

Simultaneous occurrence of selected food-borne bacterial pathogens on bovine hides, carcasses and beef meat

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Abstract

The aim of this study was to determine the simultaneous occurrence of *Salmonella* spp., *L. monocytogenes*, verotoxigenic *E. coli* (VTEC), and *Campylobacter* spp. in slaughtered cattle and in beef meat subjected for human consumption. A total of 406 bovine hides and 406 corresponding carcasses were used to collect the samples with a swab method after exsanguination and evisceration of animals, respectively. Furthermore, 362 beef meat samples were purchased in local retail shops over the same period of time as for the bovine samples. Food-borne bacterial pathogens were identified with standard ISO methods with some modification by the use of PCR for VTEC. The isolated bacteria were then molecularly speciated (*Campylobacter*), serotyped (*L. monocytogenes*) and characterized for the presence of several virulence marker genes (VTEC and *Campylobacter*). It was found that 49 hide (12.1%) and 3 (0.7%) carcass samples were contaminated with more than one bacterial pathogen tested. Most of the hides were positive for *Campylobacter* spp. and VTEC (27 samples) and *Campylobacter* spp. together with *L. monocytogenes* (12 samples). Eight bovine hides contained *L. monocytogenes* and VTEC while *L. monocytogenes* and *Salmonella* spp. were detected in one sample. Furthermore, 3 pathogens (*Campylobacter* spp., *L. monocytogenes* and VTEC) were simultaneously identified in one bovine hide tested. In case of bovine carcasses 2 samples contained *Campylobacter* spp. and VTEC whereas one carcass was positive for *L. monocytogenes* and VTEC. On the other hand, 10 out of 362 (2.8%) minced beef samples were contaminated with at least two pathogens tested. The majority of these samples were contaminated with *L. monocytogenes* and *Salmonella* spp. (6 samples). It was noticed that equal number of *C. jejuni* and *C. coli* were found, irrespective of the origin of the samples. Most of the strains possessed more than one pathogenic factor as identified by PCR. Molecular serotyping of *L. monocytogenes* revealed that the majority of the isolates (27 out of 31; 87.1%) belonged to 1/2a serogroup. It was found that most of the VTEC isolates possessed the Shiga toxin *stx2* gene (12 strains) whereas only 2 strains were *stx1*-positive. The enterohemolysin and intimin markers were identified only in 7 and 2 isolates, respectively. PCR analysis revealed that 4 VTEC belonged to O91 serogroup, 2 strains were O145 and 1 isolate was identified as O113. None of the VTEC detected in the study was O157 serogroup.

Key words: bovine hides and carcasses, beef meat, food-borne pathogens, molecular characterization, food safety

Introduction

Contamination of beef during slaughter and processing is a major risk of subsequent food-borne infection of the consumers (Norung and Buncic 2008). Cattle may be reservoir of several bacterial pathogens that are present in their gastrointestinal tract without any clinical signs in animals (Bell 1997). Shedded microorganisms in the feces may infect other animals as well as contaminate hides in abattoirs. Furthermore, the bacteria can be also transferred to the carcasses during the slaughter and dressing processes (Bell 1997). The most zoonotic pathogenic bacteria found in cattle, that may be potentially pathogenic for humans, are *Salmonella* spp., *Listeria monocytogenes*, verotoxigenic *Escherichia coli* (VTEC), and *Campylobacter* spp. According to the European Food Safety Authority (EFSA) report, the two most frequently reported food-borne diseases in humans in the European Union over last years were *Campylobacter* and *Salmonella* infections, with incidences of 40.7 and 26.4 per 100,000 people in 2008, respectively (www.efsa.europa.eu). The infection with *Campylobacter* spp., especially with *C. jejuni*, is one of the leading causes of bacterial diarrhea worldwide. The disease is usually self-limiting and lasts a few days. Infrequently, extra-intestinal infections or post-infection complications such as reactive arthritis and neurological disorders occur (Wassenaar and Blaser 1999).

