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Regional Differences in Presence of Shiga toxin-producing *Escherichia coli* Virulence-Associated Genes in the Environment in the North West and East Anglian regions of England

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SIGNIFICANCE AND IMPACT OF STUDY (100 words)

Several outbreaks within the UK have highlighted the danger of contracting Shiga toxin-producing *E. coli* from contact with areas recently vacated by livestock. This is more likely to occur for STEC infections compared to other zoonotic bacteria given the low infectious dose required. While studies have determined the prevalence of STEC within farms and petting zoos, determining the risk to individuals enjoying recreational outdoor activities that occur near where livestock may be present is less researched. This study describes the prevalence with which *stx* genes, indicative of STEC bacteria, were found in the environment in the English countryside.

ABSTRACT

Shiga toxin-producing *E. coli* is carried in the intestine of ruminant animals, and outbreaks have occurred after contact with ruminant animals or their environment. The presence of STEC virulence genes in the environment was investigated along recreational walking paths in the North West and East Anglia regions of England. 720 boot sock samples from walkers' shoes were collected between April 2013 and July 2014. Multiplex PCR was used to detect *E. coli* based on the amplification of the *uid*A gene and investigate STEC-associated virulence genes *eae*A, *stx*1 and *stx*2*.* The *eae*A virulence gene was detected in 45.5% of the samples while *stx*1 and/or *stx*2 was detected in 12.4% of samples. There was a difference between the two regions sampled, with the North West exhibiting a higher proportion of positive boot socks for *stx* compared to East Anglia. In univariate analysis, ground conditions, river flow and temperature were associated with positive boot socks. The detection of *stx* genes in the soil samples suggests that STEC is present in the English countryside and individuals may be at risk for infection after outdoor activities even if there is no direct contact with animals.

KEYWORDS Shiga toxin-producing *E. coli*, virulence genes, environmental sampling, multiplex PCR, boot socks, transmission routes **(6)**

INTRODUCTION

Shiga toxin-producing *E. coli* cause diarrhea, often bloody, that can progress to anaemia and kidney failure. An estimated 30% of cases require hospitalization during the course of infection (Byrne et al., 2015). In England, there is an average of 900 cases a year identified by Public Health England, but the real number of infections is probably much higher (Adams et al., 2016). The first outbreak of STEC was attributed to ground beef patties at a fast food restaurant chain in the United States in 1982 (Riley et al., 1983), and meat products are still associated with causing many STEC outbreaks (Heiman et al., 2015, Adams et al., 2016). However, several outbreaks have been attributed to the presence of people on fields recently vacated by ruminant animals (Crampin et al., 1999, Howie et al., 2003). Additionally, all published case-control studies performed on sporadic infections within the UK have identified contact with animals or their environments as a significant contributor to sporadic infections (Kintz et al., 2017). A recent study reports significant associations between livestock density and the spatial distribution of STEC infections in England (Elson et al., 2018). All this raises interesting questions about the likelihood of acquiring a STEC infection by participating in recreational outdoor activities in the countryside.

Cows and other ruminant animals are able to carry STEC asymptomatically as part of their normal intestinal flora since their epithelial cells lack the receptors for internalising the Shiga toxin. Studies on cows in the UK have demonstrated that, at any time, 4-15% of the animals in a herd may be carrying STEC (Chapman et al., 1997, Mechie et al., 1997, Omisakin et al., 2003, Paiba et al., 2003). The bacteria can survive in cow pats up to 21 months (Kudva et al., 1998, Hutchison et al., 2005, Fremaux et al., 2007), and within soil, STEC can be detected up to 200 days after inoculation (Maule, 2000, Jiang et al., 2002, Bolton et al., 2011). STEC requires only a small number of bacteria, between 10-100, to be ingested to cause illness (Tuttle et al., 1999, Strachan et al., 2002). Its extended survival time under different conditions and the low infectious dose increases the likelihood that individuals may become ill after encountering STEC in the environment.

STEC shares its zoonotic transmission with other gastrointestinal bacterial pathogens such as *Campylobacter* and *Salmonella*. Therefore, many techniques have been developed to screen for these pathogens within farms in an effort to curtail transmission from animals to consumers. One of these methods is using boot socks to cover the shoes and collecting samples from the floor or ground as the individual walks around the premises (Caldwell et al., 1998, Skov et al., 1999, McCrea et al., 2005). These boot socks can then be analysed for the presence of the pathogens, indicating colonized animals are present. This method has been demonstrated to be just as, if not more, sensitive than the older drag swab method developed for use on chicken farms (Buhr et al., 2007, Lungu et al., 2012). Boot socks have also recently been used to sample for pathogens in the wider environment (Brena et al., 2016, Jones et al., 2017). The aim of this research was to analyse boot socks generated during the Enigma project for *Campylobacter* to determine the frequency with which STEC-associated virulence genes were detected in the English countryside (Jones et al., 2017). Two different locations, the North West of England and East Anglia, encompassing different land uses, climates, and geographies, were chosen for the study (Jones et al., 2017).

