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Note

A quantitative real time PCR assay to detect and enumerate *Escherichia coli* O157 and O26 serogroups in sheep recto-anal swabs

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ABSTRACT

A quantitative PCR method is described for the detection and quantification of *E. coli* O157 and O26 in sheep recto-anal junction swabs. The method incorporated a short enrichment step (5 h) and the use of a developed standard calibration curve relating the real time PCR cycle threshold (Ct) values to the initial concentration of pathogen in the sheep sample.

Shiga toxin-producing *E. coli* (STEC) is a significant zoonotic pathogen and ruminants, including cattle and sheep, are considered to be the main reservoir. Shedding of STEC in faeces is reported to be variable in terms of both temporal shedding patterns and numbers shed (Elson et al., 2018; Stein and Katz, 2017). Many studies have focused on bovine shedding of *E. coli* O157 and it is reported that cattle can excrete *E. coli* O157:H7 at levels of $\geq 10^4$ CFU g^{-1} of faeces and have been referred as super-shedders (Cobbold et al., 2007; McCabe et al., 2019; Stein and Katz, 2017; Wang et al., 2017) and present a high risk for pathogen transmission in the agri-food chain. In bovines, the main colonization site of STEC is located at the distal end of the gastrointestinal tract, called the recto-anal junction (RAJ) (Martorelli et al., 2018). While the super-shedding phenomenon has also been considered in sheep (McPherson et al., 2015), there are limited studies on STEC shedding dynamics in sheep populations (Fegan and Desmarchelier, 1999; Kudva et al., 1997). The lack of studies with quantitative data on STEC is largely related to the availability of methods, which can be used to accurately and sensitively quantify the pathogen in large scale field surveillance studies.

Culture-based quantification methods and nucleic acid-based methods have been recently reported for quantification STEC in agri-food matrices (De Boer and Heuvelink, 2000; Sethulekshmi et al., 2018) and cattle faeces (Lawal et al., 2015; Shridhar et al., 2016; Stromberg et al., 2018; Verhaegen et al., 2016). However, the applicability of the real-time PCR assays in sheep faeces has not been evaluated, including their potential use for the identification of super shedder animals.

In this study, the quantitative PCR approach of Lawal et al. (2015) for quantifying *E. coli* O157 and O26 in bovine RAJ samples was adapted and applied to sheep RAJ swabs. Sheep RAJ samples may differ from those of the bovine RAJ in terms of the microbiome and matrix so it was necessary to apply and develop a new standard calibration curve relating the real time PCR cycle threshold (Ct) values against the initial concentration of pathogen in the sheep samples. The aim of this study was therefore to validate that this approach was suitable for the rapid quantification of serogroups O157 and O26 and identification of super shedders in sheep populations.

Recto-anal swabs were collected from sheep at a commercial sheep abattoir following slaughter and before dressing. Duplicate foam over cotton swabs (VWR, International Ltd. Dublin), were pre-soaked in 10 mL of maximum recovery diluent (MRD) (Oxoid, Basingstoke, UK) and inserted approximately 3–5 cm into the RAJ of each animal using a rapid in and out motion, and stored at 5 °C during the transportation to the laboratory. One of the RAJ samples was tested to confirm absence of natural occurring *E. coli* O157 or O26 and in those confirmed as negative, the duplicate RAJ swab sample was used in the method development. The swab was placed in 29.7 mL of modified tryptone soya broth (Oxoid, Basingstoke, UK) supplemented with novobiocin (16 mg/L) (mTSBn) (Oxoid, Basingstoke, UK) and inoculated with 300 µl of an *E. coli* O157 or *E. coli* O26 strain (Table 1) at a range of different concentrations from Log_{10} 1 to 9 CFU $swab^{-1}$. The study included three replicates of the dilutions for each strain (O157s1-s3 and O26s1-s3). The inoculation counts were confirmed by plate counts on Rhamnose MacConkey agar supplemented with cefiximine tellurite (CT-RMAC) (Oxoid, Basingstoke, UK) for serogroup O26 and Sorbitol MacConkey

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Table 1
E. coli O157 and O26 strain used to inoculate sheep recto-anal junction swabs.