Human salmonellosis is usually characterised by the acute onset of fever, abdominal pain, nausea, and sometimes vomiting. Symptoms are often mild and most infections are self-limiting, lasting a few days. However, in some patients, the infection may be more serious and the associated dehydration can be life-threatening. Salmonellosis has also been associated with long-term and sometimes chronic sequelae, e.g. reactive arthritis (Hohmann 2001).

Verotoxigenic *Escherichia coli* (VTEC) are a group of *E. coli* that are characterised by the ability to produce toxins that are designated verocytotoxins (VTs). Human pathogenic VTEC usually harbour additional virulence factors that are important for the development of the disease in man (Duffy et al. 2006). A large number of serogroups of *E. coli* have been recognised as verocytotoxin producers. Of these, the O157:H7 and the O157:H- serogroups (VTEC O157) are the ones most frequently reported to be associated with the human disease (www.efsa.europa.eu). Animals are the reservoir for VTEC, and VTEC (including VTEC O157) have been isolated from many different animal species, especially from cattle (Elder et al. 2000). The gastrointestinal tract of healthy ruminants seems to be the foremost important reservoir for VTEC and foods of bovine and ovine origin are frequently reported as a source for human VTEC infections (www.efsa.europa.eu).

L. monocytogenes is an ubiquitous organism that is widely distributed in the environment, especially in plant matter and soil. The principal reservoirs of *Listeria* are soil, forage and water. Other reservoirs include infected domestic and wild animals. The main route of transmission to both humans and animals is believed to be through consumption of contaminated food or feed, respectively. In humans severe illness mainly occurs in the unborn child, infants, the elderly and those with compromised immune systems. Symptoms vary, ranging from mild flu-like symptoms and diarrhea to life threatening infections characterised by septicaemia and meningoenephalitis (Osek 2005, Swaminathan and Gerner-Smidt 2007).

Several studies have been conducted to determine the prevalence of the above mentioned pathogens along the food chain (Madden et al. 2001, Zhao et al. 2001, Beach et al. 2002, Reid et al. 2002, Duffy et al. 2006, Rhoades et al. 2009). However, most of them usually focused on the identification of one of the zoonotic agent, either in live animals or on carcasses in slaughterhouses during processing. Little research has been done on the simultaneous prevalence of more than one pathogenic bacteria in beef, both at the slaughterhouse and retail levels.

The aim of this study was to determine the simultaneous incidence of *Salmonella* spp., *L. monocytogenes*, VTEC, and *Campylobacter* spp. in slaughtered cattle and in beef meat destined for human consumption available in shops. Furthermore, the isolated pathogenic bacteria have been characterized by identification of several virulence marker genes that are potentially involved in the pathogenesis of food-borne diseases.

Materials and Methods

Sample collection

A total of 406 cattle slaughtered during March 2007 – September 2009 in 3 slaughterhouses in the eastern part of Poland were used in the study. The samples from bovine hides were collected using a swab method and sponges from the brisket area after exsanguination of animals. The sponges were placed in autoclavable stomacher bags, pre-moistened with 10 ml of Maximum Recovery Diluent (MRD, Oxoid, UK; 0.1% peptone, 0.85% NaCl) and autoclaved at 121°C for 15 min. Four sites (100 cm² each) were rubbed with 4 sterile sponges, 10 times vertically and 10 times in a horizontal direction. Samples from the corresponding bovine carcasses were collected with the same method. All swabs were then placed in a cooler box and immediately transported to the laboratory. In the laboratory, all four sponges used for swabbing either hide or carcass were placed together

into 200 ml of MRD and then stomached for 3 min. Each sample was then divided into 4 aliquots of 40 ml each and centrifuged at 1000 g for 15 min. at 5°C. Finally, each pellets were resuspended in 100 ml of appropriate enrichment broth for *Salmonella* spp., *L. monocytogenes*, VTEC, or *Campylobacter* spp., respectively.