RESULTS AND DISCUSSION

Presence of STEC virulence genes in boot sock samples

There were six walk locations: three in the North West and three in East Anglia. The walks were completed between April 2013 – July 2014, occurring every week from April through July and every three weeks during August through March, leading to a total of 40 walk dates for each location. For each walk, three different walkers wearing boot socks were present. This meant a total of 720 boot socks were collected (6 locations x 40 walks per location x 3 walkers). After the walks were completed, the walkers sent the boot socks in sterile plastic bags for processing. Further details on the walks and boot sock collection can be found in Jones *et al.,* 2017.

Multiplex PCR was used to detect three different STEC virulence genes: the shiga-toxins *stx*1 and *stx*2 and the intimin *eae*A as a marker for the locus of enterocyte effacement (LEE). This method was chosen as it has previously successfully detected the presence of STEC from a variety of different sources and would lend itself to quickly screening a large number of environmental samples (Deng and Fratamico, 1996, Paton and Paton, 1998a, Noll et al., 2015). The *uid*A gene was also included as an indicator that *E. coli* was present in the culture grown up from the frozen boot socks samples (McDaniels et al., 1996). Of the 720 samples, 592 (82.2%) of the samples amplified *uid*A*,* indicating *E. coli* had grown in the overnight culture. Only 14 samples amplified virulence genes (10 *eae*A and 4 *stx*2) in the absence of *uid*A; these were still included in the subsequent analyses given the low numbers of this occurrence compared to total boot sock samples. The breakdown of the virulence genes detected in the 720 boot socks samples is shown in Figure 1. In total, 45.5% of samples were positive for the *eae*A and 12.4% postive for either of the *stx* genes. For *stx* genes, *stx*2 was detected more often than *stx*1 with 9.2% of the samples positive for only *stx*2 and 2.1% of the samples positive for only *stx*1; 1.1% of the samples contained both *stx* genes. Furthermore, the majority of the *stx* positive samples were also positive for *eae*A, with only 2.2% of the samples containing only *stx*1 and/or *stx*2*,* but 10.1% of the samples containing either *stx* gene and *eae*A.

When considering only the *stx*-postive samples, a majority (83%) amplified the *stx*2 gene. Compared to *stx*1, presence of *stx*2 in STEC bacteria is associated with more severe disease and a higher likelihood to progress to haemolytic uremic syndrome (Boerlin et al., 1999, Tarr et al., 2005). Furthermore, 82% of *stx*-positive samples also amplified the *eae*A gene. STEC strains that carry the LEE pathogenicity island are associated with more severe disease (Paton and Paton, 1998b, Boerlin et al., 1999). If the virulence genes detected on these walks are associated with live bacteria, these results indicate there would be the possibility for these strains to cause potentially severe disease if individuals became infected.

While the use of culturing from the boot sock samples suggests that the detected virulence factors came from live bacteria, it does not necessarily mean that these bacteria are an immediate threat to human health. One caveat to the method of detection for the boot socks as performed in this study is that there is no manner to quantify the amount of pathogenic bacteria that may have been encountered during a walk or that the bacteria would be ingested by the individual. Another caveat is that the detection method used does not guarantee that the detected virulence genes came from the same bacteria; *eae*A is also associated with several other types of pathogenic *E. coli* that may have also transferred to the boot socks and grown under the culturing conditions. Additionally, it is possible that free *stx* phage was present on the boot sock and this then infected the *E. coli* growing in the overnight sample, leading to *stx*-positive samples that did not initially contain STEC bacteria.

Distribution of stx by region and walk location

As *eae*A is associated with other pathogenic *E. coli*, further characterization of the boot sock samples focused on the *stx* virulence genes. Overall, 89 of the 720 boot socks were positive for at least one *stx* gene. The results were broken down by region and walk location to see if there were any differences in the presence of the *stx* genes in the environment between the two regions. The regional analysis demonstrated that the number of *stx* positive boot socks was much lower in East Anglia region compared to the North West, with 0.8%-5.8% of the 120 boot socks per location demonstrating the presence of an *stx* gene in East Anglia compared to 15.8%-25% in the North West (Figure 2). As far as the number of walks that exhibited at least one *stx* positive boot sock, at least one of the walkers on 2.5%-15% of the walks in East Anglia walked through soil that later allowed for amplification of one of the *stx* genes. In the North West, this was between 32.5%-42.5% of the walks.