Serogroup	Strain id	*vtx1 / vtx2 / eae* pattern	Reference
O157	O157s1	*vtx 1-/vtx2-/eae+*	NCTC12900
O157	O157s2	*vtx 1 + /vtx2 + /eae +*	ATCC35150
O157	O157s3	*vtx 1 + /vtx2 + /eae +*	ATCC43895
O26	O26s1	*vtx 1 + /vtx2 + /eae +*	CDC03-3014
O26	O26s2	*vtx 1 + /vtx2 + /eae +*	Thomas et al. (2012)
O26	O26s3	*vtx 1 + /vtx2 + /eae +*	Thomas et al. (2012)

agar supplemented with CT for serogroup O157 (CT-SMAC) (Oxoid, Basingstoke, UK).

The RAJ samples were incubated for 5 h at 41.5 °C followed by total genomic DNA extraction protocol using InstaGene Matrix (Bio-Rad, Hercules, CA). Briefly, 1 ml of the enriched cultures were centrifuged at 10,500 ×*g* for 10 min and the pellets washed with phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK), followed by another cycle of centrifugation. The sample pellet was resuspended in 100 µl of the commercial chelex-based solution and heated at 56 °C for 30 min and then at 95 °C for 15 min. The extracted DNA was centrifuged for 5 min at 15,000 ×*g* to eliminate cell debris and then examined by the real time PCR method using the protocol of Lawal et al. (2015).

The results obtained were used to develop standard calibration curves relating the PCR Ct value and the inoculum levels of *E. coli* O157 or O26 (Fig. 1). A good linear relationship was shown for both O157 (R^2 = 0.80) and O26 (R^2 = 0.84). The range of quantification for STEC O157 was Log_{10} 1.3–8.7 CFU swab^{-1} and Log_{10} 1.0–8.1 CFU swab^{-1} for STEC O26. The performance of the generated standard curves was validated by analysing additional RAJ samples (n = 60) inoculated with *E. coli* O157 (strain O157s1) or O26 (strain O26s1) and plotting the predicted counts obtained from the developed standard calibration curves against the inoculum level. Fig. 2 shows the correlation was good with r^2 = 0.83 for *E. coli* O157 and r^2 = 0.82 for *E. coli* O26, comparing favourably with the method of Lawal et al. (2015) for cattle (R^2 = 0.86 and 0.88 respectively).

In this study, we developed and evaluated the performance of a novel strategy to obtain quantitative data suitable to identify and enumerate STEC O26 and STEC O157 from sheep RAJ swab samples. RAJ samples have a low water content (Furet et al., 2009; Moriarty and Gilpin, 2014), combined with a complex microbiota (Huang et al., 2018) that can interfere with other molecular identification methods. In

