Isolation of Shiga toxin-producing strains of Escherichia coli from beef and swine carcasses and the characterization of their genes

O. M. Berhilevych*, V. V. Kasianchuk*, O. M. Deriabin**, M. D. Kukhtyn***

*Sumy State University, Sumy, Ukraine
**State Scientific Control Institute of Biotechnology and Strains of Microorganisms, Kyiv, Ukraine
***Ternopil Ivan Puluj National Technical University, Ternopil, Ukraine

Introduction

Escherichia coli is a bacterium that normally inhabits the intestines of humans and warm-blooded animals. Most strains of E. coli are not harmful for them, but some strains are pathogenic causing gastrointestinal infections (Dhama et al., 2013; Ray & Bhunia, 2014; Shuhong et al., 2015; Awadallah et al., 2016). Nowadays, more than 700 different serotypes of E. coli have been identified and divided into different serotypes according to their “O” (somatic or lipopolysaccharide) and “H” (flagellar) antigens. Pathogenic strains of E. coli are divided into six groups (enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, enterocytotoxigenic and diffuse-adhering) based on their ability to produce toxins and to adhere and to invade intestinal epithelial cells (Rani et al., 2017; Vijayan et al., 2017). But the most pathogenic strains of E. coli of all known strains are enterohemorrhagic E. coli (EHEC). Serogroup O157:H7 of EHEC is the most frequently associated with serious food poisoning, which is accompanied by severe bloody diarrhea, hemorrhagic uremic syndrome (HUS) and can be lethal for humans.

Other serogroups (O26, O45, O103, O111, O121, O145) of EHEC are less common. One of the main characteristics of all of these serogroups is the production of Shiga-toxin (Stx), which is an important virulence factor, responsible for HUS. Because all enterohemorrhagic E. coli produce Shiga-toxin, they are also known as Shiga-toxicigenic E. coli (STEC) or verotoxigenic E. coli (VTEC) because of their cytotoxic effect on Vero cells (Montusz & Jarzab, 2013; Haugum et al., 2014, Ray & Bhunia, 2014; Sudeshlan et al., 2014).

There are two types of Shiga-like toxins (Stx1 and Stx2) produced by STEC. Shiga-toxicigenic E. coli can produce Stx1 only, Stx2 only, or both. The production of these toxins is regulated by bacteriophages which carry the stx genes and which lyosgenize the bacteria to produce toxins. For E. coli O157:H7, the toxins are silent during lysogeny; however, if the phages are induced to enter the lytic cycle, phage and toxin production will occur. Induction to the lytic cycle can occur after exposure of the bacteria to DNA damaging agents, such as low iron conditions, UV light or mitomycin C, or to antibiotics (Pattalingamma et al., 2012; Rashid et al., 2013; Ray & Bhunia, 2014).
Bhunia, 2014). In addition to Shiga toxin production, another virulence factor expressed by STEC is intimin. Intimin is a membrane protein produced by all attaching enteric pathogens including STEC as an adherence factor for attachment to the intestinal epithelial cells. The eae gene codes produce this protein. STEC also have other adherence factors such as fimbriae, autotransporter, flagella, and EbgE and EbgL-1 adhesin. The most virulent strains of STEC have all these genes. However, some strains of STEC are capable of having the gene that codes the first toxin (stx1), or the gene that codes the second toxin (stx2), or both genes (stx1and stx2) at the same time, or other combinations of three virulence genes (stx1, stx2 and eae) (Croxon et al., 2013; Awadallah et al., 2016; Soledad-Cadona et al., 2018).

Humans primarily become infected by Shiga toxin-producing E. coli through consumption of food of animal origin (Ju et al., 2012; Monttaz & Jamshidi, 2013; Lozinak et al., 2016). Consumption of undercooked ground beef is the main source of infection as the meat can be easily contaminated with cattle feces during slaughtering and butchering (Tay et al., 2015; Abdissa et al., 2017; Premarathne et al., 2017; Vijayan et al., 2017; Omoruyi et al., 2018). Although beef meat is considered as the main source of STEC for people, alimentary infections caused by STEC have also been described after consumption of swine meat (Troz-Williams et al., 2012; Tseng et al., 2014).

