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Detection of Pathogenic *Escherichia coli* Strains Pollution in Red Deer Meat in Latvia and Determination the Compatibility of *VT1, VT2, eaeA* Genes in their Isolate

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***Abstract*—**Tasks of the work were study the possible *E.coli* contamination in red deer meat, identify pathogenic strains from isolated *E.coli*, determine their incidence in red deer meat and determine the presence of VT1, VT2 and *eaeA* genes for the pathogenic *E.coli.* 8 (10%) samples were randomly selected from 80 analysed isolates of *E.coli* and PCR reaction was performed on them. PCR was done both on initial materials – samples of red deer meat - and for already isolated liqueurs. Two of analysed venison samples contain verotoxin-producing strains of *E. coli.* It means that this meat is not safe to consumer. It was proven by the sequestration reaction of *E. coli* and by comparison of the obtained results with the database of microorganism genome available on the internet that the isolated culture corresponds to region 16S rDNS of *E. coli* thus presenting correctness of the microbiological methods.

***Keywords*—**Deer meat, pathogenic *Escherichia coli*

I. Introduction

ESCHERICHIA coli is a Gram-negative facultative anaerobic non-spore-forming bacterium and members of *Enterobacteriaceae* family. *E.coli* is a member of human and almost all mammals intestinal microflora. Of the total content of colon (approximately 10^4-10^9 kvv/g) and eubiotic intestinal microflora *E.coli* occupies up to 1%. Therefore describe it as a “additional microflora”. However, some strains may be pathogenic. An ability of *E.coli* to produce verotoxins and cause foodborne infections was first detected in 1982 in USA, when after eating hamburger severe human disease and death cases were observed [1]. Karch et al. [2], when first describe *Escherichia coli* that caused enterohemorrhage (EHEC or VTEC), indicate these microorganisms as a new group of enteropathogenic bacteria; according to Bindu Kiranmayi et al. [3] it is considered as the most pathogenic and dangerous. In human and animals, the pathogenic strains of *E.coli* can cause not only diarrhea but also hemolytic-uremic syndrome (HUS), hemorrhagic colitis (HC) and thrombocytopenic purpura (TTP), as well as other serious diseases [4]. Several VTEC serotypes, including *E.coli* O157:H7, are associated with human illnesses with clinical symptoms ranging from diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome – severe condition that may lead to fatal outcome [5].

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In opinion of, Strockbine et al. [6] verotoxigenic *E.coli* (VTEC) includes more than 100 serotypes, but according to the studies conducted by Blanco et al. [7] there can by up to 400 different VTEC serotypes in the gut microflora of healthy ruminants, mostly cattle.

These are sometimes described as Shiga-toxins producing (STEC) because of similarity with an action of toxins produced by *Shigella dysenteriae* VTEC occurrence in the feces of sick animals gives a possibility to contaminate environment, water bodies, obtained production (milk and meat) and using an infected fertilizer - also fruits and vegetables. VTEC can be transmitted also by direct contact - when contacting with sick people or animal [8].

VTEC is occasionally detected also in wild animals: in the fecal samples of mouse (*Alces alces),* in Canada and North America, of reindeer (*Rangifer tarandus)*, Lahti et al. [9] in Norway and Finland, and of red deer in Spain [10] VTEC occurrence in wild animals can be connected with the use of contaminated, collected with cattle, so-called "shared pasture" where alternately domestic animals and deer are herded [11]. VTEC transmission in deer gardens or between gardens is not known. It is detected that meat and milk products contaminated with feces is an important transmission factor of VTEC in human; it causes many exacerbations of disease or sporadic cases [12]. Studies conducted by Rabatsky-Ehr et al. [13] confirms that the consumer's illness is connected with contaminated game meat. Miko et al. [14] has revealed that game animals can be a reservoir of pathogenic VTEC and the meat obtained from them - a cause of possible consumer's disease. After examination to VTEC by using selective culture and PCR test for detection of intimin (*eae*) and verotoxin (VT) gene [10] it was detected that fifteen of 124 (12.1%) of *E.coli* isolates were *eae* gene-positive but the VT gene was found in none of them.

Although scientists are interested in VTEC infections since early 1980s, greater attention to the epidemiologic role of wild ruminants is paid only recently. Game is primary reservoir of the verotoxigenic *E.coli* and there are concerns that the game meat could be a source of infections dangerous for human and that its role in the transmission of colibacillosis is inadequately assessed [15].