A total of 362 bovine meat samples were purchased in local retail shops over the same period of time as for the bovine samples. The meat (25 g) was then used for identification of the above bacteria with the same methods as for the swabs.

Detection of pathogenic bacteria

Salmonella spp., *L. monocytogenes*, VTEC, and *Campylobacter* spp. were identified with the standard ISO 6579, 11290-1, 16654, and 10272-1 methods, respectively, except VTEC identification when the ISO 16654 standard was modified by the use of PCR for the identification of O157 and other serogroups as described earlier (Osek 2008). The suspected *L. monocytogenes* and *Salmonella* spp. colonies were subjected to biochemical analysis using API® Listeria and ID 32E tests (bioMérieux, France), respectively. The identified and confirmed bacteria isolates were stored at -80°C for further analyses.

Characterization of the isolates

DNA from *L. monocytogenes*, VTEC, and *Campylobacter* spp. was extracted using the Genomic-Mini kit (A&A Biotechnology, Poland) according to the manufacturer's instruction. *Campylobacter* species determination was done using multiplex PCR for the simultaneous detection of *C. jejuni* and *C. coli* in a single reaction tube as described by Wiczorek and Osek (2005). *L. monocytogenes* serotyping was performed using the PCR method as described (Doumith et al. 2004). *Campylobacter* and VTEC virulence genes were also examined with PCR (Tatarczak et al. 2005, Wiczorek and Osek 2008, Wiczorek et al. 2009). The sequences of all primers (synthesized by Symbios, Poland) and PCR conditions used for the amplification of virulence genes were described previously (Tatarczak et al. 2005, Wiczorek and Osek 2008). The following *Campylobacter* spp. genes were identified: *cadF*, *flaA*, *cdtA*, *cdtB*, *cdtC*, *virB11*, *iam*, *flhA*, *ciaB*, *docA*, and *wlaN*. In case of VTEC *eaeA*, *stx1*, *stx2*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, *lpfA-141* and *lpfA-154* were detected.

Results

Prevalence of bacterial pathogens in the sample tested

During the study period it was found that 49 out of 406 hide samples analyzed (12.1%) were contaminated

with more than one bacterial pathogen tested. Most of the hides (Fig. 1A) were positive for *Campylobacter* spp. and VTEC (27 samples) and *Campylobacter* spp. together with *L. monocytogenes* (12 samples). Eight bovine hides contained *L. monocytogenes* and VTEC while *L. monocytogenes* and *Salmonella* spp. were detected in one sample. Furthermore, 3 pathogens (*Campylobacter* spp., *L. monocytogenes* and VTEC) were simultaneously identified in one bovine hide tested during the study.