More *stx*-positive boot socks were found positive for the North West region compared to East Anglia. This correlates with the higher amount of livestock that are present in the North West compared to East Anglia (Elson et al., 2018) and also suggests that there is a significant reservoir for *stx*-containing bacteria in such environments. There are also more STEC infections in the North West compared to East Anglia (Byrne et al., 2015, Visham, 2019). It is reassuring for using boot socks as a sampling method that the results reflect both the trends in potential animal reservoirs and the recorded number of human infections.

Seasonality of STEC virulence genes in the environment

STEC infections exhibit a seasonal peak, with the number of human cases rising during summer months (Byrne et al., 2015). To see if the detection of the *stx* virulence factor in the environment followed the seasonal trends for STEC infections, the number of positive boot socks from each region was plotted against the month of the walks occurring (Figure 3). Too few boot socks in East Anglia were positive for *stx* to give any indication of seasonal differences of *stx* in the environment. However, the North West demonstrated an increase in the presence of *stx* over the summer months, with the number of positive boot socks dipping in October 2013 then increasing again in May 2014.

Environmental conditions associated with positive boot socks

Using information collected during the walks and data recorded from local weather stations on the days of the walks, regression analysis was used to determine if there was any association with positive boot socks and environmental conditions. In univariate analyses, state of the ground, mean river flow and mean daily temperature were associated with the number of positive boot socks per walk. In particular boggy ground and river flow were negatively associated with *stx* positive boot socks while the 7 day mean temperature was positively associated with *stx*-postive samples (Table 1). In the final model only mean daily temperature was significantly associated with the number of positive boot socks (IRR=1.20 [1.12-1.28], p < .001); the only other variable in the final model after removing all predictors with a p > 0.2 was mean river flow in the previous 7 days (IRR = 0.89 [0.777-1.02], p = 0.094).

Consistency of boot socks within walks

Since three different boot socks were worn during each walk, the number of walks where the boot socks all exhibited the same result (either all positive or all negative for *stx*) was calculated (Figure 4). 181 of all walks had zero positive boot socks while only 7 of the walks, all occurring in the North West, found all three boot socks positive. This means that less than a quarter of all walks (21.7%) exhibited variation in the presence or absence of STEC-related virulence factors on the boot socks collected.

Conclusions

Using multiplex PCR, we were able to successfully detect the virulence factors *eae*A and *stx* genes, associated with STEC, from boot socks collected during walks in the English countryside~~.~~ The samples for this study were initially collected to investigate the presence of *Campylobacter* in the environment (Jones et al., 2017). This initial analysis of the boot socks found 47.1% positive for *Campylobacter*. Similar to the results of our study, *Campylobacter*-positive boot socks were also detected more frequently in the North West over East Anglia. Overall, 41 (5.7%) of the 720 boot socks collected demonstrated the presence of *Campylobacter* and either the *eae*A and *stx* virulence genes. The results of both of these studies indicate that boot socks can be used successfully for collecting environmental samples across a wide sampling area and then used to detect multiple different pathogens. Given the convenience of this method, it is now being used by Public Health England to assist with sampling during outbreak investigations (McFarland et al., 2017).

MATERIALS AND METHODS

Preparation of samples from boot socks

Details on the choice of walking routes and use of citizen scientists for sample collection detailed in Jones *et al.* (Jones et al., 2017). For processing the boot socks, 100 ml of room temperature buffered peptone water was added to the sterile sample bag the boot socks were received in. These were palpated to resuspend any material and 4 ml removed and frozen in cryovials. This was undertaken for all boot socks received during the original study period from April 2013 through July 2014.

