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Subtyping of Stx and 16s rRNA Gene Analysis of Some Bulgarian Shiga Toxin Producing *Escherichia coli* Isolates

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Abstract

In this study, among five positive isolates of *stx1* isolated from raw milk and chicken meat, one isolate showed a positive result for subtype *stx1d* and the other four isolates were positive for subtypes *stx1d* and *stx1a*. An isolate positive for *stx2* isolated from pork was found to carry an *stx2g* subtype. Besides subtyping, we performed phylogenetic analysis based on 16s RNA gene sequencing data. Based on the mutations identified in the isolates, we sought for restriction enzymes to distinguish the strains from one another. The results for the combination of *stx1* subtypes are in agreement with other reports, being the most common combination in *Escherichia coli* isolates with an origin from pigs. Interestingly, the pork isolate subtyped as *stx2g* by us is in contrast with studies according to which the most common subtype of this source is *stx2e.*
Key words: *Escherichia coli, stx1, stx2,* 16sRNA, PCR

Резюме

При изследване на пет положителни изолати за *stx1*, изолирани от сурово мляко и от пилешко месо установихме, че един от изолатите показа наличие на субтип *stx1d,* а останалите четири - наличие на два субтипа, съответно *stx1d* и *stx1a*. Изолат, положителен за *stx2* и изолиран от свинско месо се установи, че притежава субтип *stx2g*. Освен субтипизиране, анализирахме данни на 16s DNA гена, изготвихме филогенетичен анализ и въз основа на установените мутации при рзличните изолати потърсихме рестриктазни ензими за разграничаването им. Установихме, че нашите резултати от комбинация на субтипове за *stx1* се припокриват с резултатите от проучвания на други автори, като най-често срещана при изолати *E.coli,* изолирани от свине, като в същото време изолатът от свинско месо и субтипизиран за *stx2g* от нас е в разрез с изследвания, според които най-често срещаният субтип от този източник е *stx2e*.

Introduction

Escherichia coli is a facultative anaerobe, part of the normal microflora in mammals, birds and reptiles. Studies of 5S and 16S ribosomal RNA have revealed that *Escherichia* occurred 120–160 million years ago (Ochman and Wilson, 1987), evolving with mammals to the relationships we know today. Most *E. coli* strains play an important role in animals and humans, but there are pathogenic types of *E. coli* that can cause urinary tract infections; sepsis/meningitis, pneumonia, gastrointestinal infections, osteomyelitis and soft tissue infections. Shiga toxin-producing *E. coli*, which cause infections (STEC/EHEC) (Mathewson *et al.*, 1985) through consumption of contaminated food and water, are of greatest importance. Their major reservoir is ruminants which can also be a vector of these pathogens to the human food chain and may cause diseases in humans (Mainil, 1999). In recent years, the consumption of raw green vegetables as well as sprouted seeds has gained popularity, resulting in numerous outbreaks of diseases after

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consumption of such products (Hou *et al.,* 2013). The variants of *stx* differ according to their biological activity as well as the presence or absence of cross-reactivity with the *stx1* and *stx2* antiserum and some of the variants are characterized by lower affinity for the glycolipid Gb3 receptor caused by the exchange of amino acids in the B subunit of the protein (Melton-Celsa *et al*., 1998). The lack of serological confirmation for *stx* that was positive in a PCR study may be due to the lack of *stx* expression or to its expression at a level below the detection limit of the method (Zhang *et al.*, 2005). According to the subtyping nomenclature suggested at the 7th International Symposium on Shiga toxin-producing *E. coli* in 2009, there are three *stx1* subtypes (*stx1a, stx1c* and *stx1d*) and seven *stx2* subtypes (*stx2a, stx2b, stx2c, stx2d, stx2e, stx2f* and *stx2g*) and not all of them could be detected in different assays, but some strains reportedly may have poor expression of some *stx* subtypes (Ahmad *et al.,* 2006).

Often O157:H7, as well as other pathogenic EHEC are carriers of the *stx2a* subtype. At the same time, however, these strains also have additional virulence factors, since *stx* itself cannot cause severe symptoms such as bloody diarrhea and haemolytic uremic syndrome (Jelacic *et al*., 2003).

Subtype *stx2g* from STEC was originally isolated from cows (Leung *et al.*, 2003) and then from human isolates. It has been found that in most of these isolates, the *stx2g* subtype gene is not expressed (Prager *et al.*, 2011).