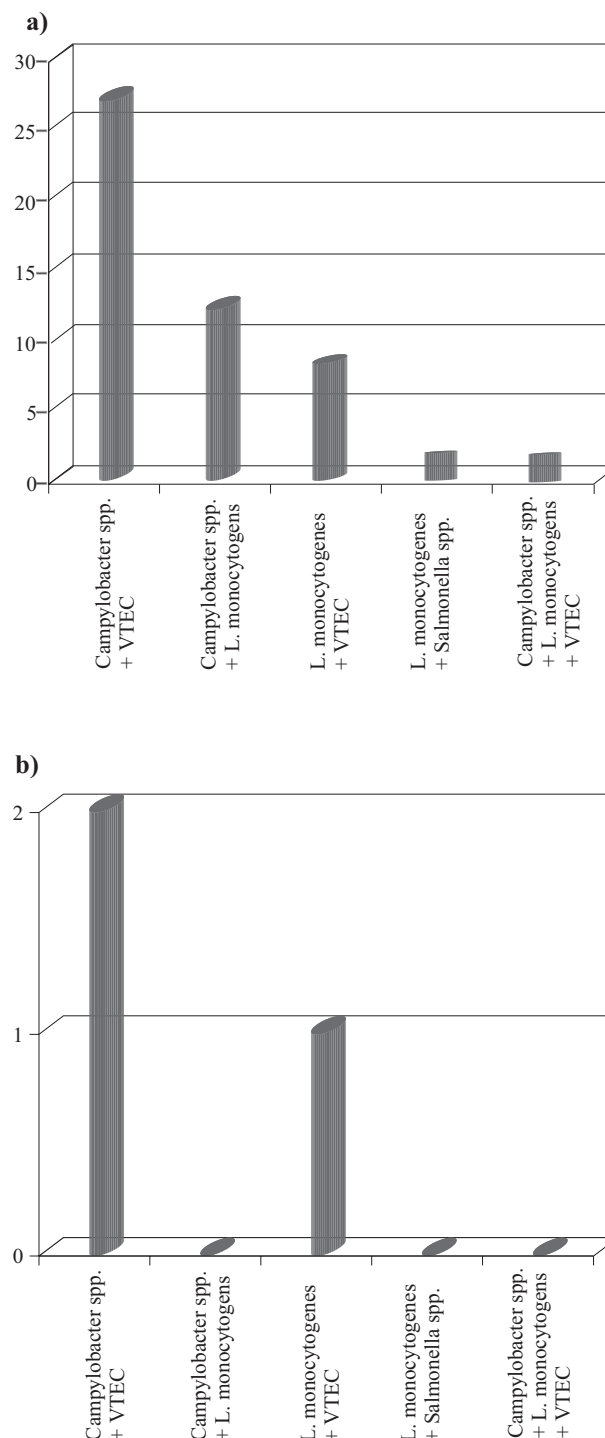


Fig. 1 a, b

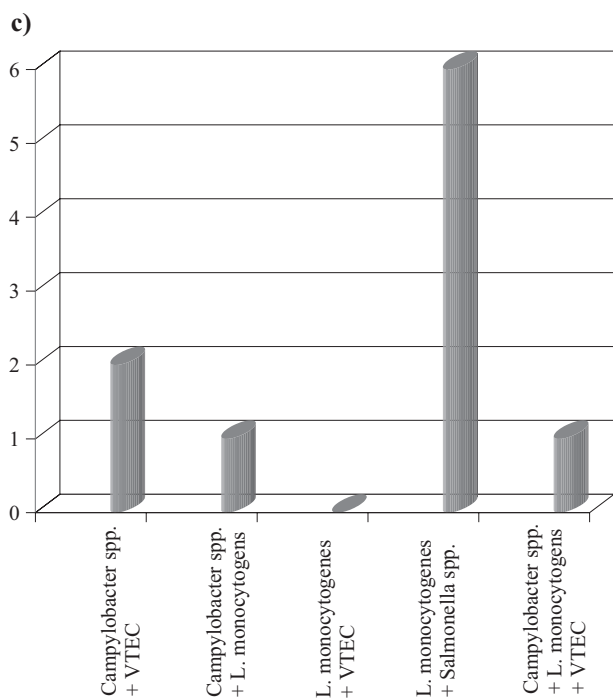


Fig. 1. Number of samples positive for multiple pathogens on hide (a), carcasse (b) and in minced beef (c).

Taking together, 63 samples analyzed during this study were simultaneously contaminated with more than one bacterial pathogen. Most of the positive samples contained *Campylobacter* and VTEC (31; 49.2%), *Campylobacter* and *L. monocytogenes* (13; 20.6%) or *L. monocytogenes* and VTEC (9; 14.3%). However, it must be pointed out that the number of samples contaminated with VTEC ($n = 42$) were counted on the basis of the PCR test which allows identification of the *stx* Shiga toxin gene. Only in case of 13 samples (9 of hide origin, 3 from carcass and 1 from meat) VTEC isolates were recovered and detailly characterized.

Molecular characterization of the isolates

Two main *Campylobacter* species were identified among all isolates ($n = 46$) detected in the study. It was found that equal number of *C. jejuni* and *C. coli* were detected, irrespective of the origin of the samples (Table 1).

