**Доклади на Българската академия на науките**
**Comptes rendus de l'Académie bulgare des Sciences**
*Tome 66, No 7, 2013*

*BIOLOGIE*
*Biologie moléculaire*

# GENETIC VARIATIONS IN THE *STX1* AND *EAE* GENES OF BULGARIAN MILK *ESCHERICHIA COLI* ISOLATES

**Ivo Sirakov, Ralitsa Popova, Irina Alexandar*, Atanaska Marinova-Petkova**

(*Submitted by Academician A. Atanassov on April 1, 2013*)

**Abstract**

Several types of *Escherichia coli* could cause intestinal and extra-intestinal diseases in humans and animals. Shiga toxin (Stx) is the virulence factor which causes haemorrhagic colitis. The *eaeA* gene gives the ability of the bacteria to induce lesions on the host colonic epithelial cells. The aim of this study is to compare the regions of subunit A of *stx1* and *eae* genes of *E. coli* isolates with known ones described up to now in NCBI GenBank. Specific primers for the bacterial *stx1* and *eae* genes, conventional PCR, sequencing PCR and nucleic acid phylogenetic analysis were used in this study. Three isolates of *E. coli* possessing *stx1* gene, and one isolate – the *eae* gene, have been used. Sequencing analysis of the amplified fragments showed several point mutations, specific for the Bulgarian isolates. Nucleotide phylogenetic trees were constructed and they showed that the Bulgarian *stx1 E. coli* isolates formed an independent group, while *eae* isolate differentiated into an independent branch.

**Key words:** *E. coli*, *stx1* gene, *eae* gene, mutations

**Introduction.** *Escherichia coli* (*E. coli*) is an intestinal inhabitant, usually harmless and rarely causes diseases. However, several types of strains can cause intestinal and extra-intestinal diseases in humans and animals by horizontal transfer of virulence factors [1]. Enterohaemorhagic *E. coli* (EHEC) is considered as one of the most pathogenic types of *E. coli*, which can cause serious complications as haemolytic uremic syndrome (HUS) [2]. Shiga toxin (Stx) is the virulence factor responsible for the induction of haemorrhagic colitis [3]. The *stx1* gene has two subunits A and B. Additionally, some typical EHEC strains possess pathogenicity island, called "the locus of enterocyte effacement" (LEE),

which encodes a set of proteins. One of the proteins is an adhesin called intimin and is encoded by the *eaeA* gene. It gives the ability of the bacteria to induce attaching and effacing (A/E) lesions on the host colonic epithelial cells [4]. The LEE has been found in Enteropathogenic *E. coli* (EPEC), which cause diarrhoea in infants [5]. The presence of pathogenic *E. coli* in food is a public health concern. Transmission of virulence factors among *E. coli* strains contributes to their increased diversity due to its capacity for horizontal gene transfer. Under certain circumstances, such a gene transfer could lead to emergence of new pathogenic strains [6].

The aim of our study is to analyze the *E. coli* isolates by comparing specific regions of the *eae* gene and subunit A of *stx1* for nucleotide changes in their sequences.

**Material and methods.** ***Isolates and reference data.*** Three different isolates of *E. coli* containing the *stx1* gene (lab numbers: 6, 298–3, 298–4), and one with the *eae* gene (lab number 7) were used. The *stx1* and *eae* GeneBank reference sequences were used for the purposes of our study (Table 1). The *stx1* reference sequence AF461169.1 and the *eae* reference sequence AJ877229.1 were used for comparing our sequence results of the studied regions.

