Original Study • DOI: 10.15212/ijafr-2020-0122 IJAFR • 60(1) • 2022 • 12-18

Irish Journal of Agricultural and Food Research

A preliminary study of *Salmonella* spp., *Listeria monocytogenes, Escherichia coli* O157, *Enterococcus faecalis* and *Clostridium* spp. in Irish cattle

L. Russell[1,2], C.P. Galindo[1,2], P. Whyte[2], D. Bolton[1†]

[1]Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland
[2]School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

Abstract

Although Salmonella spp., Escherichia coli O157, Listeria monocytogenes, Enterococcus faecalis and Clostridium spp. present a significant food safety and/or spoilage issue for the beef sector, there are limited data on their prevalence in Irish cattle. The objectives of this preliminary study were to investigate the distribution (percentage of farms positive) of Salmonella spp., E. coli O157, L. monocytogenes, E. faecalis and Clostridium spp. and the overall prevalence (%) of these bacteria in cattle on a small cohort of Irish beef farms. A total of 121 fresh bovine faecal samples were obtained on 10 randomly selected beef farms in the Northeast of Ireland and tested for the target pathogens using standard culture-based methods. Presumptive positives were confirmed using previously published polymerase chain reaction (PCR) methods. Salmonella were not detected in any of the samples. E. coli O157, L. monocytogenes, E. faecalis and Clostridium spp. were present on 50%, 40%, 100% and 100% of farms, respectively, with overall (all farms) prevalence rates in cattle of 9%, 8.2%, 61.9% and 87.6%, respectively. This study suggests that E. coli O157 may be more prevalent than previously thought and L. monocytogenes, E. faecalis and Clostridium spp. are widespread in Irish beef animals.

Keywords

Bovine • Escherichia coli O157 • faeces • Listeria monocytogenes • Salmonella

Introduction

Salmonella spp. are rod-shaped Gram-negative bacteria and the second most common cause of foodborne illness in the European Union (EU), with over 91,000 cases of salmonellosis reported in 2018 (EFSA and ECDC, 2019). According to Irish public health surveillance data for the same year, there were 363 confirmed cases in Ireland, 135 of which were hospitalised (HPSC, 2019). *Salmonella* spp. naturally colonise the intestinal tract of humans and animals, and human infection can occur when contaminated food or water is ingested, causing diarrhoea, cramps, vomiting and/or fever. *Salmonella* contamination of beef presents a significant risk in terms of food safety, with 3.2% of strong-evidence (there is strong evidence implicating a particular food vehicle) food outbreaks attributed to meat and meat products and 2.6% of these linked to bovine meat (EFSA and ECDC, 2019).

Escherichia coli O157 is also a Gram-negative rod-shaped bacteria and a Shiga toxin-producing *E. coli* (STEC), which can cause foodborne illness characterised by diarrhoea, haemorrhagic colitis (bloody diarrhoea) and in more severe cases, haemolytic uraemic syndrome. This pathogen has a low infectious dose, with 966 confirmed STEC cases reported in Ireland in 2018 (EFSA and ECDC, 2019), an increase from the previous year's figure of 923 confirmed cases (205 of which were attributed to O157), 295 hospitalisations and 4 deaths (HPSC, 2018). Contaminated, undercooked ground beef is commonly associated with *E. coli* O157-related foodborne illness, while water and ready-to-eat products contaminated with bovine faeces are also important sources (EFSA and ECDC, 2019).

Listeria monocytogenes is a Gram-positive, facultative anaerobe. Although *L. monocytogenes* is not a common foodborne pathogen, almost half of listeriosis cases result in hospitalisation, and approximately 15% end in fatality (EFSA and ECDC, 2019). There were 22 confirmed cases in 2018 in Ireland (HPSC, 2019). Listeriosis causes fever and diarrhoea similar to other foodborne pathogens, but in immunocompromised individuals, elderly people and pregnant women, more severe issues including preterm labour, miscarriage and/or death may occur (CDC, 2019).

†Corresponding author: D.J. Bolton
E-mail: declan.bolton@teagasc.ie

Enterococci, and more specifically *Enterococcus faecalis,* are Gram-positive cocci and opportunistic pathogens that are naturally present in the intestinal tract of humans and animals. While enterococci have been linked to waterborne outbreaks, the incidence is significantly lower than most bacterial pathogens under surveillance in the EU, with just one incidence reported in 2018 (EFSA and ECDC, 2019).