The disease caused by the toxin usually has very serious consequences for human health and can manifest by three different syndromes: hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura and which can even cause death (Majowicz et al., 2014; Smith et al., 2014; ECDC, 2016). Due to the danger of STEC at the international level and to ensure food safety, it is officially recognized as a requirement to carry out research on the testing of these microorganisms in meat and meat products (Tafida et al., 2014; ECDC, 2016). It is possible to determine STEC by bacteriological methods that include the preenrichment of studied samples in broth with antibiotics, then isolation on agar typical colonies and then serotyping. To control STEC in each country, culture-dependent methods are different, but PCR – methods are the most rapid, sensitive and highly specific. Therefore, development and modifying a general technique of PCR – methods for detection of Shiga toxin-producing strain of E. coli are the focus of attention of scientists in different countries. Often, for rapid detection of the presence of several virulence factors of STEC in the samples, polymerase chain reaction (PCR) in multiplex version is used (Puttalingamma et al., 2012; Rantsiou et al., 2012; Haugum et al., 2014; Hara-Kudo et al., 2016).

It should be noted that at present in Ukraine rapid control techniques for STEC, including PCR, have not been developed yet, which is a deterrent to ensuring access of Ukrainian goods to foreign markets, especially the EU. In this regard, it is important to modify or adapt a general specific and rapid method of detection and identification Shiga toxin-producing strains of E. coli (STEC). Therefore, the aim of this study was to identify the presence of the Shiga toxin-producing strains of E. coli on beef and swine carcasses in Ukraine and investigate their genetic characteristics. The following tasks were pursued in this study: (i) isolation of STEC from beef and swine carcasses and (ii) modification of multiplex PCR method to detect the stx1, stx2, and eae genes (iii) to find out main the genetic characteristics of STEC from beef and pork carcasses.

Materials and methods

Samples collection. The work was performed in the Microbiology Laboratory of Center "Ecomedium" in Sumy State University (Sumy, Ukraine), in the State Research Institute of Laboratory Diagnostics and Veterinary Expertise (Kyiv, Ukraine) and in the State Scientific Control Institute of Biotechnology and Strains of Microorganisms, (Kyiv, Ukraine). A total of 230 samples of swab from beef (130) and swine (100) carcasses were obtained from 5 slaughterhouses in Ukraine (Kyiv (3) and Sumy (2) regions) between 2012 and 2015. Swabs were collected with sterile tampons in sterile saline solution. Area selection of swabs was 100 cm² from each carcass according to requirements ISO 17604. Samples of swabs from carcasses were selected randomly at the final point of the process after the final processing and washing of the carcass, from the following areas: distal hind limb, abdomen (lateral and medial) from swine carcasses, brisket, flank and flank groin areas from beef carcasses. After sampling, swabs were delivered to the laboratory at 4–5 °C in refrigeration bags.

Methods of isolation and identification of E. coli from samples. Samples were examined for 2–12 hours. Swab samples were examined by standard methods: serial dilutions of samples were plated on the surface of commercial medium "Compact Dry™ EC" for isolation of E. coli in Petri dishes (NISUI Pharma). It is a ready-to-use chromogenic medium for performing E. coli and coliform counts. Petri dishes with isolates were incubated for 24 h at 37 °C. Interpretation of the results was performed by the following indicators: blue colonies were considered as E. coli.

Oligonucleotide primers. Alignment of nucleotide sequences and their homology analysis was performed by the module Clustalw software "Vector NTI" v.10.0.1 (Invitrogen) and Blast-analysis resource www.ncbi.nlm.nih.gov (National Center for Biotechnology Information, USA). Primers for PCR multiplex variant were calculated by the software "Vector NTI" v.10.0.1 and synthesized in the "Lytech" (Russia). Lysophilized primers were diluted to a concentration of 100 pmol/μl "Ultra Pure Distilled Water" (Invitrogen, Cat. # 10977-023, USA) and stored at –20 °C until use. Specific oligonucleotide primers that were used for the detection of STEC are shown in Figure 1.