***Primers, PCR and sequencing.*** The genes were amplified by the primers described by Paton and Paton [7]. PCR reactions were run with PCR Master-Mix (Geneshun Biotech, China). The following programme was used described by Paton and Paton [7] and modified for this study with initial denaturation of 95 °C – 1 min, 40 cycles of 95 °C – 1 min, 62 °C – 1.5 min, 72 °C – 1.5 min, final

Table 1

Reference sequences from GeneBank for *stx1* and *eae* genes included in our study

| | | | | | |
|---|---|---|---|---|---|
| NCBI accession number for *stx1* source | AJ314839.1<br>*E. coli* ovine | AJ312232.1<br>*E. coli* human | Z36901.1<br>*E. coli* Australia | AF461169.1<br>*E. coli* human | M19437.1<br>*S. dysenteriae* |
| NCBI accession number for *stx1* source | JQ907525.1<br>*E. coli* feces | AY170851.1<br>*E. coli* bovine | AB050958.1<br>*E. coli* bovine | AY986982.1<br>*E. coli* shellfish | AB050959.1<br>*E. coli* bovine |
| NCBI accession number for *E. coli eae* source | AJ877229<br>human | DQ523610<br>no data | U59502 AF043226<br>rabbit bovine | AB647561<br>*Hirundo rustica* | AF253560 AB647615<br>goat kids human |

extention of 72 °C – 7 min. The PCR products were purified through PCR Gel Purification kit (Geneshun Biotech, China). The GeneQuant II spectrophotometer (Pharmacia LKB, Biochrom, UK) has been used to control nucleic acid quantity and purity. The obtained PCR products were sequenced using MegaBACE 1000 (Amersham Biosciences) by DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Giles, UK) according to the manufacturer's instructions. Each product was sequenced twice with each of the primers (forward and reverse). Two controls were included – control for the reaction and control for the detection.

***Data processing.*** ClustalW [8] for multiple alignments of nucleotide sequences was used. JModelTest 0.1.1 [9] was used to find the best model for construction of phylogenetic trees, based on nucleotide sequences. PhyML 3.0 [10] by Phylemon 2 [11] and 1000 bootstrap replications were used to build phylogenetic trees. FigTree software has been used for a graphical view of phylogenetic trees (`http://tree.bio.ed.ac.uk/`). BLAST (National Center for Biotechnology and Information) was used for searching a similarity between sequences.

**Results.** The sequenced products for the *stx1* were positioned between 746 and 925 bp of the gene region and were 180 bp in length. The *eae* sequenced region was positioned between 40 bp and 396 bp and was 357 bp in length. The *eae* gene nucleotide sequence of lab number 7 isolate was deposited in GeneBank under the number of KC196849. Similarity between 95 and 99% with other *stx1* and *eae* *E. coli* isolates has been established by using the BLAST. The sequence analysis demonstrated point mutations for Bulgarian isolates. Nine point mutations were established for the *stx1* gene region and four point mutations for the *eae* gene (Table 2).

K80 and TPM3uf were found as the best models for constructing phylogenetic trees for the *stx1* and *eae* genes respectively. The phylogenetic analysis

T a b l e 2

Point mutations detected into the *stx1* and *eae* genes of *E. coli* isolates

| *E.coli* isolates | GenBank Accession number | Positions of point mutations of the region 746–925 bp of the *stx1* gene | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 784 | 791 | 796 | 820 | 829 | 865 | 874 | 880 | 904 |
| 6 | | G | T | C | A | A | G | C | C | T |
| 298-3 | | G | T | C | A | A | G | C | C | T |
| 298-4 | | G | T | C | A | A | G | C | C | T |
| EK921 | AF461169.1 | A | C | T | G | G | A | G | A | G |
| | | Positions of point mutations of the region 40–396 bp of the *eae* gene | | | | | | | | |
| | | 120 | | 201 | | 315 | | 394 | | |
| 7 | KC196849 | T | | A | | A | | C | | |
| CB 9786 | AJ877229.1 | C | | G | | G | | G | | |