Clostridium spp. are a genus of spore-forming, obligate anaerobic Gram-positive bacteria that are naturally part of the gut microbiota of animals and include species that are of pathogenic and food spoilage concern. *Clostridioides difficile*, *Clostridium perfringens* and *Clostridium botulinum* are important pathogens, while species such as *Clostridium estertheticum* and *Clostridium gasigenes* cause significant spoilage of vacuum-packaged beef (Reid *et al*., 2017).

Bacteria in bovine faeces can be readily transferred to carcasses during slaughter and processing (Monaghan *et al*., 2012). Previous Irish studies have reported that 0.75% of hides (Khen *et al*., 2014), 0.25% (McEvoy *et al*., 2003a) and 7.6% (Khen *et al*., 2014) of carcasses and 3.8% of beef products (Khen *et al*., 2014) are contaminated with *Salmonella* spp. The corresponding figures for *E. coli* O157 are 17.6% on hides and 0.7–3.2% on carcasses (Thomas *et al*., 2012) with 2.4–3.0% of beef products being contaminated with this pathogen (Cagney *et al*., 2004; Carney *et al*., 2006). *L. monocytogenes* has also been reported on bovine hides (14%), carcasses (27%) and in beef products (29%) (Khen *et al*., 2015), but there are little or no data for *E. faecalis* or *Clostridium* spp.

The objectives of this preliminary study were to investigate the distribution (percentage of farms positive) of *Salmonella* spp., *E. coli* O157, *L. monocytogenes*, *E. faecalis* and *Clostridium* spp. and the overall prevalence (%) of these bacteria in cattle on a small cohort of Irish beef farms.

Materials and methods

Sampling

Ten beef farms in county Meath in the Northeast of Ireland participated in this study including nine privately owned beef farms (randomly selected from the Teagasc client database), and the Teagasc beef research farm at Grange, Dunsany, Co. Meath. Testing took place during the late Spring and Summer months when the animals were on grass. All samples were obtained from the same herd in a single field. Ten freshly voided faecal samples (the first 10 observed by the research team) were obtained on eight of the farms. As one of the private farms and the Teagasc farm were larger than the others with multiple herds, 16 and 25 samples were obtained, respectively. Samples were obtained using a sterile scoop (Sterileware; Fisher Scientific, Dublin, Ireland) taking care to only remove material from the top of the faecal pile. All samples were transported in sterile containers (VWR, Dublin, Ireland) to the laboratory in a cool box at 2–4°C within 2 h and processed the same day.

Microbiological analysis

Salmonella spp.

Exactly 25 g of faeces was added to 225 mL Buffered Peptone Water (BPW; Oxoid, Fannin Ltd., Dublin, Ireland) and stomached (Colworth Seward Stomacher 80, Worthing, West Sussex, England) for 60 s. Following incubation at 37°C for 24 h, 100 µL of pre-enriched sample was plated onto Modified Semi-Solid Rappaport Vassiliadis (MSRV; Oxoid, Fannin Ltd., Dublin, Ireland) medium with Novobiocin supplement (20 mg/L; Oxoid, Fannin Ltd., Dublin, Ireland). Samples were incubated at 42°C for 24 and 48 h. Presumptive *Salmonella* colonies that exhibited haloed growth were streaked onto Xylose Lysine Deoxycholate (XLD) agar (Oxoid, Fannin Ltd., Dublin, Ireland) and incubated at 37°C for 24 h (ISO 6579-1:2017). Plates were inspected for growth, and typical colonies (red colonies with some having black centres) were plated on tryptone soya agar (TSA). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) and frozen at –80°C for subsequent confirmation by polymerase chain reaction (PCR). Isolates were subjected to real-time PCR using the Lightcycler 480 (Roche Diagnostics, Basel, Switzerland) as described by Pathmanathan *et al*. (2003).

E. coli O157

Enrichment for *E. coli* O157 was carried out using modified tryptone soya broth (mTSB) with cefixime (50 µg/L) and vancomycin (6 mg/L) (Oxoid, Fannin Ltd., Dublin, Ireland). A 25-g quantity of each sample was added to 225 mL of mTSB and stomached for 60 s. The samples were incubated at 37°C for 24 h. Following this, immunomagnetic separation (IMS; Dynal®BeadRetriever, Thermo Fisher Scientific, Waltham, MA, USA) was carried out, and samples were plated on Sorbitol MacConkey agar supplemented with cefixime-tellurite (CT-SMAC; Oxoid, Fannin Ltd., Dublin, Ireland) before incubation at 37°C for 24 h. Suspect colonies (colourless round colonies) were streaked onto both eosin methylene blue agar (EMB; Oxoid, Fannin Ltd., Dublin, Ireland) and plate count agar (PCA; Oxoid, Fannin Ltd., Dublin, Ireland) and incubated at 37°C for 24 h. The EMB plates were inspected for a green metallic sheen, and corresponding colonies on PCA plates were used to carry out agglutination testing using the Sifin Anti-coli O157 sera test (Cruinn Diagnostics Ltd., Dublin, Ireland) (ISO 16654:2001(R2018)). Colonies that exhibited a green sheen and were agglutination positive were transferred to phosphate-buffered saline (PBS) and stored at –20°C until extraction. DNA extraction was carried out using the Qiagen

DNeasy Blood & Tissue Kit and tested by PCR as described by Paton & Paton (1998).