Isolation of genomic DNA and multiplex PCR

The samples frozen in peptone-buffered water were thawed and 0.5 mls added to 4.5 mls modified Tryptone Soy Broth (Oxoid CM0989) to enrich for *E. coli*. Cultures were grown overnight at 37°C, 1 ml removed and the bacteria pelleted. Genomic DNA isolation was performed using Qiagen's DNA mini kit (Qiagen, Manchester UK). Primers were purchased from Sigma-Aldrich. The *uid*A primers were based on McDaniels et al., 1996 while the *eae*A and *stx* primers were from Son et al., 2014 (McDaniels et al., 1996, Son et al., 2014). Qiagen's HotStar Taq Plust Master Mix kit, supplemented to 2.5mM MgCl2, was used to perform all multiplex PCR reactions according to manufacturer's directions using 0.2 ul of gDNA. Cycling conditions included an initial 10 min at 95°C to activate the polymerase followed by 25 cycles of 95°C for 1 minute, 54°C for 1 minute, and 72°C with an final extension step of 7 minutes at 72°C. Samples were run on 2% TAE gel electrophoresis with ethidium bromide and visualized with UVP's ChemiDoc-IT^2 imager. The expected sample sizes were 623 bp for *uid*A, 482 bp for *stx*2, 306 bp for *stx*1 and 245 bp for *eae*A.

Statistical analysis

During the walks, information was collected on the condition of the footpath and the number of livestock seen by the walkers. Information was also collected from local weather stations on the flow of nearby rivers and the average temperatures and amount of rainfall in the 7 days up to and including the day of the walk. These were used in a longitudinal panel negative binomial regression univariate analysis using the number of boot socks as count data. All variables with $p < 0.2$ in the univariate analysis were combined in a multiple variable analysis. Using a backwards step-wise process, variables were removed if $p > 0.2$ until only variables with $p < 0.2$ remained in the model. All statistical analyses were performed in STATA 14.

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CONFLICT OF INTEREST

No conflict of interest declared.

REFERENCES

Adams, N.L., Byrne, L., Smith, G.A., Elson, R., Harris, J.P., Salmon, R., Smith, R., O'brien, S.J., Adak, G.K. and Jenkins, C. (2016) Shiga Toxin-Producing Escherichia coli O157, England and Wales, 1983-2012. *Emerging Infectious Diseases* **22**, 590-597.

Boerlin, P., Mcewen, S.A., Boerlin-Petzold, F., Wilson, J.B., Johnson, R.P. and Gyles, C.L. (1999) Associations between virulence factors of Shiga toxin-producing Escherichia coli and disease in humans. *J Clin Microbiol* **37**, 497-503.

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. *J Appl Microbiol* **111**, 484-90.

Brena, M.C., Mekonnen, Y., Bettridge, J.M., Williams, N.J., Wigley, P., Sisay Tessema, T. and Christley, R.M. (2016) Changing risk of environmental Campylobacter exposure with emerging poultry production systems in Ethiopia. *Epidemiol Infect* **144**, 567-75.

Buhr, R.J., Richardson, L.J., Cason, J.A., Cox, N.A. and Fairchild, B.D. (2007) Comparison of four sampling methods for the detection of Salmonella in broiler litter. *Poult Sci* **86**, 21-5.

Byrne, L., Jenkins, C., Launders, N., Elson, R. and Adak, G.K. (2015) The epidemiology, microbiology and clinical impact of Shiga toxin-producing Escherichia coli in England, 2009-2012. *Epidemiol Infect* **143**, 3475-87.

Caldwell, D.J., Hargis, B.M., Corrier, D.E. and Deloach, J.R. (1998) Frequency of isolation of Salmonella from protective foot covers worn in broiler houses as compared to drag-swab sampling. *Avian Dis* **42**, 381-4.

Chapman, P.A., Siddons, C.A., Gerdan Malo, A.T. and Harkin, M.A. (1997) A 1-year study of Escherichia coli O157 in cattle, sheep, pigs and poultry. *Epidemiol Infect* **119**, 245-50.

Crampin, M., Willshaw, G., Hancock, R., Djuretic, T., Elstob, C., Rouse, A., Cheasty, T. and Stuart, J. (1999) Outbreak of Escherichia coli O157 infection associated with a music festival. *Eur J Clin Microbiol Infect Dis* **18**, 286-8.

Deng, M.Y. and Fratamico, P.M. (1996) A Multiplex PCR for Rapid Identification of Shiga-Like Toxin-Producing Escherichia coli O157:H7 Isolated from Foods (double dagger). *J Food Prot* **59**, 570-576.

Elson, R., Grace, K., Vivancos, R., Jenkins, C., Adak, G.K., O'brien, S.J. and Lake, I.R. (2018) A spatial and temporal analysis of risk factors associated with sporadic Shiga toxin-producing Escherichia coli O157 infection in England between 2009 and 2015. *Epidemiol Infect* **146**, 1928-1939.

Fremaux, B., Delignette-Muller, M.L., Prigent-Combaret, C., Gleizal, A. and Vernozy-Rozand, C. (2007) Growth and survival of non-O157:H7 Shiga-toxin-producing Escherichia coli in cow manure. *J Appl Microbiol* **102**, 89-99.