The *Campylobacter* isolates were then screened for the presence of 11 virulence marker genes. The results

Table 1. Prevalence of virulence genes in *Campylobacter* strains.

Source of isolates	<i>Campylobacter</i> species	Number of strains	Virulence marker gene – number of positive strains										
			<i>cadF</i>	<i>flaA</i>	<i>cdtA</i>	<i>cdtB</i>	<i>cdtC</i>	<i>virB11</i>	<i>iam</i>	<i>flhA</i>	<i>ciaB</i>	<i>docA</i>	<i>wlaN</i>
Hides	<i>C. jejuni</i>	20	20	20	20	20	20	0	7	20	20	20	5
	<i>C. coli</i>	20	20	17	8	8	8	0	20	20	4	4	0
Carcasses	<i>C. jejuni</i>	1	1	1	1	1	1	0	0	0	1	1	1
	<i>C. coli</i>	1	1	1	0	0	0	0	1	1	0	0	0
Beef meat	<i>C. jejuni</i>	2	2	2	2	2	2	0	0	0	2	2	0
	<i>C. coli</i>	2	2	2	1	1	1	0	2	2	0	0	0
Total		46	46	43	32	32	32	0	30	43	27	27	6

In case of bovine carcasses (Fig. 1B), only 3 samples (0.7%) were found to be positive for more than one pathogenic bacteria: two contained *Campylobacter* spp. and VTEC whereas one carcass was contaminated with *L. monocytogenes* and VTEC (Fig. 1).

On the other hand, 10 out of 362 (2.8%) minced beef were contaminated with at least two pathogens tested (Fig. 1C). The majority of these positive samples were contaminated with *L. monocytogenes* and *Salmonella* spp. (6 samples). The remaining beef meats were positive for *Campylobacter* spp. and VTEC (2 samples), *Campylobacter* spp. and *L. monocytogenes* (1 sample) as well as for 3 pathogens – *Campylobacter* spp., *L. monocytogenes*, and VTEC (1 sample).

are shown in Table 1. It was found that most of the strains possessed more than one pathogenic factor as identified by PCR. It was found that all samples, irrespective of the origin, possessed the *cadF* gene. The vast majority of the isolates were also positive for the *flhA* and *flaA* markers (43 samples of each gene). Furthermore, many *Campylobacter* strains had the cytolethal descending toxin and *iam* genes (*cdtA*, *cdtB*, and *cdtC*; 32 isolates) as well as the *ciaB* i *docA* markers (27 strains). On the other hand, none of the *Campylobacter* spp. was positive for the *virB11* gene and only 6 isolates were found to possess the *wlaN* putative virulence marker. It was noted that *cdt* toxin genes were predominant in *C. jejuni* strains (100% positive strains) whereas

Table 2. Serotypes of *L. monocytogenes* identified in the study.

Source of isolates	Number of strains	Serotype of <i>L. monocytogenes</i> – number of positive strains			
		1/2a	1/2b	1/2c	4b
Hides	22	19	0	1	2
Carcasses	1	1	0	0	0
Beef meat	8	7	0	1	0
Total	31	27	0	2	2

Table 3. Prevalence of virulence marker genes among isolated VTEC.

Source of isolates	Number of strains	Virulence marker gene – number of positive strains									
		<i>eaeA</i>	<i>stx1</i>	<i>stx2</i>	<i>stx2c</i>	<i>stx2d</i>	<i>stx2e</i>	<i>stx2f</i>	<i>ehlyA</i>	<i>lpfA-141</i>	<i>lpfA-154</i>
Hides	9	2	0	9	3	2	0	0	6	0	0
Carcasses	3	0	1	3	1	0	1	0	1	1	0
Beef meat	1	0	1	0	0	0	0	0	0	0	0
Total	13	2	2	12	4	2	1	0	7	1	0

this marker was seldom identified in *C. coli* isolates (9 out of 23 strains identified). The same observation was made for the *ciaB* and *docA* genes where *C. jejuni* isolates were much often positive than *C. coli* (23 versus 4 strains, respectively).