L. monocytogenes

Exactly 25 g of faeces was added to 225 mL of half-strength Fraser Broth (Oxoid, Fannin Ltd., Dublin, Ireland) and incubated overnight at 30°C. Following incubation, 0.1 mL was placed into 10 mL of full-strength Fraser Broth (Oxoid, Fannin Ltd., Dublin, Ireland) prior to incubation at 37°C for 48 h. After 24 and 48 h, broths were streaked on *Listeria* Selective Oxford Agar (Oxoid, Fannin Ltd., Dublin, Ireland) and Brilliance *Listeria* Agar (Oxoid, Fannin Ltd., Dublin, Ireland). Following incubation at 37°C for 48 h, presumptive positive colonies (black colonies with a black halo and sunken centre on the former and blue/green colonies with a halo on Brilliance *Listeria* Agar) were streaked onto PCA and incubated at 37°C for 24 h. Presumptive colonies were selected, DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit and tested using the PCR protocol of Terzi *et al*. (2015).

E. faecalis

Exactly 25 g of each faecal sample was added to 225 mL BBL Enterococcosel Broth (Becton Dickinson, Limerick, Ireland) and incubated at 37°C for 24 h. The enrichment broths were plated on Slanetz and Bartley Agar (Oxoid, Fannin Ltd., Dublin, Ireland) and incubated at 37°C for 24 h, followed by 44°C for an additional 24 h. Pink colonies were streaked on PCA and stabbed in rows into well-dried Bile Aesculin Agar (BAA; Oxoid, Fannin Ltd., Dublin, Ireland) plates. The PCA plates were then incubated at 37°C for 24 h, while BAA plates were incubated at 44°C for the same time period. Colonies that exhibited aesculin hydrolysis (more than half the agar plates turned dark brown) were considered presumptive positives and single colonies from corresponding PCA were selected for DNA extraction. Extraction was carried out using the Qiagen DNeasy Blood & Tissue Kit, and samples were frozen at –80°C prior to PCR (Dutka-Malen *et al*., 1995).

Clostridium spp.

Exactly 25 g of each faecal sample was added to 225 mL of Maximum Recovery Diluent (MRD), stomached and a serial dilution prepared, before plating on Reinforced Clostridial Agar (RCA; Oxoid, Fannin Ltd., Dublin, Ireland). Plates were incubated anaerobically, using Oxoid Anaerogen sachets (Oxoid, Fannin Ltd., Dublin, Ireland) and BioMérieux GENbox jars (bioMérieux UK Ltd., Hampshire, UK), at 37°C for 48 h. Suspect colonies were transferred to Columbia Blood Agar (CBA; Oxoid, Fannin Ltd., Dublin, Ireland) supplemented with 5% defibrinated horse blood (Cruinn Diagnostics, Dublin, Ireland). Following anaerobic incubation of these plates at 37°C for 24 h, colonies were inspected for haemolysis, and suspect colonies selected for DNA extraction and PCR. DNA extraction was carried out using Qiagen DNeasy Blood & Tissue Kit and subjected to real-time PCR using group-specific primers (TibMolBiol, Berlin, Germany) to target and detect the 16S rRNA of Cluster 1 *Clostridium* spp. (Song *et al*., 2004).

Results

The individual results for each farm are shown in Table 1. *Salmonella* spp. were not detected on any of the farms. Bovine faecal samples on Farm 1 tested positive for *E. faecalis* and *Clostridium* spp. but the other target bacteria were not detected. A similar pattern was obtained on Farms 4, 6, 7 and 8. *E. coli* O157, *L. monocytogenes*, *E. faecalis* and *Clostridium* spp. were detected in the faecal samples on Farms

Table 1: The number and percentage of pathogen-positive samples in bovine faecal samples on each farm

