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Emerging Verocytotoxigenic *Escherichia coli* (VTEC) on Irish beef farms

Key external stakeholders:

Irish beef farmers, beef processors, FSAI, DAFF, public health personnel, epidemiologists and scientists interested in VTEC research.

Practical implications for stakeholders:

This study discovered that VTEC were widespread on Irish beef farms and some serotypes were capable of causing serious illness in humans. A range of different VTEC serotypes were also detected on cattle hides and carcasses in the abattoir. New, more virulent serotypes are emerging and will join *E. coli* O157 in causing serious disease outbreaks in the future.

Main results:

- VTEC are widespread on Irish beef farms
- VTEC are present on hides and carcasses in the abattoir
- VTEC survive well in Irish clay and sandy soils
- Several serotypes of potential clinical significance are emerging

Opportunity / Benefit:

The data generated, especially on non-O157 VTEC will be used to formulate new risk based meat inspection procedures and in the development of public health protection policy. It strongly supports the case for expanding current microbiological criteria in meat monitoring and identifies novel VTEC that should be tested for in seriously ill patients not infected with O157.

Collaborating Institutions:

UCD; UUJ; USDA-ARS

Contact Declan Bolton Email: declan.bolton@teagasc.ie.

Teagasc project team: Dr. Declan Bolton (PI)
External collaborators: Prof. Seamus Fanning , University College Dublin
Prof. Torres Sweeney, University College Dublin
Prof. David McDowell, University of Ulster at Jordanstown
Dr. Pina Fratamico, United States Department of Agriculture-Eastern Region Research Centre (USDA-ERRC), USA

1. Project background:

This project addressed the emergence of a range of emergent verocytotoxigenic pathogens in cattle in Ireland. The discovery of new zoonotic cytotoxic pathogens, with multiple antibiotic resistances, is of major importance, since they have the potential to entering the food chain through beef carcass contamination. There is a real lack of information on these emergent pathogens and this project focused on establishing the presence of these new pathogens on Irish bovine farms and abattoirs and the consequence of infecting consumers, through the consumption of contaminated beef. The project was aimed at generated knowledge of benefit to the beef sector industry by informing them of the potential hazards associated with these pathogens and the risk posed by their emergence.

2. Questions addressed by the project:

- What methods should be used to detect non-O157 VTEC?
- What is the prevalence, distribution, serotypes and virulence gene profiles of VTEC on Irish beef farms?
- What are the VTEC antibiotic resistance profiles?
- How long does these pathogens survive in soil?

3. The experimental studies:

The first part of this project focused on the design of suitable methodologies for culturing and isolating the different VTEC serotypes from bovine faeces, soil, bovine hide and bovine carcass samples. All VTEC isolates were found to be commonly resistant to two antibiotics (Streptomycin & sulfamethazine). These antibiotics were used to fortify different media (SMAC, Chromocult coliform agar, Eosin methylene blue agar (EMB) and Chromocult tryptone bile x-glucuromide agar (Chrom-TBX)). These different agars were validated using a range of different VTEC isolates. The media that best recovered all the VTEC serotypes was used in the farm and abattoir study.

A PCR isolation technique was used to determine the serogroup of each suspect isolate. Teagasc worked in collaboration with USDA in Philadelphia to amplifying and sequencing the entire O-antigen gene cluster of each of VTEC O2 and O63. The location of the *wzx* and *wzy* genes was determined and serogroup specific primers were designed from these genes. The specificity of these primers were validated by Pennsylvania state university. The equivalent primers for O148, O149, O174 and O26 were designed published in the peer reviewed scientific literature by other research teams. Using all of these primers specific PCR assays were developed by optimising PCR conditions (DNA denaturation, annealing and elongation temperatures and DNA concentrations). These O-antigen specific primers were used to screen farm and abattoir isolates.