Fig. 1. Specific oligonucleotide primers specific to the toxin gene stx1 (a), stx2 (b) and intimin (eae) (c) that were used for the detection of Escherichia coli (STEC)

DNA isolation. DNA isolation was performed by three different methods: 1st – a colony of E. coli cultures was taken in a test tube with 0.5 microliters of sterile deionized water and was heated for 3 minutes at 100 °C, after that the tube was immediately transferred to ice; 2nd – selection using a commercial kit "DNA Sorb-B" (Amphyl-sens, Russia); 3rd – using silica-modified (~ 15 nm) magnetic particles (synthesized and provided by N. Volkova, Institute of Physics and Biophysics NAS) with a concentration of 10 mg ml⁻¹ and saturation magnetization of 37 emu (Am²/kg), purification of bacterial DNA for spectrophotometric analysis was performed using a set of "Ultra Clean DNA Purification Kit" (Cat. # 12500-100; Mobio, USA). The concentration and purity of drugs obtained DNA was measured on a spectrophotometer "NanoDrop 2000c" (USA).
Polymerase chain reaction (PCR). PCR was performed in termo-
cycles “Tertsyk” (DNA technology, Russia) and “T1” (Biometra,
Germany). The reaction was carried out by “hot” start in a volume of
0.025 cm³. In order to minimize the formation of nonspecific dimers
primer matrix and its amplification the method of preparation of the
reaction mixture with the physical separation of PCR components was used.

To prepare the “lower” reaction mixture, nucleoehydroxyphosphate
(2 mM) was mixed with appropriate primers in one tube at the rate of
0.025 cm³ each (final concentration from each primer 10–15 pmol/
sample). After mixing in a vortex, the mixture was dropped in
preparation for PCR microtubes in volume 0.005 cm³ in each and on the
top of it molten wax in volume 0.015 cm³ was added. After solidifica
ion of wax in the tube, the “upper” reaction mixture in volume 0.017
cm³ and 2 drops of mineral oil were added. The “upper” reaction
mixture (1 sample calculation) consisted of 0.005 cm³ (x5) PCR buffer;
0.0025 cm³ 50 mM MgSO4; 0.009 cm³ 1H2O MilliQ and 0.0005 cm³
Taq-polymerase (5 units/ml). Samples of bacterial DNA were placed
under oil in the volume 0.003 cm³.

Thermal cycling parameters were as follows: 95 °C for 3 min,
followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for
30 s; and a terminal extension step of 72 °C for 4 min. Negative
control was a non-pathogenic E. coli strain.

Electrophoretic analysis of PCR products. Analysis of amplifica
tion products was performed by separation of DNA fragments in a
1.5% (w/v) agarose gel with containing 0.5 μg/cm² ethidium bromi
des (Sigma, USA). Electrophoresis was performed at a constant
voltage of 10 V/cm to confirm the presence of the amplified DNA.
Location of obtained DNA stripes and their registration was perfor
med by using gel documentation system "Molecular Image Gel Doc XR+" (BioRad, USA).

Results

Modifying of PCR for identification of stx1, stx2 and intimin (eae)
genesis in isolated STEC. For the modifying of PCR for detection
and identification of Shiga toxin-producing E. coli in beef and swine
carcasses, first we performed research on the products received in
PCR with oligonucleotide primers specific to the toxin gene stx1, stx2
and intimin (eae), which are pathogenicity markers for STEC.
To design specific oligonucleotide primers, gene sequences in the
databases GenBank, EMBL (European Molecular Biology Library),
DDBJ (Japanese database nucleotide sequences) and PDB sequences
were analyzed. As a basis, the following sequence had been selected:
AB647443, AF022236, BA000007, ECOSLLII, AB647559,
AB647430, AB647374, AB334567, AJ308552.1, AB647553.1,
DQ523611.1, AB647374.1, H19BLS1A, KF771380.1, AB647493.1,
AB647432.1, AB647437.1, AB647449.1, DQ523603.1, AB647365.1,
EU700490.1, EF441598.1, EF441588.1.

As a basis, we have chosen a pair of oligonucleotide primers for
the detection of E. coli O157:H7, which had been developed by Putta
lingamma et al. (2012). We found that aforementioned primers
contain palindromes and loops, and they influence the formation of
numerous homo- and heterodimers that generally affect the specificity
of the reaction. Simultaneous use of these primers in PCR multiplex
option is not effective. The correct choice of oligonucleotide primers
for the detection of E. coli is very important because it determines the effectiveness and reproducibility of PCR.