Farm	*N*	Location	*Salmonella* spp.	*Escherichia coli* O157	*Listeria monocytogenes*	*Enterococcus faecalis*	*Clostridium* spp.
1	10	Lobinstown (Meath)	ND	ND	ND	7 (70%)	10 (100%)
2	10	Castletown (Meath)	ND	1 (10%)	5 (50%)	6 (60%)	10 (100%)
3	10	Castletown (Meath)	ND	2 (20%)	ND	5 (50%)	10 (100%)
4	10	Rathcore (Meath)	ND	ND	ND	3 (30%)	5 (50%)
5	10	Moynalty (Meath)	ND	1 (10%)	1 (10%)	7 (70%)	10 (100%)
6	10	Wilkinstown (Meath)	ND	ND	ND	6 (60%)	10 (100%)
7	10	Howthstown (Meath)	ND	ND	ND	1 (10%)	10 (100%)
8	10	Fennor (Meath)	ND	ND	ND	4 (40%)	10 (100%)
9	16	Castletown (Meath)	ND	1 (6%)	1 (6%)	12 (75%)	16 (100%)
10	25	Grange (Meath)	ND	6 (24%)	3 (12%)	24 (96%)	25 (100%)

N = number of samples tested; ND = not detected.

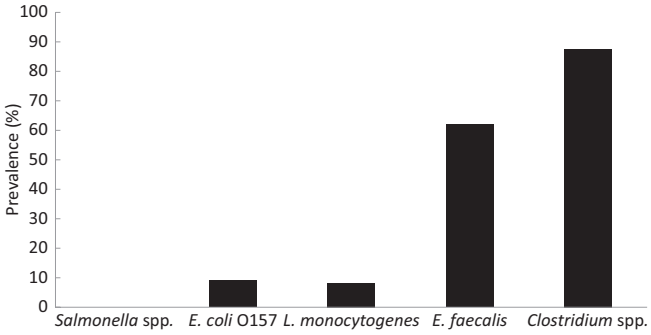

Figure 1. The overall prevalence (%) of *Salmonella* spp., *Escherichia coli* O157, *Listeria monocytogenes*, *Enterococcus faecalis* and *Clostridium* spp. in the faecal samples.

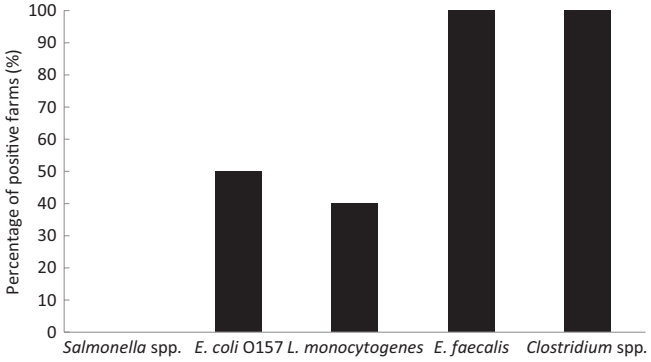

Figure 2. The percentage of Irish beef farms positive for *Salmonella* spp., *Escherichia coli* O157, *Listeria monocytogenes*, *Enterococcus faecalis* and *Clostridium* spp.

2, 5, 9 and 10. With the exception of *L. monocytogenes*, these bacteria were also present on Farm 3. The overall prevalence (%) of *Salmonella* spp., *E. coli* O157, *L. monocytogenes*, *E. faecalis* and *Clostridium* spp. in the faecal samples is shown in Figure 1 and the percentage of farms positive for a given pathogen is shown in Figure 2. *Clostridium* spp. were particularly common, being detected in 87.6% of samples followed by *E. faecalis* (61.9%), *E. coli* O157 (9%), *L. monocytogenes* (8.2%) and *Salmonella* spp. (0%). *Clostridium* spp. and *E. faecalis* were widely distributed being detected on all farms, *E. coli* O157 on half and *L. monocytogenes* on 40% of farms.

Discussion

Even allowing for the small sample size (121 samples), the absence of *Salmonella* spp. in the samples tested was unexpected. McEvoy *et al*. (2003a) reported a prevalence of 2% for *Salmonella* spp. in cattle in the Republic of Ireland, while Madden *et al*. (2007) obtained a 3% rate in Northern Ireland. In the United Kingdom, where bovine animal breeds, husbandry practices and weather conditions are similar to Ireland, Hutchison *et al*. (2004) reported a 7.7% *Salmonella* prevalence in fresh bovine faeces. In contrast, *Salmonella* prevalence as low as 0.2% has been previously reported in bovine faeces in the USA (Rodriguez *et al*., 2006). While season, feed type and animal husbandry all influence *Salmonella* carriage and shedding in bovine animals (Rhoades *et al*., 2009), sampling isolated individual faecal deposits in fields also results in lower detection rates as these are not cross-contaminated by waste from other animals (Arnold *et al*., 2015). Moreover, the impact of good animal husbandry, biosecurity and hygiene practices by Teagasc client farmers cannot be ruled out, although validation would require further investigation.