The aim of this study was to subtype isolates that we found to be positive for *stx*, as well as to sequence and analyze data on the 16s DNA gene, *stx 1* and *stx 2* genes. The main objective is to determine to what extent these isolates could cause human illnesses based on their subtype.

Materials and Methods

Genomic DNA extraction, primers and PCR

Genomic DNA was extracted using the Bacteria Genomic DNA Fast Mimi Kit (Geneshun Biotech, Guangzhou, China) and was used as the template for PCR. The specific regions of *stx1, stx2* and *eae* genes were amplified by means of PCR. The reaction was conducted in 50 μL volumes, using Prime Taq™ DNA Polymerase (GeNet Bio, Korea). The primers used and the reaction conditions are presented in Table 1 and are according CRL VTEC procedure for Identification of the subtypes of Verocytotoxin encoding genes (vtx) of *E. coli* by conventional PCR.

The quality and quantity of the extracted DNA and the PCR products were verified by 2% agarose gel (SeaKem® LE *Agarose,* Lonza, USA) electrophoresis at 120 V, 80 mA and 40 min at room temperature. We used DNA Ladder 100 bp (Bio-Lab, New England) and HyperLadder™ 1kb (Bioline, UK).

We used MEGA7 and Restriction Mapper (Blaiklock, 2009) to select suitable restriction enzymes by which to distinguish the isolates based on the 16s rRNA gene.

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Sequencing and data processing

The PCR products were prepared for sequencing by column purification and an agarose gel DNA Extraction Kit (Geneshun Biotech, Guangzhou, China), according to the manufacturer's instructions. Purified products and primers at a concentration of 10 pmol were sent for sequencing to Macrogen Europe, Netherlands. The sequences we have obtained were processed through MUSCLE (Edgar, 2004) by MEGA 7 software (Kumar *et al*., 2016), which was used for multiple alignments and construction of phylogenetic trees based on nucleotide and amino acid sequences. To build the phylogenetic trees, 1000 bootstrap replications were used.

Results

When analyzing the isolates positive for *stx1* and *stx2*, respectively, we found that one of the isolates positive for *stx1* showed presence of subtype *stx1d* and the other four isolates showed two other subtypes, *stx1d* and *stx1a*. The isolate positive for *stx2* was found to have the *stx2g* subtype (Fig. 1).

After processing the sequences, we obtained the 1369 nucleotides suitable for analysis of the 16S RNA gene. The HKY (Hasegawa-Kishino-Yano) + G nucleotide substitution model was proved to be the most appropriate for phylogenetic analysis by the Maximum Likelihood Method (ML).

The results from the nucleotide distribution frequency are presented in Table 2. Using Restriction Mapper and MEGA7, we were not able to detect

Table 1. Primers and PCR parameters. ID – initial denaturation/polymerase activation; D – denaturation; A – annealing; E – elongation; FE – final elongation; C – cycles