Molecular serotyping of *L. monocytogenes* performed by the PCR method revealed that the majority of the isolates belonged to 1/2a serogroup (27 out of 31; 87.1%). Only few strains were of 1/2c or 4b serogroups (2 isolates of each) (Table 2).

VTEC isolates recovered from hides (n = 9), carcasses (n = 3) as well as from minced beef (n=1) were characterized using the PCR method. Several virulence marker genes were identified (Table 3). It was found that most of the isolates possessed the Shiga toxin *stx2* gene (12 strains) whereas only 2 strains were *stx1*-positive. Some VTEC had also other toxin gene variants – *stx2c*, *stx2d* or *stx2e*. The enterohemolysin and intimin markers were identified only in 7 and 2 isolates, respectively. VTEC strains were also serotyped using the classical agglutination (O157 serogroup) and molecular-based PCR methods. It was found that none of the strains tested belonged to O157 serogroup. PCR analysis revealed that 4 VTEC belonged to O91 serogroup, 2 strains were O145 and 1 isolate was identified as O113. The remaining 8 VTEC could not be typed with the PCR test used in the study.

Discussion

In this study simultaneous prevalence of four bacterial pathogens were identified on bovine hides and carcasses as well as in beef meat available in retail. The

survey focused on *Salmonella* spp., *L. monocytogenes*, VTEC, and *Campylobacter* spp. because these pathogens are responsible for most of the human food-borne infections in Poland and worldwide (www.efsa.europa.eu). A significant number of samples was used to assess the prevalence of these bacteria both at the process and retail levels. Furthermore, the bacterial isolates recovered in the study were characterized by the determination of several known or putative virulence marker genes using the PCR approach. It was found that 12.1% of bovine hides were contaminated with multiple pathogenic bacteria species; most of the samples were positive for *Campylobacter* spp. and VTEC. On the other hand, only 0.7% of the corresponding carcasses were contaminated with more than one pathogen tested, again mainly with *Campylobacter* spp. and VTEC.

Cattle hides have been identified as a source of general microbial contamination on carcasses. It has also been shown that some pathogenic bacteria, including VTEC and *Salmonella*, can be transferred from hides to carcasses during processing (Elder et al. 2000, Reid et al. 2003, Hussein and Bollinger 2005). The prevalence of pathogens in cattle identified during the slaughter process has varied considerably in the previously described surveys (Bell 1997, Scanga et al. 2000, Madden et al. 2001, Barkocy-Gallagher et al. 2003, Rivera-Betancourt et al. 2004, Brichta-Harhay et al. 2008, Bosilevac et al. 2009, Wiczorek et al. 2009, 2010). The number of positive samples strongly depends on sampling strategies, time of the year, and the laboratory methods used during the studies. Furthermore, most of the studies have focused on the identification of separate bacterial pathogens, therefore it is difficult to compare those data with the present results when only samples with multiple

bacteria were taken into account. Reid et al. (2002) found that the most prevalent pathogen on the cattle hides was VTEC O157 (28.8% of the animals tested) followed by *Salmonella* spp. (17.7%). Although it is known that healthy cattle are both reservoirs and excretors of *Campylobacter* spp. (Bell 1997), this pathogen was not detected on any animal hide examined by Reid et al. (2002). However, in that survey the simultaneous occurrence of the bacterial pathogens was not the main subject of the analysis. In another study of Barkocy-Gallagher et al. (2003) the seasonal prevalence of VTEC and *Salmonella* spp. was monitored at 3 beef-processing plants in the USA. It was found that these bacteria were recovered from as many as 60.6% and 92.0% hide samples, respectively. On the other hand, the corresponding bovine carcasses were contaminated at the level of 96.0% and 12.7% for VTEC and *Salmonella* spp., respectively.