The overall prevalence of *E. coli* O157 of 9% in bovine faeces was higher than the 0.7–2.4%, previously reported in Ireland (McEvoy *et al*., 2003b; Thomas *et al*., 2012) but similar to the 7.1–8.3% reported in the UK (Gunn *et al*., 2007; Smith *et al*., 2009, 2016). STEC, especially serogroup O157, are a common cause of gastrointestinal illness around the world and are frequently associated with severe illness including haemorrhagic colitis and haemolytic uraemic syndrome. Bacteria in faeces contaminate hides and are readily transferred to the carcasses during hide removal in the abattoir (Tergny & Bolton, 2006). Beef is therefore a major source of *E. coli* O157 infections in the EU (EFSA, 2020) and the relatively high prevalence of these bacteria observed in this study is a cause for concern.

Data on the prevalence of *L. monocytogenes* in bovine faeces are also limited. The *L. monocytogenes* prevalence obtained in this study (8.2%) is midway between the prevalence of 4.8% reported in a Northern Irish Study (Madden *et al*., 2007) and 12% reported in dairy cows in Ireland (Fox *et al*., 2009). In contrast, Nightingale *et al*. (2004) reported a 29.4% overall faecal prevalence in 323 bovine faecal samples examined as part of a US study. Previous studies reported that *L. monocytogenes* prevalence in minced beef products in Dublin ranged from 4.7% to 16% (Sheridan *et al*., 1994, 1997). However, this research is more than 20 years old and to the best of our knowledge more up-to-date data are not available. As there are multiple sources of *L. monocytogenes* along the beef processing chain, the impact of bovine faecal contamination rates on the risk to public health is unknown.

E. faecalis was present in 61.9% of bovine faecal samples tested. This organism is not widely studied in cattle, and to the best of our knowledge, comparative data are not available for Ireland or the UK. However, prevalence rates of 13.3% and 43.3% have been previously reported for Belgian cattle (de Jong *et al*., 2018) and Latvian calves (Terentjeva *et al*., 2019), respectively. Enterococcal species are common constituents

of the intestinal flora of many animal species. However, in recent years, enterococci, especially multiple antibiotic-resistant *E. faecalis*, have been associated with nosocomial bloodstream, urinary tract and surgical wound infections, within the hospital environment, rather than food, serving as the source of infection (Moellering, 1992). Thus, the high prevalence in bovine faeces may not present a direct risk of infection but may serve as a source of antibiotic resistance determinants for other pathogens (Weigel *et al*., 2003).

Of all the organisms examined in our study, *Clostridium* spp. were the most frequently detected, with 87.6% of samples testing positive. PCR confirmation focused on targeting Cluster 1 clostridia, which includes pathogenic species: *C. botulinum*, *Clostridium tetani*, *C. perfringens*, as well as *C. difficile*, and spoilage organisms, *C. estertheticum* and *C. gasigenes* (Song *et al*., 2004). Comparison of this figure with previously published data is difficult, as no other studies focused on Cluster 1 clostridial species, but specifically targeted *C. difficile*, *C. botulinum* or *C. perfringens* (Cox *et al*., 2005; Bartels *et al*., 2010; Kruger *et al*., 2012). However, previous Irish research reported *C. estertheticum* and *C. gasigenes* in 17.9% and 25.4% of bovine faecal samples, respectively (Moschonas *et al*., 2009). A more recent study found *C. difficile* in 6.7% of bovine faeces (Marcos *et al*., 2021). Further research is required to characterise the *Clostridium* spp. in bovine faeces before the potential impact of such high prevalence on food safety and spoilage can be assessed.

It was concluded that, although differences in study design, animal age, diet, seasonality and the detection method affect the prevalence in specific bacteria in bovine faecal surveys (Bolton *et al*., 2012), *Salmonella* spp., *E. coli* O157 and *L. monocytogenes* may be present in beef animals but usually at a low (<10%) prevalence. In contrast, *E. faecalis* and *Clostridium* spp. are more common, perhaps reflecting their ubiquitous distribution in farming environments.

Acknowledgements

This study was funded by the Food Institute Research Measure administered by the Department of Agriculture, Food and the Marine (Project 14/SF/487). Ms. Lauren Russell was supported by the Teagasc Walsh Scholarship Programme (number 2014239).

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