DEVELOPMENT OF A RAPID PCR METHOD TO QUANTIFY *E. COLI* (O157 AND O26) IN BOVINE FAECES

Dolapo Lawal[1,2], Catherine Burgess[1], Evonne McCabe[1], Paul Whyte[2] and Geraldine Duffy[1*].

[1]Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland
[2] University College Dublin, Belfield, Dublin 4, Ireland
*geraldine.duffy@teagasc.ie

Abstract – Bovine animals are reservoirs for a range of VTEC, including VTEC belonging to serogroups O157 and O26. Whilst shedding dynamics of the pathogens is highly variable, it is recognised that 'super-shedders' may excrete high numbers (>10,000 colony-forming units (CFU)/g) in faeces subsequently increasing the risk of VTEC contamination at farm and slaughter level. The frequency and underlying causes of super-shedding are poorly understood due to the lack of robust and sensitive enumeration methods in screening studies. This method consists of a short pre-enrichment in modified TSB followed by a spin column DNA extraction and finally detection and quantification of O157 and O26 serogroups by real-time PCR in bovine faeces as a tool for investigating shedding dynamics. Independent assays for *E. coli* O157 and O26 were developed in parallel, and resulted in the generation of calibration curves by plotting PCR Ct values against the starting concentration in the swab sample for each serogroup. A calibration curve for *E. coli* O157 with a R^2 value of 0.85 was achieved whilst for *E. coli* O26 the R^2 value was 0.87 demonstrating the developed method is effective for collection of quantitative data on *E. coli* O157 or O26 serogroups found in bovine faecal samples.

I. INTRODUCTION

Verocytotoxigenic *E. coli* (VTEC) is a zoonotic pathogen that has caused major outbreaks worldwide. Cattle are recognised as a principal reservoir for VTEC [1]. These pathogens are generally transient members of the intestinal micro-flora and only rarely do they cause disease in young, weakened calves. While cattle have been shown to harbour this pathogen on occasion in their rumen, it is found more frequently in the distal portion of the bovine gastro intestinal tract, with the rectal anal junction identified as the predominant colonisation site for O157: H7 [2, 3]. Shedding of VTEC by cattle is generally intermittent with herd members remaining negative for months with only a proportion sporadically becoming positive for a few weeks at a time [4, 5]. It has also been hypothesised that high level carriage of these microorganisms is a consequence of intestinal colonisation while low levels within individual animals may be a result of environmental exposure with no significant colonisation [6, 7]. The typical pattern of shedding in a herd is sporadic with intense periods of shedding interspersed with periods of non-shedding [8]. Ogden *et al* (2004) have also reported that concentrations of *E. coli* O157 being shed in the faeces of positive cattle were highest during summer months [9]. The number of VTEC (CFU g^{-1}) being shed in the faeces of individual animals is considered important in the context of hide, environmental and subsequent carcass contamination. The phenomenon of 'super shedding' animals (those shedding >10^4 CFU/g faeces) is thought to be a significant contributor in the dissemination of O157 VTEC within and between herds and within abattoirs [10-12]. However, quantitative data and the frequency of super-shedders are few relative to prevalence data, as the routine detection methods generally employed in large surveys, are designed to yield data only on presence of the pathogen and not on the numbers present. Generation of quantitative data has been hampered by a lack of enumeration methods which are robust and sensitive enough to be applied in large scale animal screening studies.

In our study we set out to develop a strategy to detect and quantify *E. coli* O157 and O26 in bovine faeces through the combination of a short enrichment period and a quantitative real time PCR, with a developed calibration curve to correlate starting concentration (CFU) in the sample with the PCR Ct value to screen and identify super-shedders of these serogroups.

II. MATERIALS AND METHODS

Independent real time assays for *E. coli* O157 and O26 were developed in parallel in pure culture. The PCR primers used for the amplification of O antigen specific genes for *E. coli* O157 and O26 were *rfbE* and *wzx* respectively as described in ISO13136:2012. In order to determine the optimal enrichment time samples spiked with different initial concentrations of *E. coli* O26 or O157 (Log_{10} 1 CFU to Log_{10} 6 CFU) were enriched in modified tryptone soya broth (mTSB) for various periods of time to assess when a differential Ct could be obtained, the enrichment times trialled were 4, 5, 6 and 8h at 41.5°C and the DNA was extracted with Qiagen Blood and Tissue Kit. The Ct value being the value that measures the linear relationship between the crossing point of fluorescence produced and the log of the input DNA start molecules. The objective was to ascertain if a correlation existed between starting concentration of the pathogen (CFU) and the PCR Ct value .To validate the assay, swabs were then taken from the bovine recto-anal junction (RAJ) in cattle presented for slaughter. These were inoculated with various levels (< log_{10} 1 CFU/g to > log_{10} 6 CFU/g) of either *E. coli* O157 (n= 81) or *E. coli* O26 (n=77). Three strains of each serogroup were used. The swabs were enriched in mTSB prior to DNA extraction. These DNA samples were used as a template in real time PCR reactions using the serogroup specific primers and probes outlined in ISO13136:2012. A calibration curve was constructed for each serogroup, plotting PCR Ct value against the starting concentration (CFU) in the swab sample.