So far there are few studies about exacerbations of the diseases associated with consumption and processing of game meat. However, despite the more stringent control of animal origin food and better quality of hygienic standards, human infection with game meat still occurs.

Objective of the study: to find out the distribution of *E.coli* and prevalence of VT1 and VT2-producing strains of *E.coli* isolated from samples of red deer meat in the deer farms in Latvia.

The aims of the work were:

1. Study the possible *E.coli* contamination in red deer meat;
2. Identify pathogenic strains from isolated *E.coli* and determine their incidence in red deer meat;
3. Determine the presence of VT1, VT2 and *eaeA* genes for the pathogenic *E.coli* ;
4. Determine the compatibility of VT1, VT2 and eaeA genes in one E.coli isolate.

II. Materials and Methods

According to the accepted methods for determination of the total number of microorganisms, the contamination of mesophilic aerobic and facultative anaerobic organisms in 80 samples of red deer meat was determined. In the further studies, the contamination of *E.coli* was isolated and pathogenic *E.coli* was determined randomly (in 10% of cases – in 8 samples). According to the article 12.2 of the standard LVS NE ISO 7218:2007, 8 pure cultures of *Escherichia coli* were obtained for comparison of the samples. The cultures were biochemically verified and compared with reference pure culture ATCC 25922. Separate cultures of *E.coli* were inoculated in bars on non-selective nutrient agar (Biolife, Italy). The agar plates were incubated at +37 °C for 24 ± 2 hours. After checking the homogeneity of the inoculated culture, it was re-inoculated in tubes on sloping agar, incubated at the previous regime and used in the further work.

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A. Methodology of Polymerase Chain Reaction (PCR)

PCR was done both on initial materials – isolated pure cultures – and for samples of red deer meat. To perform PCR, DNA was extracted first from chosen samples. According to the manufacturer instructions, it was done by using the tissue sample "DNeasy Blood and Tissue Kit" (Qiagen) and the set for the isolation of Gram-negative microorganisms. After the extraction of DNA, concentrations and purity of the extracted DNAs was tested measuring by spectrophotometer NanoDrop (Applied Biosystems). The pure cultures obtained by using inhibiting concentration, as well as DNA of the meat samples were used for PCR. DNA samples of the pure culture were diluted before PCR up to the concentration that is optimal for performing the reaction (approximately 100 ng/µl).

The DNA extract was stored at – 20 °C up to use. A commercial kit (Genekam Biotechnology AG) was used for performing of the common conventional PCR for amplification of highly conserved region of the *E.coli* genes. The reaction and amplification was performed according to the manufacturer instructions including the positive and negative control in the kit and using the amplificator TProffesional Gradient (Biometra, USA).

Agarose gel electrophoresis (HE 99X; Amersham Pharmacia Biotech, USA) was done after the end of reaction. The PCR products were brought to the 1.8% agarose gel together with the DNA heaviness marker (100 bp DNA Ladder, Fermentas). Providing amperage of 100V, electrophoresis was done for 45 minutes. The results were viewed in the UV transiluminator (Gene Genius; Syngene; United Kingdom). At first, the samples of red deer were analysed. Then the samples of the pure cultures of *E.coli* were examined by PCR analysis by using GeneRuler 100 bp DNA Ladder from Fermentas (Lithuania) as a marker.

Classical PCR was also performed to clarify if some of 8 isolated samples of *E.coli* DNA do not contain verotoxin-producing genes VT1, VT2 and intimin gene *eaeA* which serves as a binding site for toxins. The reaction was done in accordance with the reaction description in manual of 2004 (OIE Manual**:** chapter 2.9.11, 2004). For determining the verotoxin-producing genes, the amplification reaction was done in the volume of 25 µl. Primers selected according to the manual (OIE Manual**:** chapter2.9.11, 2004) were purchased from the company „Operon". The nucleotide sequences of primers and product sizes are showed in methodology (see Table 1).

After the end of the reaction, an agarose gel electrophoresis was performed. The PCR products were transferred to 1.8% agarose gel together with a DNA heaviness marker. Electrophoresis was done for 45 minutes at the amperage of 100V. The results were viewed in a UV transiluminator. Following the manufacturer directions and using a commercial kit „MicroSeq® 16S rDNA Bacterial Sequencing Kit", (Applied Biosystems), a sequencing reaction was performed.