Genes	Primer sequences 5'– 3'	Thermal profiles	Product size (bp)	Positions of primers	Source of primers
16s RNA	27F- AGAGTTTGATCMTGGCTCAG	ID – 95˚C – 2 min, C - 30	~ 1502	8-27	Lane, 1991
		D - 95˚C – 1 min			
	1492R - GGTTACCTTGTTATGACTT	A – 56˚C – 1 min		1492-1510	Harris et al. 2004
		E – 72˚C -2 min			
v t x 1 a - F 1	CCTTTCCAGGTACAACAGCGGTT	ID – 95˚C – 15 min, C - 35	478	362-384	EU RL VTEC
vtx1a-R2	GGAAACTCATCAGATGCCATTCTGG	D - 94˚C –50 sec		815-839	
		A – 64 C – 40 sec			
v t x 1 c - F 1	CCTTTCCTGGTACAACTGCGGTT	E – 72˚C -1 min	252	362-384	EU RL VTEC
vtx1c-R1	CAAGTGTTGTACGAAATCCCCTCTGA	FE – 72˚C -3 min		588-613	
v t x 1 d - F 1	CAGTTAATGCGATTGCTAAGGAGTTTACC		203	50-78	EU RL VTEC
vtx1d-R2	CTCTTCCTCTGGTTCTAACCCCATGATA			225-252	
v t x 2 a - F 2	GCGATACTGRGBACTGTGGCC	ID – 95˚C – 15 min, C - 35	349	754-774	EU RL VTEC
v t x 2 a - R 3	CCGKCAACCTTCACTGTAAATGTG	D - 94˚C –50 s		1079-1102	
vtx2a-R2	GGCCACCTTCACTGTGAATGTG	A – 62 C – 40 s	347	1079-1100	
		E – 72˚C -1 min			
v t x 2 b - F 1	AAATATGAAGAAGATATTTGTAGCGGC	FE – 72˚C -3 min		968-994	EU RL VTEC
vtx2b-R1	CAGCAAATCCTGAACCTGACG		251	1198-1218	
v t x 2 c - F 1	GAAAGTCACAGTTTTTATATACAACGGGTA			926-955	EU RL VTEC
vtx2c-R2	CCGGCCACYTTTACTGTGAATGTA		177	1079-1102	
v t x 2 d - F 1	AAARTCACAGTCTTTATATACAACGGGTG			927-955	EU RL VTEC
v t x 2 d - R 1	TTYCCGGCCACTTTTACTGTG		179	1085-1105	
vtx2d-R2	GCCTGATGCACAGGTACTGGAC		280	1184-1206	
v t x 2 e - F 1	CGGAGTATCGGGGAGAGGC		411	695-713	EU RL VTEC
vtx2e-R2	CTTCCTGACACCTTCACAGTAAAGGT			1080-1105	
v t x 2 f - F 1	TGGGCGTCATTCACTGGTTG		424	451-475	EU RL VTEC
vtx2f-R1	TAATGGCCGCCCTGTCTCC			856-874	
v t x 2 g - F 1	CACCGGGTAGTTATATTTCTGTGGATATC		573	203-231	EU RL VTEC
vtx2g-R1	GATGGCAATTCAGAATAACCGCT			771-793	

Table 2. Nucleotide frequencies of investigated Bulgarian isolates (in bold) and five NCBI accessions sequences.

Isolates	**Nucleotide frequencies:**				
	T(U)	**C**	**A**	**G**	**Total**
6RP stx1	19.7	22.9	25.0	32.4	1369.0
22RP stx1	19.7	23.2	25.0	32.1	1369.0
390RP stx1	19.7	23.2	25.0	32.1	1369.0
983RP stx1	19.7	23.2	25.0	32.1	1369.0
984RP stx1	19.7	23.2	25.0	32.1	1369.0
348RP stx2	19.9	23.2	24.8	32.1	1369.0
KT824776	19.7	23.2	25.0	32.1	1369.0
KX421365	19.7	23.2	25.0	32.1	1369.0
KY367384	19.7	23.2	25.0	32.1	1369.0
KY887787	19.6	22.7	25.5	32.2	1363.0*
KY678497	19.8	23.2	24.9	32.1	1369.0