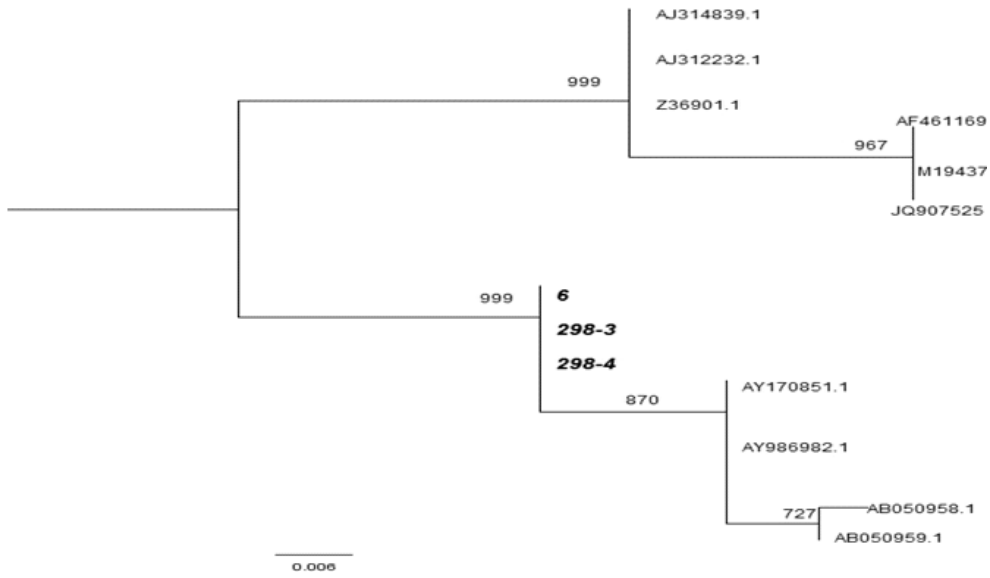


Fig. 1. Nucleotide phylogenetic analysis of Bulgarian *stx1* isolates (bold) and reference *E. coli* strains. It was used the K80 model by PhyML3.0, Phylemon 2 and bootstrap 1000. FigTree software has been used to a graphical view of phylogenetic tree

demonstrated that the examined Bulgarian *E. coli* isolates carrying the *stx1* genes differentiated into an independent group (Fig. 1), while *eae* differentiated into an independent branch (Fig. 2).

**Discussion.** Three of the studied isolates contained the *stx1* gene responsible for the Stx expression. The sequencing of the 180 bp fragments of the *stx1* gene showed several point mutations, the same in all three isolates. Thus these isolates differ from the other published until now and form an independent branch of the phylogenetic tree. The isolates were found in the same region and this could explain the same point mutations, which shows that most probably these are three individual isolates from the same strain. Detected point mutations could lead to structural differences of the toxin and its hydrophobic characteristics as it has been established for some viruses [12]. The mutated gene could have different effect on the protein expression and host organism.

The fourth *E. coli* isolate contained the *eae* gene. Sequencing of the 357 bp fragment showed several point mutations. These mutations probably affect the molecule of *intimin* like a part of the LEE and lead to a change in A/E lesions on the host colonic epithelial cells.

The mutations found in the studied fragments of the *stx1* and *eae* genes could be explained like single nucleotide polymorphisms – SNPs [13]. This could