B. Methodology for Real-time PCR reaction (RT-PCR) for Escherichia coli O157:H7

For finding out whether some of isolated samples belongs to the serotype O157:H7 of the VTEC group, the Real-time PCR was done by the Bio–Rad CFX96.

TABLE I
Sequences of the Used Primers for Approval of Verotoxin Genes

Target gene	Accession nr.	Primer name	Sequence	Amplicon length
VT1	M19437	VT1-1	5'-CGCTCTGCAATA GGTACTCC-3'	256 bp
		VT1-2	5'-CGCTGTTGTACC TGGAAAGG-3'	
VT2	X07865	VT2-1	5'-TCCATGACAACG GACAGCAG-3'	185 bp
		VT2-2	5'-GCTTCTGCTGTG ACAGTGAC-3'	
eaeA	X60439	eaeA1	5'-GCTTAGTGCTGG TTTAGGATTG-3'	618 bp
		eaeA2	5'-CCAGTGAACTAC CGTCAAAG-3'	

Using the commercial kit from the company PrimerDesign^{TM}Ltd., the quantitative determination of the *Escherichia coli* O175:H7 gene of translocated intimin receptor was performed The principle of hydrolyze probe (TaqMan®) was used in the study. Using the RT–PCR programs, RT-PCR standard curves and positive amplification curves was calculated for determining of the presence of *E. coli* 0157:H7 and *E. coli*.

III. Results

E.coli cultures were isolated from the tested 80 samples of red deer meat. In 10% of cases 8 pure cultures of *E. coli* were selected randomly from 8 samples of red deer meat, respectively. By PCR, the high-purity DNA was extracted from the mentioned samples both from pure cultures of *E. coli* and samples of red deer meat.

The results of analysis shows that the pathogenic *E.coli* O157:H7 was isolated from the first sample of pure culture and from the first sample of red deer meat; at the same time the stain contains pathogenic verotoxins, but the sample 6 of red deer meat and the pure culture obtained from it contained verotoxin VT2. The results of other samples of pure culture and red deer meat were negative (see Table II).

TABLE II
IDENTIFICATION OF PATHOGENIC E. COLI STAIN O157:H7 AND VEROTOXINS IN THE SAMPLES OF PURE CULTURE AND RED DEER MEAT

Sample No.		*E. coli*	*E. coli* O157:H7 VTEC	VT1	VT2	eaeA
Samples of *E. coli* pure culture	Samples of red deer meat					
1	1	+	+	-	+	+
2	2	+	-	-	-	-
3	3	+	-	-	-	-
4	4	+	-	-	-	-
5	5	+	-	-	-	-
6	6	+	-	-	+	-
7	7	+	-	-	-	-
8	8	+	-	-	-	-

The successfully performed PCR showed that *E.coli* DNA corresponding to a fragment of 670 base pairs (bp) was present in all 8 samples of red deer meat and in all 8 samples of *E.coli* pure cultures isolated from them. Between 8 samples of red deer meat and 8 samples of pure cultures obtained from them, the first meat sample and the first sample of *E.coli* pure culture obtained from it, as well as the sixth meat sample and the sixth sample of *E.coli* pure culture obtained from it contained *VT2* verotoxin gene. *VT2* corresponds to a fragment of 185 base pairs (bp) and *eaeA* corresponds to a fragment of 618 base pairs (bp). The first sample of red deer meat and the first sample of pure culture that was a pathogenic *E.coli O157:H7* also showed positive result to eaeA intimin gene.

The RT–PCR results confirmed again that between 8 examined samples of red deer meat and 8 samples of pure culture there was an *E. coli* serotype *O157:H7* in the first meat sample and the first sample of pure culture isolated from. Since DNA copy standards were included in the examination, the number of DNA copies was also determined in each sample. The results showed that there were thousands times more DNA copies in each sample of pure culture comparing with the corresponding sample of red deer meat. Such result could be explained by the fact that the bacterial material in the isolated pure culture is repeatedly multiplied, as well as the PCR inhibitors are diluted, but the last ones are present in large amount in the red deer meat.

IV. DISCUSSION

Additionally to classical microbiological methods PCR technique was used in the work to confirm the microbiological result because it is difficult to distinguish pathogenic and non-pathogenic strains of *E.coli* using classical microbiological selective and differential cultivation methods [3]. To find out and confirm a human diseases caused by verotoxin-producing pathogenic *E.coli,* detect contamination of raw materials of food and production molecular biology methods should be used.