The first task in the development of PCR was to improve the
current method of detecting DNA of Shiga toxin-producing E. coli
(STEC) by changing the composition of nucleotide sequences in
primers for amplification of specific nucleic acid fragments. The de
veloped primers could be used also in multiplex variant for detection
of specific DNA fragments (stx1, stx2 and eae) of Shiga toxin
producing E. coli (STEC) in the samples.

Primer specificity was confirmed in test strains of heterologous
microorganisms Salmonella enterica, Listeria monocytogenes, Bacil
lus anthracis, Campylobacter jejuni, Pasteurella multocida and Yersi
nia enterocolitica. To evaluate specificity and sensitivity of the reaction,
optimal annealing temperature of the primers was determined (Fig. 2).

To determine the sensitivity of the developed primers, 10-fold serial dilutions of purified bacterial DNA were prepared. Concentration of puri
fied DNA was determined with a spectrophotometer. Sensitivity for
eae gene of O145 strain was 0.220 and 0.021 ng for genes stx1 and
stx2 of O157 strain.

Results of isolation and identification of E. coli from samples.
Among the 230 swabs, a total of 97 E. coli isolates (42.2%) with
typical cultural properties (blue colonies) was isolated by using
commercial medium “Compact Dry” for isolation of E. coli in Petri
dishes. The study was conducted on 62 isolates from the surfaces of
beef and 35 isolates from swine carcasses (Table 1). More positive
samples were detected from surfaces of beef (47.7%) than from swine
carcasses (35.0%).

| Table 1 |
|-----------------------|
| Sample type | No. investigated samples | No. positive samples | Percentages, % |
|-----------------------|
| Swabs from beef | 130 | 62 | 47.7 |
| Swabs from swine | 100 | 35 | 35.0 |
| Total | 230 | 97 | 42.2 |

These 97 isolates were further subjected to polymerase chain
reaction (PCR). Multiplex PCR was used for detection of three target
genesis stx1, stx2 and eae in 97 E. coli isolates. Only 7 E. coli isolates
(7.2%) had at least one of 3 genes. Most of the isolates (6/85.7%,
5/71.4%) carried the stx1 or/and stx2 genes and only one isolate had
the eae gene. Fragments of positive result of detection of PCR products
genesis of stx2 and gene eae are shown on Figure 2.

![Fig. 2. Detection of PCR products of fragment gene of stx2 and gene eae after optimization of primer annealing temperature: M – marker “100 bp Plus DNA Ladder” (Thermo Scientific); gene stx2 – 1 – 63 °C, 2 – 64 °C, 3 – 65 °C; gene eae – 4 – 63 °C, 5 – 64 °C, 6 – 65 °C.](image-url)
All positive STEC isolates from swine carcasses were negative for virulence eae genes (Table 3).

**Table 2**

Results of incidence of virulence genes of STEC isolated from the examined samples (n = 97)

| Sample type               | No. investigated samples (%) | Virulence genes | stx1 | stx2 | eae |
|---------------------------|-----------------------------|----------------|------|------|-----|
| Swabs from beef carcasses | 5 (8.1%)                    |                | 4    | 3    | 1   |
| Swabs from swine carcasses| 2 (5.7%)                    |                | 2    | 2    | 0   |
| Total                     | 7 (7.2%)                    |                | 6    | 5    | 1   |

**Table 3**

The combinations of virulence genes of STEC (stx1, stx2, eae) in isolated strains (n = 7)

| Combination of virulence genes | No. investigated samples | No. positive samples |
|--------------------------------|--------------------------|----------------------|
| stx1+ stx2+ eae               | 0                        | 0                    |
| stx1+ stx2                  | 5                        | 3                    |
| stx1 + eae                   | 1                        | 1                    |
| stx2+ eae                    | 0                        | 0                    |
| stx1                         | 1                        | 1                    |
| stx2                         | 0                        | 0                    |
| eae                          | 0                        | 0                    |

Discussion

According to international epidemiological data, in all countries of the world, cases of foodborne illness among people are increasing. Therefore, within the framework of trade agreements between Ukraine and European countries, food and raw food of animal origin must be tested for safety before being shipped for export. One of the important criteria of current microbiological safety of raw meat and meat products (especially beef and pork) is the control of Shiga toxin-producing *E. coli* (STEC), because they are food-borne pathogens that are very serious threats to public health. STEC infection is associated with sporadic outbreaks of clinical diseases in humans, including severe hemorrhagic colitis, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, which can even cause death (Majowicz et al., 2014; Smith et al., 2014; Sadershan et al., 2014; ECDC, 2016).