III. RESULTS AND DISCUSSION

Cattle are recognised as a principal reservoir for VTEC [1] and Rhoades et al (2009) extensively reviewed the prevalence of VTEC in the beef chain and the faecal prevalence of *E. coli* O157 in cattle and showed it varied from 0% to 48.8% [13]. However, quantitative data are few relative to prevalence data as the routine detection methods generally employed in surveys, are designed to yield data only on presence of the pathogen and not on the numbers present. A study by [14] examined faeces and hide for concentrations of six VTEC serogroups and showed that the vast majority of samples had counts below the limit of detection of the count method and samples with detectable counts ranged from 60 to 100 CFU/cm^2 on hide and 100 to 1300 CFU g^{-1} in faeces. A UK abattoir study found that 70% of *E. coli* O157-positive animals shed <100 cfu $g^{-1}$of faeces but in some individual concentrations could be as high as 10^6 cfu g^{-1} of faeces [15]. These authors also showed that the 9% of the animals shedding *E. coli* O157:H7 at slaughter produced over 96% of the total O157:H7 faecal load for the group. The phenomenon of 'super shedding' animals (those shedding >10^4 CFU/g faeces) is thought to be a significant contributor in the dissemination of O157 VTEC within and between herds and within abattoirs [10-12] but there is limited information on how often this occurs in cattle, with such studies limited by lack of a robust method to rapidly screen, detect and enumerate key VTEC serogroups in bovine faeces.

Therefore, in this study we aimed to develop a method based on quantitative PCR to rapidly detect and enumerate *E. coli* O157 or O26 in bovine faeces. In the first part of the study, it was shown that following five hours enrichment in mTSB there was a direct correlation between the initial number of *E.*

coli O157 or O26 (CFU/ml) in the broth and the PCR Ct value for that sample. This demonstrated that a standard calibration (r^2 value of 0.99) could be set up relating CFU and PCR Ct value and used to calculate the number of VTEC in the tested sample. This approach based on enrichment, real time PCR and the use of a standard calibration curve, is the similar to the one used by Krämer *et al*, (2011) to detect and enumerate *Salmonella* in porcine samples. The approach has the advantage of giving a higher sensitivity than previous quantitative approaches which were based on direct application of the PCR to the sample [16, 17].

The approach was further tested using swabs taken from the bovine rectal junction that were inoculated with O157 and O26. From the results obtained it was found that each assay provided satisfactory correlations between the Ct values obtained in the PCR reactions and the starting concentration of VTEC in the swab samples. For *E. coli* O157 an R^2 value of 0.85 was achieved whilst for *E. coli* O26 the R^2 value was 0.87. These constructed calibration curves allow the estimation of the number (CFU) VTEC O26 or O157 in the sample from the PCR Ct value. This compares to an r^2 value of 0.94 reported for the enumeration of *Salmonella* in porcine samples using a similar approach (Kramer *et al*, 2011). This developed method will support large scale screening studies on the numbers of *E coli* O157 and O26 serogroups shed by bovine animals and will progress investigations on shedding dynamics of VTEC and on the frequency and causes of the super shedding phenomenon. Additionally, as the start of the assay, ie. the enrichment procedure is similar to that used in ISO13136:2012 (presence/ absence test), any samples testing positive by the developed method can then be reverted back into the ISO method for cultural isolation of the strains.

IV. CONCLUSION

The developed method provides a rapid qualitative and quantitative method for *E. coli* O157 and O26 in bovine faecal samples and will support studies on shedding of these serogroups in cattle including super shedding dynamics.

ACKNOWLEDGEMENTS

This project is part funded by Food Institutional Research Measure (FIRM) administered by the Irish Department of Agriculture, Food and the Marine.

REFERENCES

[1] A. Caprioli, S. Morabito, H. Brugere, and E. Oswald, "Enterohaemorrhagic Escherichia coli: emerging issues on virulence and modes of transmission," *Veterinary research,* vol. 36, pp. 289-311, May-Jun 2005.

[2] S. W. Naylor, J. C. Low, T. E. Besser, A. Mahajan, G. J. Gunn, M. C. Pearce, I. J. McKendrick, D. G. Smith, and D. L. Gally, "Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic Escherichia coli O157:H7 in the bovine host," *Infection and immunity,* vol. 71, pp. 1505-12, Mar 2003.

[3] T. M. Arthur, D. M. Brichta-Harhay, J. M. Bosilevac, N. Kalchayanand, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie, "Super shedding of Escherichia coli O157:H7 by cattle and the impact on beef carcass contamination," *Meat science,* vol. 86, pp. 32-7, Sep 2010.