Similar study was recently performed by Brichta-Harhay et al. (2008) who investigated the prevalence of VTEC O157:H7 and *Salmonella* spp. on hides and carcasses of cattle slaughtered in different time of the year. No significant seasonal effect was detected for contamination of hides and carcasses with *Salmonella*, which ranged from 86.1% to 93.8% and 44.2% to 55.3% of the animals tested, respectively. The prevalence of *E. coli* O157:H7 investigated at the same sampling points was lower, ranging from 38.7% to 55.7% and from 14.2% to 19.5% for VTEC and *Salmonella*, respectively. Unfortunately, simultaneous identification of both bacterial pathogens has not been tested.

A broad survey on the prevalence of VTEC O157, *L. monocytogenes*, and *Salmonella* spp. in beef processing plants have been performed by Rivera-Betancourt et al. (2004). A total of 1,032 bovine hides and the same number of corresponding carcasses were tested. The prevalence of bacterial pathogens on the hides was very high and ranged, depending on the plant, from 68.1% to 55.9% for VTEC, 91.8 – 50.3% for *Salmonella*, and 0.8 – 18.7% for *L. monocytogenes*, respectively. The corresponding carcasses were contaminated at a lower level: 3.1 – 10.9% for VTEC, 0 – 1.1% for *Listeria*, and 0 – 0.8% for *Salmonella*. Furthermore, the samples were also analyzed for the multiple pathogen prevalence and it was found that in case of hides two microorganisms had been detected in 46.4% and 3 bacterial species in 23.4% of the samples tested. For the carcasses the corresponding values were 4.5% and 0.5%, respectively. However, the authors did not describe which bacterial species had been simultaneously identified in both kind of samples, therefore, it is difficult to compare their data with the results obtained in the present study.

There is very few information concerning the simultaneous contamination of beef meat at the retail level with the four bacteria tested in the present study.

Instead, several authors have described the prevalence of one bacterial pathogen or one group of bacteria (Zhao et al. 2001, Crowley et al. 2005, Samadpour et al. 2006, Rhoades et al. 2009). Crowley et al. (2005) identified the presence of *Enterobacteriaceae* microorganisms and VTEC O157:H7 in retail beef in Ireland. Among 1,303 beef meat samples purchased in local shops 43 (3.3%) were contaminated with verotoxigenic *E. coli* O157. Zhao et al. (2001) analyzed 210 beef meat samples for the presence of *Salmonella* spp., VTEC, and *Campylobacter* spp. and identified 4 (1.9%), 40 (19.0%), and 1 (0.5%) samples contaminated with the respective bacterial species. Some retail meats were contaminated with more than one food-borne pathogen. In another study of Scanga et al. (2000) beef meat samples were tested for the presence of *Salmonella* spp. and *L. monocytogenes* and 5.4% and 5.3% were positive, respectively. However, simultaneous occurrence of these two bacterial species was not analyzed.

In conclusion, the results of the present study indicate that beef meat, both at the slaughterhouse and retail levels, may be contaminated with more than one food-borne bacterial species that are potentially pathogenic for the consumers. It was noted that identified VTEC had several virulence marker genes responsible for production of Shiga toxin, enterohemolysin, or/and intimin, the factors that play a crucial role in the pathogenesis of human infection. Furthermore, other bacteria found in beef meat, especially *Campylobacter* spp. also harboured several virulence markers that make them potentially pathogenic for humans. Since more than one bacterial pathogen was identified in the samples analyzed in the present survey, it may suggest that beef may be potentially a significant public health concern. To diminish *Campylobacter*, VTEC, *L. monocytogenes*, and *Salmonella* contamination rates in retail meats, it is critical that risk reduction strategies are used throughout the food chain. These strategies include on-farm practices that reduce pathogen carriage, increased hygiene at both slaughter and meat processing, and increased consumer education efforts. Additionally, consumption of undercooked beef meat or meat products and cross-contamination during food handling and preparation must be avoided. Further research focusing on effective prevention of food-borne illness is essential for developing intervention strategies to reduce the presence of food-borne bacterial pathogens at the retail level.

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