STEC strains associated with human disease and they are used as a markers for STEC (Jeon et al., 2013; Bonardi et al., 2015; Chui et al., 2015). The higher prevalence of STEC in our study was found in swabs from beef (8.1%) in comparison with results from pork swabs (3.0%). Our result was higher than results reported by other researchers. In some reports STEC was detected from beef carcass at processing plants with a lower percentage: 0.5%, 3.3%, 4.5% (Abdisa et al., 2017; Beyi et al., 2017; Anu et al., 2018). But, the largest number of Shiga toxin producing strains of *E. coli* from samples of beef carcass was 30.0% (Omoruyi et al., 2018). As other researchers have highlighted, pork carcasses sampled were positive in 4.1% (Colello et al., 2016). Even though these results differ from each other, they are consistent with the assumption that beef and pork can be potential sources of STEC.

Often, for rapid detection of the presence one or more pathogenic factors of STEC in samples, polymerase chain reaction in multiplex version is used. Two types of Shiga toxin, stx1 and stx2 (encoded by stx1 and stx2 genes) are bacterial virulence STEC determinants that are associated with human disease and they are used as a markers for multiplex PCR (Puttalingamma et al., 2012; Rantsiou et al., 2012; Haugum et al., 2014; Hara-Kudo et al., 2016). STEC strains producing Stx2 are considered more virulent than Stx1 producers (Kavalaiuskiene et al., 2017; Stromberg et al., 2018). Besides Shiga toxin production, another virulence factor of STEC is known. It is a membrane protein intimin, which causes attaching of STEC to the intestinal epithelial cells. The eae gene codes produce this protein. But, many experts contend that the most virulent strains of STEC have all these genes (stx1, stx2 and eae) or other combinations (Croxon et al., 2013; Awadallah et al., 2016; Soledad-Cadona et al., 2018).

In the basis of our task, the multiplex PCR for the detection of stx1, stx2, eae virulence genes out of 97 isolates which were identified as *E. coli* by culture-dependent method (specific blue colonies on commercial medium Compact Dry) for isolation of *E. coli* was developed and used. We synthesized six pairs of original oligonucleotide primers for this method. Their homology and position on the target DNA was chosen. The corresponding genes are shown in Figure 1.

In the present work, no samples showed positive results for all 3 target genes (stx1, stx2, eae) in one sample. Most of the STEC isolates carried the stx1 gene (6 from 7 isolates, 85.8%) and stx1 gene (5 from 7 isolates, 71.4%). One isolate (14.3%) carrying the eae gene was found from one examined sample of beef carcass only. The stx1 gene was the predominant gene detected in all STEC positive samples from beef and swine carcasses. Some authors have reported similar
findings (Troz-Williams et al., 2012; Colell et al., 2016). But prevalence of STEC isolates carrying virulence genes stx1, stx2, intimin (eae) has been reported in lactating cows and in contact workers in dairy farms at Sharkia Province, Egypt (Merwad et al., 2014). Three isolates from swabs of beef carcass carried both stx1 and stx2 genes, one isolate showed association between stx1 and eae genes, one isolate had positive result for stx1 genes only (Table 3). In contrast to this study, some results have shown that E. coli O157:H7 was absent in raw minced beef samples in Tripoli, Lebanon by using real-time PCR-based method (Omari et al., 2018).