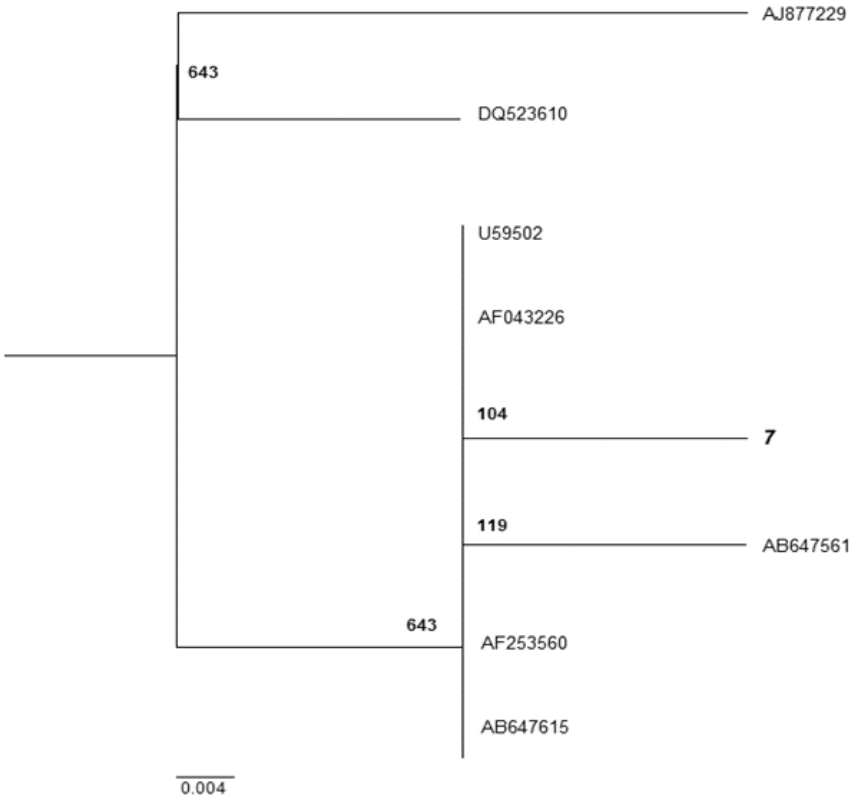


Fig. 2. Nucleotide phylogenetic analysis of the Bulgarian *eae* isolate of *E. coli* and reference strains. TPMuf model was used by PhyML3.0, Phylemon 2 and bootstrap 1000. FigTree software has been used to a graphical view of phylogenetic tree

be used as a biological marker and a rapid diagnosis of pathogenic *E. coli* isolates in illness progression.

Our next work will be to confirm the SNPs found in this study by another method. We are going to find out if these are synonymous mutations or not, what their significance for the protein structure is and their interactions with receptor molecules of the host cells.

**Conclusions.** Following sequencing research demonstrated several point mutations (SNPs) which are not established in another isolate up to now. The constructed phylogenetic tree suggested that the Bulgarian *E. coli* isolates form independent phylogenetic branches due to their point mutations in the studied regions. The studied fragment of the *eae* gene was deposited in the GeneBank under the number of KC196849.

**Acknowledgements.** The authors are thankful to Dr. Doris D'Souza (Director, Center for Detection Technology, NAFSS, USA) for the support in adapting the protocol for detection of *stx1* and *eae* genes by PCR in our lab.

## REFERENCES


[1] Dobrindt U. Int. J. MedMicrobiol., **295**, 2005, No 1, 357–371.
[2] Caprioli A., S. Morabito, H. Brugere, E. Oswald. Vet. Res., **36**, 2005, No 2, 289–311.
[3] Nataro J. P., J. B. Kaper. Clin. Microbiol. Rev., **11**, 1998, No 1, 142–201.
[4] Kaper J. B., J. P. Nataro, H. L. Mobley. Nat. Rev. Microbiol., **2**, 2004, No 2, 123–140.
[5] Jores J., L. Rumer, L. H. Wieler. Int. J. Med. Microbiol., **294**, 2004, Nos 2–3, 103–113.
[6] Donnenberg M. S., T. S. Whittam. J. Clin. Investig., **107**, 2001, No 5, 539–548.
[7] Paton A. W., J. C. Paton. J. Clin. Microbiol., **36**, 1998, No 2, 598–602.
[8] Thompson J. D., D. G. Higgins, T. J. Gilbson. Nucleic. Acids Res., **22**, 1994, No 22, 4673–4680.
[9] Posada D. Mol. Biol. Evol., **25**, 2008, No 7, 1253–1256.
[10] Guindon S., O. Gascuel. Syst. Biol., **52**, 2003, No 5, 696–704.
[11] Sanchez R., F. Serra, J. Tarraga et al. Nucleic. Acids Res., **39**, 2011 (Web Servar issue), 408.
[12] Deininger P. L., P. Laporte, T. Friedmann. J. Virology, **37**, 1981, No 3, 871–875.
[13] Worth C. L., D. Burke, T. L. Blundell. Beilstein-Institut Proceedings, 2007, 11–26.



*Prof. Dr G. Pavlov National Diagnostic and Research Veterinary Medical Institute*
*15, Pencho Slaveykov Blvd*
*1606 Sofia, Bulgaria*
*e-mail*: insirakov@gmail.com

**Institute of Molecular Biology*
*Bulgarian Academy of Sciences*
*Acad. G. Bonchev Str., Bl. 21*
*1113 Sofia, Bulgaria*