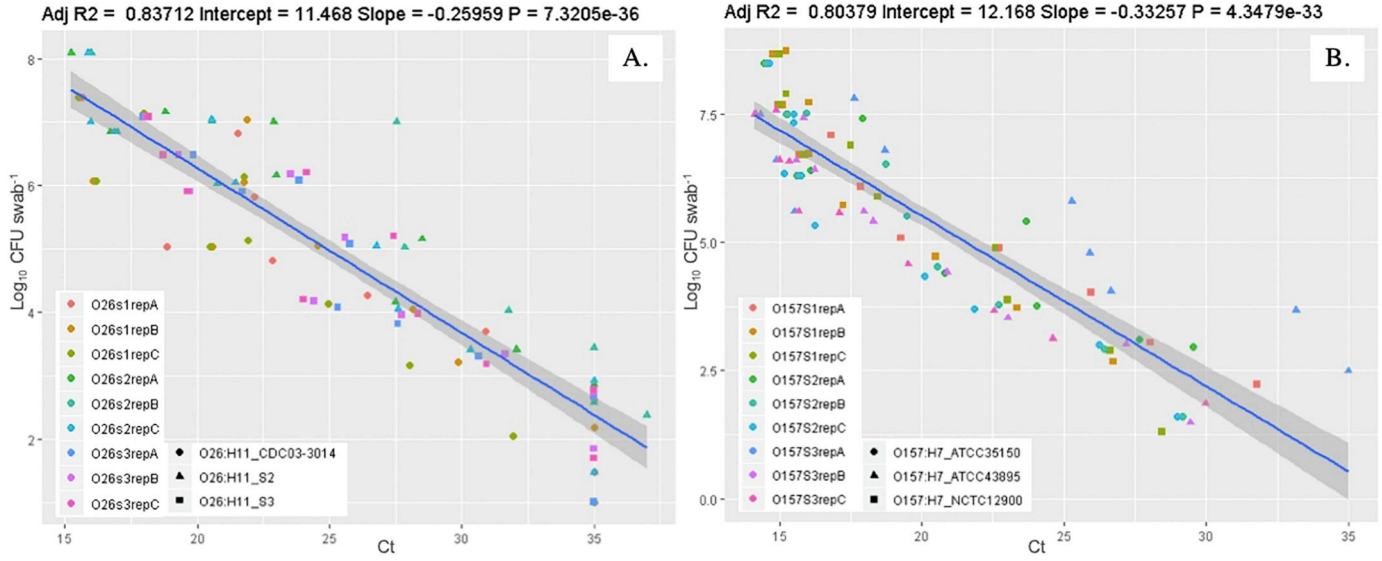

Fig. 1. Standard calibration curves relating the PCR C_t values and inoculum in sheep recto-anal junction swab samples for (A) STEC O26 and (B) O157.

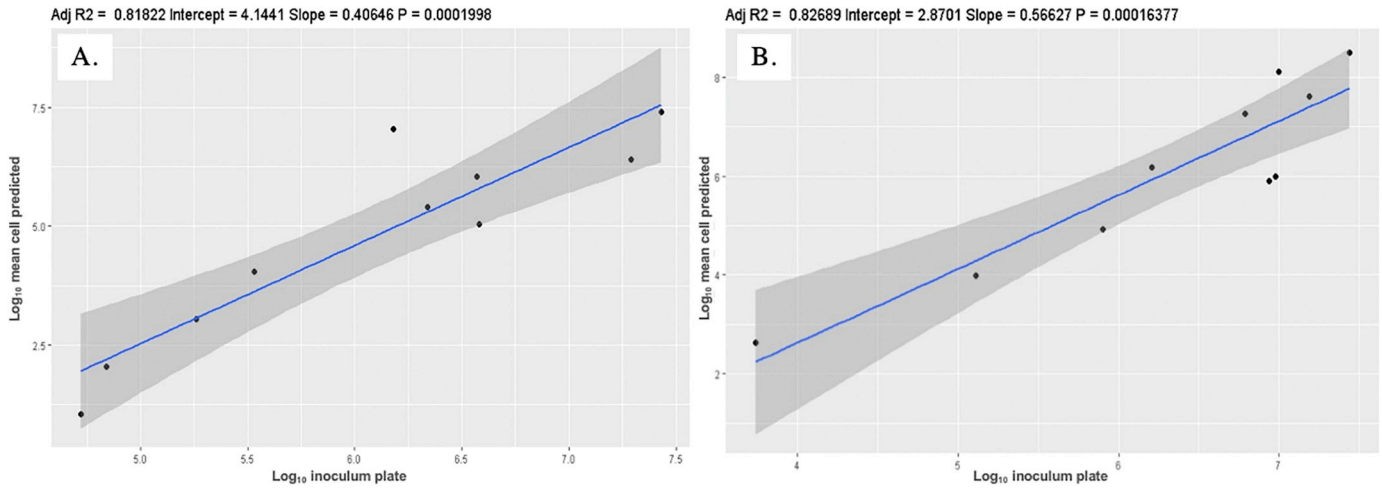

Fig. 2. Validation curve relating the predicted counts obtained from the standard calibration curve for sheep recto-anal junction swabs against the inoculum levels for (A) STEC O26 and (B) O157. The 10 plotted values correspond to the average of replicates of each inoculum level of the *E. coli* serogroup O157 (O157s1) (A) or *E. coli* serogroup O26 (O26s1) (A).

this method, the specificity of the primers and probes used for the qPCR allow the identification of two serogroups, with a range of quantification for STEC O26 of Log_{10} 1.0–8.1 CFU swab^{-1}, and Log_{10} 1.3–8.7 CFU swab^{-1} for STEC O157. The equation of the line generated can be used to predict the initial concentration of *E. coli* O26 or O157 from sheep RAJ samples. The method will support studies on shedding dynamics of these serogroups in sheep populations and could facilitate the screening for super-shedding animals ($\geq 10^4$ CFU swab^{-1}).

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