Louie et al. [16] pointed out that *E. coli* is considered to be a virulent and infective microorganisms for human if the organism's genome contains intimin-coding *eaeA* gene additionally to verotoxin genes. Tutenel et al. [4] disclose that the last one ensure the introduction of *E.coli* into the intestinal microflora. Hazarina et al. [17] showed that VTEC colonization in the meat is determined by verotoxin-producing genes.

To confirm correctness of the classical microbiological methods and PCR reactions, the first sample of *E.coli* pure culture was chosen for sequestration reaction that is also a commonly used method for determining bacterial infections. The results obtained in sequestered region 16S rRNA were compared with database of the National Centre for Biotechnology Information of USA (NCBI) available on internet.

The obtained sequence revealed 100% correspondence with *E.coli* rRNA isolates presented on two databases (NCBI and „BLAST Search") Thus, the correspondence demonstrates the correctness of the used microbiological and molecular-biological methods.

The study reveals that there are possibly pathogenic strains with verotoxins between isolated *E. coli* strains. In one case (sample 1 of pure culture and meat sample 1) it was *VT2* and intimin binding *eaeA* but in sample 6 of pure culture and meat sample 6 – non-pathogenic strain of indefinite antigen structure with VT2. *E.coli* O157:H7 and other pathogenic strains of *E. coli* are toxic and possibly has originate during pre-treatment of red deer, evisceration, as well as during transportation and storage. Therefore it is necessary to follow HACCP (self-control of food processing plant) system from a game acquisition place to a consumer, especially in meat processing plants. In two cases, the analyzed samples of red deer meat contained verotoxin-producing *E.coli* strain thus approving its unconformity to safe use of food.

Our studies revealed that the evaluation of contamination of red deer meat is required in the practice. Verotoxigenic *E.coli* O157:H7 is a zoonotic agent since it is isolated mainly from foods of animal origin, isolated also from vegetables and water [18]. VT1, VT2 and *eaeA* are associated with the occurrence of *E.coli* O157:H7 infection in consumers [19]. The contamination by verotoxin-producing *E.coli* was undisputedly revealed in the samples of red deer meat examined by PCR for the first time in Latvia. There was evidence that it is necessary to examine the mentioned strains for virulent genes since the consumers of infected meat could be infected. Molecular biology methods for determination of virulent genes of *E.coli O157:H7* were used by Meng et al. [20], Holland et al. [21], Keen and Elder [22].

The presence of VT1, VT2 and *eaeA* in *E. coli* O157:H7 strains isolated from domestic animals have been investigated in USA, Japan and European countries. The studies reveal that the percentage of the verotoxigenic strains is quite high and compose 30 to 70% [9], [20], [22], [23], [24], [25], [26], [27], [28].

The detected presence of VTEC *E.coli* O157:H7 in the meat of red deer obtained in Latvia during hunting confirm a need for introducing the control measures for monitoring of *E.coli* O157:H7 serotype in red deer meat. Taking into account the results of previous studies and the data obtained in this study

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we see that cervids are one of the natural reservoirs of O157:H7 in Latvia that can cause zoonotic risk for hunters, consumers of red deer meat and for owners of nontraditional livestock who contact with carcasses of red deer or their parenchymatous organs[3], [10]. According to the data of Strockbine et al. [6], the VT2 and *eaeA*- producing pure culture of *E.coli O157:H7* and the meat contaminated by it, as well as the VT2-producing pure culture of *E.coli* its contaminated meat is connected with the *E.coli* caused infectious disease.

V. CONCLUSIONS

In the conducted study *E.coli* belonging to a pathogenic *E. coli* O157:H7 strain was proven and confirmed by the classical microbiological method and PCR revealing a need for a control program for captive red deer gardens and raw materials of red deer meat. Two samples of the red deer meat contained verotoxin-producing strains of *E.coli* from which one sample (*E.coli* O157:H7 serotype) contains both a verotoxin-producing gene VT2 and intimin gene *eaeA* thus revealing that the red deer meat can be a source of infection.

The studies for detection of the contamination of red deer meat with pathogenic *E.coli* by using the molecular-biology methods were conducted in Latvia for the first time.

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