Twenty farms (both dairy and livestock) were selected throughout the country. The selected farms were visited six times over a one year period. At each of 6 visits, 10 fresh faecal and 5 soil samples were collected from each farm. On each occasion, samples of faeces and soil were enriched in mTSB (TSB + vancomycin) overnight at 37 °C. The enriched samples were PCR screened for the presence of vt_1 and vt_2 genes. The verotoxin positive samples were then serial diluted and plated mTBX (Task 1a) and incubated overnight at 37 °C. After incubation 4 colonies showing different colony morphology on mTBX were isolated by streaking each colony onto nutrient agar (NA) and EMB agar and incubated overnight at 37 °C. The DNA was extracted from each positive (green metallic sheen on EMB) *E. coli* isolates. This was carried out by boiling each isolate (loop of culture) in prepman reagent for 10 min. The suspension was cooled and centrifuged at 14000 rpm for 3 min. The DNA of each isolate was extracted and screened for verotoxin genes. The verotoxin positive isolates were stored on cryo-protect beads for further analysis.

Three bovine abattoirs were selected. The selected abattoirs were visited six times (18 Abattoir visits) over a one year period to determine if there is a seasonal incidence of the cytotoxic *E. coli* serotypes on cattle hides and beef carcasses. At each of 6 visits, 25 hides (Rump) and 25 carcasses (full carcass) were taken. Hide

Contact Declan Bolton Email: declan.bolton@teagasc.ie.

and carcass swabs were stomached in 100 ml mTSB and incubated overnight at 37 °C. The enriched samples were screened for the presence of vt_1 and vt_2 genes using the Paton and Paton (1998) PCR protocol. The verotoxin positive samples were serial diluted and plated onto mTBX (Task 1a) and incubated overnight at 37 °C. After incubation, 4 colonies showing different colony morphology on mTBX were isolated by streaking each colony onto nutrient agar (NA) and EMB agar and incubated overnight at 37 °C. The DNA was extracted from each *E. coli* isolate. The DNA of each isolate was extracted and screened for verotoxin genes. The verotoxin positive isolates were stored on cryo-protect beads for further analysis.

The verotoxin positive isolates were serotyped (O and H typing) by Pennsylvania Stat University. A virulent profile was generated for each by examining each isolate for a range of different virulence factors (previously been associated with clinical VTEC strains). Briefly, published PCR protocols were used to screen the genome of all the VTEC isolates for vt_1, vt_2, *TIR, hlyA, eaeA, saa,* $lpfA_{O113}$, $lpfA_{O157/OI-141}$, $lpfA_{O157/OI-154}$ *katP, etpD, espF, espP, espB, espA, toxB* and *iha*. Four virulent factors (vt1, vt2, eae, and saa) were typed to determine the genetic variation within each gene. PCR, Restriction fragment length polymorphism-PCR (RFLP-PCR) and sequencing techniques were used to detect genetic variations within the each of the genes.

The ability of the emerging VTEC to survive in a free-living state in different soil types was also determined. Fourteen different serotypes (ONT, O-, O2, O6, O20, O26, O86, O113, O116, O119, O136, O145, O168 and O174) cultured from both soil and faeces were assessed on their ability to survive in soil over time. These isolates were inoculated into two types (sandy and clay) of soils and incubated at 10 °C (average Irish temperature) over a 201 day period. On day 0, 12, 26, 40, 54, 75, 103, 131, 159 and 201 the inoculated soil was sampled. Soil samples were serial diluted and plated on to mTBX agar (Task 1a), after an over night at 37 °C the cfu on each plate were enumerated and the survivors were calculated.

4. Main results:

A selective medium, a modification of TBX agar, was developed and validated for the isolation and detection of the 7 target VTEC serotypes. A specific PCR based assay was also developed to detect the 7 target VTEC serotypes (O2, O63, O148, O149, O174 and O26).

Data/information was generated on the incidence/prevalence of VTEC on the farm on beef animals and their corresponding carcasses including serogroups and virulence profiles. This work discovered that VTEC are widespread on Irish farms with 17 different non-O157 serogroups detected). 40 VTEC isolates representing 12 different serogroups were discovered in the abattoir.The most common virulence factor detected in the farm isolates was vt_2 (77% of all VTEC isolates) followed by *iha* (73%), vt_1 (60%), $lpfA_{O113}$ (48%), *hlyA* (26%), *espP* (23%), *eaeA* (18%), *toxB* (16%), *Tir* (14%), $lpfA_{O157/OI-141}$ (10%), *saa* (7%), *espA* (1%) and *espB* (1%). While *KatP*, *etpD*, $lpfA_{O157/OI-154}$ and *sab* were not detected.