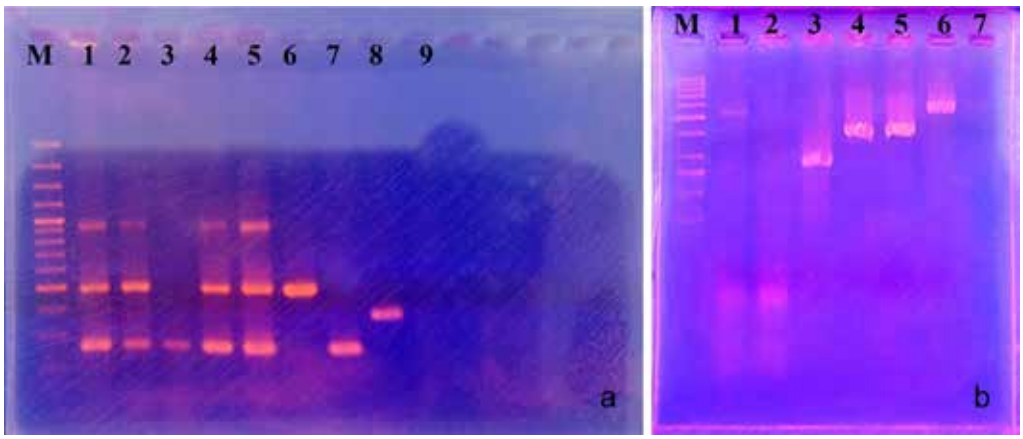

Fig. 1. Stx isolates subtyping by gel electrophoresis of PCR products.
Panel a - stx1 subtyping. Lane M: Molecular marker –100bp; Lane 1: isolate 983RP stx1; Line 2: isolate 6RP stx1; Lane 3: isolate 984RP stx1; Lane 4: isolate 22RP stx1; Lane 5: isolate 390RP stx1; Lane 6: positive control for stx1a subtype; Lane 7: positive control for stx1c subtype; Lane 8: positive control for stx1d subtype; Line 9: negative control.
Panel b - stx2 subtyping. Lane M: Molecular marker –100bp; Lane 1: isolate 348RP stx2, Lane 2: negative control; Lane 3: positive control for stx2d subtype, Lane 4: positive control for stx2e subtype, Lane 5: positive control for stx2f subtype, Lane 6: positive control for stx2g subtype; Lane 7: no sample.

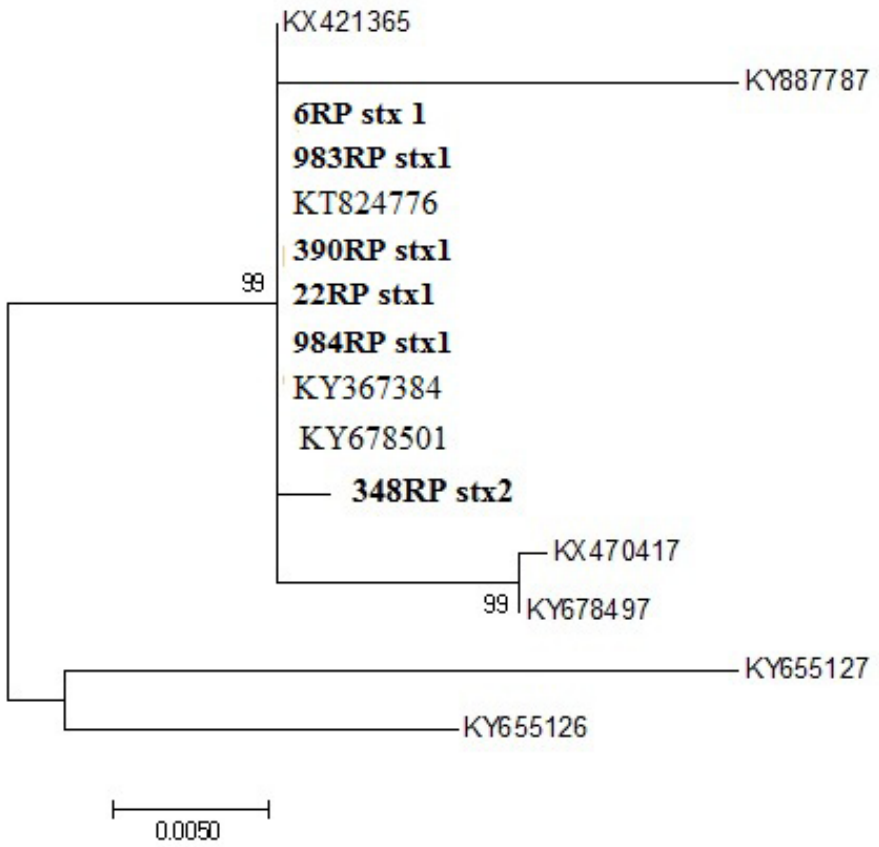

Fig. 2. Phylogenetic tree of thirteen sequencing datasets. Our isolates are in boldface. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap 1000 were used.

restriction enzymes by which to distinguish the isolates based on the mutations defining their topology in a phylogenetic analysis (Fig. 2).