Although a small number of research articles which implicate pork as a source of human infection have been reported, the results of these studies indicate that meat other than beef also can be potential vehicles of STEC transmission (Troz-Williams et al., 2012). It is important to note that in swabs from swine carcasses (2 isolates) stx1 and stx2 genes were presented. So, the results of our study indicate that swine can be a potential reservoir of STEC strains. The same researchers isolated from 465 not ready to eat pork samples 65 (14.0%) stx-positive E. coli: the stx2 gene was detected more frequently (13.3%) than the stx1 gene (1.3%) and associations of genes in pork samples were next: stx1+eae (0.4%), stx2+eae (8.0%) and stx1+stx2+eae (0.7%) (Bardasi et al., 2017). At slaughter houses in Argentina 4.1% of carcasses of swine were stx positive: 50% of isolates positive for stx2 and 16.0% for stx1/stx2. (Colello et al., 2016).

**Conclusion**

This study is the first report on the presence of Shiga toxin-producing strains of E. coli (STEC) in beef and swine carcasses in Ukraine. The results indicate that fresh raw meat (beef and pork) could be potential vehicles for transmission of enterohaemorrhagic E. coli infections to humans since the highest prevalence these pathogenic microorganisms was found in swabs from beef carcasses and swine carcasses. These data will be valuable for microbiological risk assessment and help authorities to develop strategies to mitigate health risk.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**References**

Abdissa, R., Haile, W., Fite, A. T., Beyi, A. F., Agga, G. E., Eado, B. M., Tadesse, F., Korsa, M. G., Beyene, T., Beyene, T. J., De Zutter, L., Cox, E., & Goddeeris, B. M. (2017). Prevalence of E. coli O157:H7 in beef cattle at slaughter and beef carcasses at retail shops in Ethiopia. MIC Infectious Diseases, 17(1), 277–283.

Anu, P. J., Latha, C., Vinodkumar, K., Vinod, V. K., & Sathu, T. (2015). An interlaboratory study on efficient detection of Shiga toxin-producing E. coli O157:H7 in beef and swine carcasses in Ukraine. Regul. Mech. Biosyst., 9(2), 1–9.

Ju, W., Shen, J., Li, Y., Toro, M. A., Zhao, S., Ayers, S., Najjar, M. B., & Meng, J. (2012). Non-O157 Shiga toxin-producing Escherichia coli in retail ground beef and pork in the Washington D.C. area. Food Microbiology, 32, 371–377.

Kavalaukiené, S., Dyve Lingelem, A. B., Skotland, T., & Sandvig, K. (2017). Protection against Shiga toxins. Toxins (Basel), 9(2), e44.

Loinazk, K. A., Jari, N., Gargiulina, J., Patel, I., Ekhin, C. A., Hu, Z., Kassim, P. A., Myers, R. A., & Laksanalarai, P. (2016). Investigation of potential shiga toxin producing Escherichia coli (STEC) associated with a local foodborne outbreak using multidisciplinary approaches. Food Science, 5(3), 163–166.

Majowiec, S. E., Scallan, F., Jones-Bitton, A., Sargeant, J. M., Stapleton J., Angulo, F. J., Yeung, D. H., & Kirk, M. D. (2014). Global incidence of human Shiga toxin-producing Escherichia coli infections and deaths: A systematic review and knowledge synthesis. Foodborne Pathogens and Disease, 11, 447–455.

Merwad, A., Glariere, R., & Saher, T. (2014). Occurrence of Shiga toxin-producing Escherichia coli in lactating cows and in contact workers in Egypt: Serotypes, virulence genes and zoonotic significance. Life Science Journal, 11(5), 563–571.

Microbiology of the food chain. Carcass sampling for microbiological analysis (2018). European Centre for Disease Prevention and Control. Escherichia coli factsheet Stockholm: ECDC: ISO 17604:2015.

Montaz, H., & Jannushi, A. (2013). Shiga toxin-producing Escherichia coli isolated from chicken meat in Iran: Serogroups, virulence factors, and antimicrobial resistance properties. Poultry Science, 92, 1305–1313.

Omari, K. E., Kassa, I. A., Kana-Ali, H., Dabbousi, F., & Harrae, M. (2018). Prevalence of E. coli O157:H7 in raw minced beef at slaughterhouses in Tripoli, Lebanon. Virology and Immunology Journal, 2(4), 2–4.

Omoriyui, I. M., Uwadiye, E., Mulade, G., & Omoruku, E. (2018). Shiga toxin producing strains of Escherichia coli (STEC) associated with beef products and its potential pathogenic effect. Microbiology Research Journal International, 23(1), 1–7.