All the VTEC isolates possessing the vt_1 gene did not possess any of the vt_1 variants (vt_{1c} and vt_{1d}). Therefore, there was no genetic variation between vt_1 gene of all the VTEC isolates. All (98 isolates) the vt_2 positive strains carried variants (vt_1, vt_{1c}, vt_{1d}, vt_2, vt_{2c}, vt_{2d}, vt_{2dact}, vt_{2e}, vt_{2f} and vt_{2g}) of vt_2. All the VTEC isolates containing a vt_2 also contained the variant vt_{2d}. 14% of the VTEC isolates also contained a vt_2 also contained the variants vt_{2d} and vt_{2dact}. 9% of the VTEC isolates containing a vt_2 also contained the variants vt_{2d} and vt_{2g}. 3% of the VTEC isolates containing a vt_2 also contained the variants vt_{2d} and vt_{2c}. 3% of the VTEC isolates containing a vt_2 also contained the variants vt_{2d}, vt_{2c} and vt_{2dact}. 19 VTEC farm isolates containing the intimin gene were amplified, purified and sequenced. The results found four different intimin variants present in the farm VTEC isolates. The most common intimin variant being β (14 isolates) followed by ζ (2), θ (1), γ (1) and 1 isolated was not typed. 7 VTEC abattoir isolates containing the intimin gene were amplified, purified and sequenced. The results discovered two different intimin variants present in the abattoir isolates with the most common intimin variant being β (4) followed by ζ (2) and 1 isolated was not typed. Potentially clinically significant serotypes include O13:H2, O145:H28, O150:H2, O2:H27, O26:H11, O5:H-, ONT:H27, ONT:H4, ONT:H18, O20:H19, O86;H21, ONT:H11, O33:H11, O128:H8, O109:H5, O119:H5 & O138:H48. Similar research had not previously been undertaken in Ireland and the dataset generated is proving to be a valuable resource in the identification of emerging VTEC that may represent a serious treat to public health in the future.

Data/information was also generated on survival in Irish clay and sandy soils which provided a scientific basis for the high incidence and widespread dissemination of the different VTEC serotypes observed. In brief, all serotypes tested survived for in excess of 200 days although survival rates were higher in sandy soils.

Contact Declan Bolton Email: declan.bolton@teagasc.ie.

All VTEC isolates demonstrated antimicrobial resistance, with 23 % showing resistance to 5 or more antibiotic classes. Resistance to sulphonamide-tetracycline-colistin and sulphonamide-tetracycline-ampicillin were the most common profiles observed in the abattoir and farm environments, respectively. The percentage of isolates resistant to 4 or more antibiotic classes was higher in the abattoir isolates (60 %) compared to farm isolates (41 %).

5. Opportunity/Benefit:

VTEC are emerging as a serious threat to public health and the Irish beef industry. This project provided the data required for the development of effective monitoring and control.

6. Dissemination:

The findings of this project have been extensively disseminated through oral and poster presentation at national and international workshops and conferences.

Main publications:

Fratamico, P., Yan, X., Liu, Y., DebRoy, C., Byrne, B., Monaghan, A., Fanning, S. and Bolton, D. (2010) *Escherichia coli* serogroup O2 and O28ac O-antigen gene cluster sequences and detection of pathogenic *E. coli* O2 and O28ac by PCR. *Canadian Journal of Microbiology* 56 (4), 308-316.

Bolton, D. J. (2010) Verocytotoxigenic (Shiga Toxin Producing) *Escherichia coli*: Virulence Factors and Pathogenicity in the Farm to Fork Paradigm. *Foodborne Pathogens and Disease* 8(3): 357-365

Bolton, D. J., Monaghan, A., Byrne, B., Fanning, S., Sweeney, T. and McDowell, D. A. (2011). Incidence and survival of non-O157 verocytotoxigenic *Escherichia coli* in soil. *Journal of Applied Microbiology*, 111 (2), 484-490.

7. Compiled by: Dr. Declan Bolton

Contact Declan Bolton Email: declan.bolton@teagasc.ie.