Discussion

Stx is encoded by bacteriophages, lysogenic lambdoid phages, and can be obtained by horizontal transfer (Muniesa *et al*., 2004). Based on the phylogenetic analysis and the topology of certain isolates, Asakura *et al*. (2001) suggest that it is possible for strains to circulate between humans and sheep, through phage conversion, in close contact between the two species, such as that in the wool and the meat industry. A study of *E. coli* strains isolated from pigs found that the most common subtype was *stx2e*, followed by *stx1a, stx1d* and the smallest percent was that of *stx1c* (Baranzoni *et al*., 2016). Although the *stx1a* subtype is mostly associated with human diseases, STEC that produce subtypes *stx2a, atx2c*, *stx2d* are more commonly associated with HC and HUS (Friedrich *et al*., 2002, Melton-Celsa, 2014). We found that the isolate positive for *stx2* originating from pork carries the *stx2g* subtype. Isolates from pigs belong to different STEC serogroups that are transmitted through the food chain. Most of the piglet isolates are adapted to this host species and are most likely to have a low potential to infect humans (Baranzoni *et al.,* 2016). Besides the production of *stx*, additional virulence factors are involved in the pathogenesis of human STEC-induced diseases. The most common one is the attaching and effacing lesions gene of intimin, *eae* (Kaper, 1998). Several hundred STEC serotypes are known that can produce any of the subtypes or a combination of them, but not all of them can cause disease. Production of *stx* alone without an adherent factor is not sufficient to cause serious illness. A study and characterization of 132 STEC isolates for the presence of virulent genetic factors and *stx* subtypes showed that the *stx1a* and *stx2a* subtypes were present in 22% and 56%, respectively, and were the most common ones. The second most common subtype is *stx2d*, followed by *stx2c* and only 2–3% of strains are *stx2e* and *stx2g* positive. The same study proves that most of the isolates that have *stx1* are actually carriers of the *stx1a* subtype, and that this subtype was most commonly present in combination with others, in particular, *stx2* subtypes. Pathogenic STEC strains that produce *stx1* most commonly possess the *stx1a* subtype (Feng and Reddy, 2013). The absence of adherence factor in these strains suggests that these isolates cannot cause serious diseases in humans.

Considering this and the report of Asakura *et al.* (2001), as the isolates 298-3, 4 and 6 have sheep milk origin, we could similarly speculate that our isolates that have the *stx1* subtype but do not have an additional adherent factor are not likely to cause serious diseases in humans (animal handlers and consumers), but still can be a potential risk factor for them.

It has been reported that only 2% STEC show the *stx2g* subtype, as the isolates are isolated from sprouts, spinach and coriander. For the first time, this subtype was isolated from faecal bacteriophages in contaminated water (Garciia-Aljaro *et al.,* 2006). This subtype is rarely found in isolates from humans but has been detected in food STEC isolates (Beutin *et al.,* 2007). The *stx2g* subtype is also detected in bovine faeces, and has been found to play a very minor role as a pathogen in humans (Leung *et al.*, 2003). Having in mind that the isolate positive for *stx2* and subtyped as *stx2g* originating from pork has an additional virulent factor gene for intimine, *eae*, we could suggest that despite the low virulence of this subtype, according to the literature it may still have higher virulence for humans because of the combination of *stx2g* and the gene of attaching and effacing lesions, intimin.

Stx subtyping is important not only for STEC characterization but also for the diagnosis of disease, as some shiga toxin types and subtypes are epidemiologically associated with different manifestations of STEC infection (Krüger *et al.,* 2015).

The 16s rRNA gene shows a high degree of functional consistency (Woese, 1982) and it is the most widely used and most useful molecular chronometer (Woese, 1987). In this regard, the sequencing of the 16s rRNA gene is a reliable identification method (Petti *et al.,* 2005) and can be used concurrently with the phenotypic characterization of bacteria.

Our phylogenetic analysis of the 16s rRNA gene revealed that the studied Bulgarian isolates fall into one cluster and the *stx2* positive isolate is a separate branch in it.

Some authors use the sequencing of the 16s rRNA gene to identify isolated bacteria (Petti *et al*., 2005; Atanasova-Pancevska *et al.*, 2016), while in other cases it cannot be used alone itself but as a complementary method (Orozova *et al*., 2017), due to the fact that genotypically and ecologically different bacteria have similarity in the 16s rRNA gene (Jaspers and Overmann, 2004). Kotetishvili *et al.* (2002) attempted to implement the MLST based on the 16S rRNA gene and sequencing the same for

typing and discriminating Salmonella isolates, but did not detect any differences (lack of cluster formation). This is due to the different rate of changes of the 16s rRNA gene, which is determined by the taxonomic group of the microorganism (Clarridge, 2004). In our analysis, the data from the *E.coli* isolates included in the study are of different topology, but the differences in the sequences are not pre-selective for enzymes, by which to distinguish them. Additionally, the isolates are a limited number and it can be speculated that there is a connection between the topology of isolate 348RP and the presence of the stx2 gene.

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