 0.0
Identities = 396/400 (99%), Gaps = 2/400 (0%)
Strand=Plus/Plus

Query  1    ACACACTTTCACCGAGGAAGCGCATCTGAGGGCCGGGCAGTGTTGACAAATAAAGTCGTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295  ACACACTTTCACCGAGGAAGCGCATCTGAGGGCCGGGCAGTGTTGACAAATAAAGTCGTT  354

Query  61   AAAAACTTTTTGCTTCAAACGCTCCATGATATAGATATTAGAGGTAGCGCGAGTAAAGAC  120
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  AAAAACTTTATGCTTCAAACGCTCCATGATATAGATATTAGAGGTAGCGCGAGTAAAGAC  414

Query  121  CCCGCATACGCCAGCCAGACCCGTGAAGCTATACTATCGGCAGTTTACAGCAAGTATAAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  CCCGCATACGCCAGCCAGACCCGTGAAGCTATACTATCGGCAGTTTACAGCAAGTATAAA  474

Query  181  GATCAGTATTGTAACTTGCTCATCAGCAAAGGAATCGACATAGCGCCTTTTCTTAAGGAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  GATCAGTATTGTAACTTGCTCATCAGCAAAGGAATCGACATAGCGCCTTTTCTTAAGGAA  534

Query  241  ATTGGTGAGGCTGCGCAAAATGCAGGTCTGCCCGGAGCAACCAAGAATGACGTTTTTACG  300
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  ATTGGCGAGGCTGCGCAAAATGCAGGTCTGCCCGGAGCAACCAAGAATGACGTTTTTAGC  593

Query  301  CCAAGCGGCGCAGGAGCCAATCCTTTTATAACTCCGTTGATTACATCAGCATACAGTAA   359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594  CCAAGCGGCGCAGGAGCCAATCCTTTTATAACTCCGTTGATTACATCAGCATACAGTAA   653

Query  360  GTATCCACATATGTTTACCAGTCAACATCAGAAGGCATCC  399
            |||||||||||||||||||||||||||||||||||||||
Sbjct  654  GTATCCACATATGTTTACCAGTCAACATCAGAAGGCATCC  693
```

Fig. 3: Nucleotide alignments of a part of *SopE* gene with standard isolate (L78932)

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gb|L78932.1|STYSOPE  Salmonella dublin SopE (sopE) gene, complete cds
Length=1011

 Score =  276 bits (706),  Expect = 1e-72, Method: Compositional matrix adjust.
 Identities = 131/133 (98%), Positives = 133/133 (100%), Gaps = 0/133 (0%)
 Frame = +1

Query  1    THFHRGSASEGRAVLTNKVVKNFLLQTLHDIDIRGSASKDPAYASQTREAILSAVYSKYK  60
            THFHRGSASEGRAVLTNKVVKNF+LQTLHDIDIRGSASKDPAYASQTREAILSAVYSKYK
Sbjct  295  THFHRGSASEGRAVLTNKVVKNFMLQTLHDIDIRGSASKDPAYASQTREAILSAVYSKYK  474

Query  61   DQYCNLLISKGIDIAPFLKEIGEAAQNAGLPGATKNDVFTPSGAGANPFITPLITSAYSK  120
            DQYCNLLISKGIDIAPFLKEIGEAAQNAGLPGATKNDVF+PSGAGANPFITPLITSAYSK
Sbjct  475  DQYCNLLISKGIDIAPFLKEIGEAAQNAGLPGATKNDVFSPSGAGANPFITPLITSAYSK  654

Query  121  YPHMFTSQHQKAS  133
            YPHMFTSQHQKAS
Sbjct  655  YPHMFTSQHQKAS  693
```

Fig. 4: Translation alignments of a part of *SopE* gene with standard isolate (L78932)

have a major effect on diagnostic of this bacteria. The cloned *SopE* gene from *S. dublin* was submitted to NCBI Genbank (EU399750).

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