[4] J. Blanco, M. Blanco, J. E. Blanco, A. Mora, E. A. Gonzalez, M. I. Bernardez, M. P. Alonso, A. Coira, A. Rodriguez, J. Rey, J. M. Alonso, and M. A. Usera, "Verotoxin-producing Escherichia coli in Spain: prevalence, serotypes, and virulence genes of O157:H7 and non-O157 VTEC in ruminants, raw beef products, and humans," *Experimental biology and medicine (Maywood, N.J,* vol. 228, pp. 345-51, Apr 2003.

[5] A. Monaghan, B. Byrne, S. Fanning, T. Sweeney, D. McDowell, and D. J. Bolton, "Serotypes and virulence profiles of non-O157 Shiga toxin-producing Escherichia coli isolates from bovine farms," *Applied*

and environmental microbiology, vol. 77, pp. 8662-8, Dec 2011.

[6] J. C. Low, I. J. McKendrick, C. McKechnie, D. Fenlon, S. W. Naylor, C. Currie, D. G. Smith, L. Allison, and D. L. Gally, "Rectal carriage of enterohemorrhagic Escherichia coli O157 in slaughtered cattle," *Applied and environmental microbiology,* vol. 71, pp. 93-7, Jan 2005.

[7] S. W. Naylor, D. L. Gally, and J. C. Low, "Enterohaemorrhagic E. coli in veterinary medicine," *International journal of medical microbiology,* vol. 295, pp. 419-41, Oct 2005.

[8] D. D. Hancock, T. E. Besser, D. H. Rice, E. D. Ebel, D. E. Herriott, and L. V. Carpenter, "Multiple sources of Escherichia coli O157 in feedlots and dairy farms in the northwestern USA," *Preventive veterinary medicine,* vol. 35, pp. 11-9, Apr 16 1998.

[9] I. D. Ogden, M. MacRae, and N. J. Strachan, "Is the prevalence and shedding concentrations of E. coli O157 in beef cattle in Scotland seasonal?," *FEMS microbiology letters,* vol. 233, pp. 297-300, Apr 15 2004.

[10] L. Matthews, I. J. McKendrick, H. Ternent, G. J. Gunn, B. Synge, and M. E. Woolhouse, "Super-shedding cattle and the transmission dynamics of Escherichia coli O157," *Epidemiology and infection,* vol. 134, pp. 131-42, Feb 2006.

[11] L. Matthews, J. C. Low, D. L. Gally, M. C. Pearce, D. J. Mellor, J. A. Heesterbeek, M. Chase-Topping, S. W. Naylor, D. J. Shaw, S. W. Reid, G. J. Gunn, and M. E. Woolhouse, "Heterogeneous shedding of Escherichia coli O157 in cattle and its implications for control," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, pp. 547-52, Jan 17 2006.

[12] G. Duffy, E. Cummins, P. Nally, O. B. S, and F. Butler, "A review of quantitative microbial risk assessment in the management of Escherichia coli O157:H7 on beef," *Meat science,* vol. 74, pp. 76-88, Sep 2006.

[13] J. R. Rhoades, G. Duffy, and K. Koutsoumanis, "Prevalence and concentration of verocytotoxigenic Escherichia coli, Salmonella enterica and Listeria monocytogenes in the beef production chain: a review," *Food microbiology,* vol. 26, pp. 357-76, Jun 2009.

[14] K. M. Thomas, M. S. McCann, M. M. Collery, A. Logan, P. Whyte, D. A. McDowell, and G. Duffy, "Tracking verocytotoxigenic Escherichia coli O157, O26, O111, O103 and O145 in Irish cattle," *International journal of food microbiology,* vol. 153, pp. 288-96, Feb 15 2012.

[15] F. Omisakin, M. MacRae, I. D. Ogden, and N. J. Strachan, "Concentration and prevalence of Escherichia coli O157 in cattle feces at slaughter," *Applied and environmental microbiology,* vol. 69, pp. 2444-7, May 2003.

[16] N. Kramer, C. Lofstrom, H. Vigre, J. Hoorfar, C. Bunge, and B. Malorny, "A novel strategy to obtain quantitative data for modelling: combined enrichment and real-time PCR for enumeration of salmonellae from pig carcasses," *Int J Food Microbiol,* vol. 145 Suppl 1, pp. S86-95, Mar 1 2011.

[17] D. M. Brichta-Harhay, T. M. Arthur, J. M. Bosilevac, M. N. Guerini, N. Kalchayanand, and M. Koohmaraie, "Enumeration of Salmonella and Escherichia coli O157:H7 in ground beef, cattle carcass, hide and faecal samples using direct plating methods," *Journal of applied microbiology,* vol. 103, pp. 1657-68, Nov 2007.