Permananthi, J. M. K. J. K., New, C. Y., Ubong, A., Nakaguchi, Y., Nishibauchi, M., & Son, R. (2017). Risk of Escherichia coli O157:H7 infection linked to the consumption of beef. Food Research, 1(3), 67–76.

Puttalgamunu, V., Shylaja, R., Bhat, H. V., & Bavta, A. S. (2012). A novel multiplex PCR system for the detection of virulence associated genes of E. coli O157:H7 from food system. Recent Research in Science and Technology, 4(5), 36–40.

Rani, S., Singh, Y., Gulati, B. R., & Khurana, S. K. (2017). Occurrence of enterohaemorrhagic Escherichia coli in buffalo meat. Journal of Experimental Biology and Agricultural Sciences, 3(5), 208–214.

Rantou, K., Alessandra, V., & Cossil, I. (2012). Prevalence of Shiga toxin-producing Escherichia coli in food products of animal origin as determined by molecular methods. International Journal of Food Microbiology, 154, 37–43.

Rashid, M., Kotwal, S. K., Malik, M. A., & Singh, M. (2013). Genetic profile of virulence determinants and multidrug resistance of Shigella isolates from food of animal origin. Veterinary World, 6, 139–142.

Ray, B., & Bhunia, A. (2014). Fundamental food microbiology. CRC Press, Taylor & Francis Group, New York.

Shahorg, Z., Xuemei, Z., Qiongqin, W., Jiumei, Z., Xiaoke, X., & Haigang, L. (2015). Prevalence and characterization of Escherichia coli O157 and O157:H7 in retail fresh raw meat in South China. Annals of Microbiology, 65, 1993–1999.

Smith, J. L., Fratamico, P. M., & Gunther, N. W. T. (2014). Shiga toxin-producing Escherichia coli. Advances in Applied Microbiology, 86, 145–197.

Soledad-Cadona, J., Bustamante, A. V., Gonzalez, J., & Mariel-Sanso, A. (2018). Pathogenicity islands distribution in non-O157 Shiga toxin-producing Escherichia coli (STEC) Genes, 9, 81–99.

Stromberg, Z. R., Redweik, G. A. J., & Mellata, M. (2018). Detection, prevalence, and pathogenicity of Non-O157 shiga toxin-producing Escherichia coli from cattle hides and carcasses. Foodborne Pathogens and Disease, 15(3), 119–131.

Sadeshan, R. V., Naveenkumar, R., Kishanlath, L., Bluskar, V., Polasa, K. (2014). Foodborne infections and intoxications in Hyderabad India. Epidemiology Research International, 10, 1–5.

Tafida, S. Y., Kwaga, J. K. P., Bello, M., Kabir, J., Umoh, V. J., Yakubu, S. E., & Nok, A. J. (2014). Occurrence of Escherichia coli O157 in refined-beef and related meat products in Zaria, Nigeria. Food and Nutrition Sciences, 5, 481–487.
Taye, M., Berhanu, T., Berhanu, Y., Tamiru, F., & Terefe, D. (2013). Study on carcass contaminating *Escherichia coli* in apparently healthy slaughtered cattle in Haramaya University Slaughter House with special emphasis on *Escherichia coli* O157:H7. *Journal of Veterinary Science and Technology*, 4, 132.

Troz-Williams, L. A., Mercer, N. J., Walters, L. M., Maki, A. M., & Johnson, R. P. (2012). Pork implicated in a Shiga toxin-producing *Escherichia coli* O157:H7 outbreak in Ontario, Canada. *Canadian Journal of Public Health*, 103, 322–326.

Tseng, M., Fratamico, P. M., Bagi, L., Manzinger, D., & Funk, J. A. (2014). Shiga toxin-producing *E. coli* (STEC) in swine: Prevalence over the finishing period and characteristics of the STEC isolates. *Epidemiology and Infection*, 8, 1–10.

Vijayan, C., Ajaykumar, V. J., Bhattacharya, A., & Bhanurekka, V. (2017). Detection of enterohaemorrhagic *E. coli* O157: H7 from beef and chevon sold in and around Puducherry. *Journal of Entomology and Zoology Studies*, 5(6), 1395–1403.