Heiman, K.E., Mody, R.K., Johnson, S.D., Griffin, P.M. and Gould, L.H. (2015) Escherichia coli O157 Outbreaks in the United States, 2003-2012. *Emerg Infect Dis* **21**, 1293-1301.

Howie, H., Mukerjee, A., Cowden, J., Leith, J. and Reid, T. (2003) Investigation of an outbreak of Escherichia coli O157 infection caused by environmental exposure at a scout camp. *Epidemiol Infect* **131**, 1063-9.

Hutchison, M.L., Walters, L.D., Avery, S.M., Munro, F. and Moore, A. (2005) Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. *Appl Environ Microbiol* **71**, 1231-6.

Jiang, X., Morgan, J. and Doyle, M.P. (2002) Fate of Escherichia coli O157:H7 in manure-amended soil. *Appl Environ Microbiol* **68**, 2605-9.

Jones, N.R., Millman, C., Van Der Es, M., Hukelova, M., Forbes, K.J., Glover, C., Haldenby, S., Hunter, P.R., Jackson, K., O'brien, S.J., Rigby, D., Strachan, N.J.C., Williams, N., Lake, I.R. and Consortium, E. (2017) Novel Sampling Method for Assessing Human-Pathogen Interactions in the Natural Environment Using Boot Socks and Citizen Scientists, with Application to Campylobacter Seasonality. *Appl Environ Microbiol* **83**, e00162-17.

Kintz, E., Brainard, J., Hooper, L. and Hunter, P. (2017) Transmission pathways for sporadic Shiga-toxin producing E. coli infections: A systematic review and meta-analysis. *Int J Hyg Environ Health* **220**, 57-67.

Kudva, I.T., Blanch, K. and Hovde, C.J. (1998) Analysis of Escherichia coli O157:H7 survival in ovine or bovine manure and manure slurry. *Appl Environ Microbiol* **64**, 3166-74.

Lungu, B., Waltman, W.D., Berghaus, R.D. and Hofacre, C.L. (2012) Comparison of a real-time PCR method with a culture method for the detection of Salmonella enterica serotype enteritidis in naturally contaminated environmental samples from integrated poultry houses. *J Food Prot* **75**, 743-7.

Maule, A. (2000) Survival of verocytotoxigenic Escherichia coli O157 in soil, water and on surfaces. *Symp Ser Soc Appl Microbiol*, 71S-78S.

Mccrea, B.A., Norton, R.A., Macklin, K.S., Hess, J.B. and Bilgili, S.F. (2005) Recovery and genetic similarity of Salmonella from broiler house drag swabs versus surgical shoe covers. *Journal of Applied Poultry Research* **14**, 694-699.

Mcdaniels, A.E., Rice, E.W., Reyes, A.L., Johnson, C.H., Haugland, R.A. and Stelma, G.N., Jr. (1996) Confirmational identification of Escherichia coli, a comparison of genotypic and phenotypic assays for glutamate decarboxylase and beta-D-glucuronidase. *Appl Environ Microbiol* **62**, 3350-4.

Mcfarland, N., Bundle, N., Jenkins, C., Godbole, G., Mikhail, A., Dallman, T., O'connor, C., Mccarthy, N., O'connell, E., Treacy, J., Dabke, G., Mapstone, J., Landy, Y., Moore, J., Partridge, R., Jorgensen, F., Willis, C., Mook, P., Rawlings, C., Acornley, R., Featherstone, C., Gayle, S., Edge, J., Mcnamara, E., Hawker, J. and Balasegaram, S. (2017) Recurrent seasonal outbreak of an emerging serotype of Shiga toxin-producing Escherichia coli (STEC O55:H7 Stx2a) in the south west of England, July 2014 to September 2015. *Euro Surveill* **22**, pii 30610.

Mechie, S.C., Chapman, P.A. and Siddons, C.A. (1997) A fifteen month study of Escherichia coli O157:H7 in a dairy herd. *Epidemiol Infect* **118**, 17-25.

Noll, L.W., Shridhar, P.B., Dewsbury, D.M., Shi, X., Cernicchiaro, N., Renter, D.G. and Nagaraja, T.G. (2015) A Comparison of Culture- and PCR-Based Methods to Detect Six Major Non-O157 Serogroups of Shiga Toxin-Producing Escherichia coli in Cattle Feces. *PLoS One* **10**, e0135446.

Omisakin, F., Macrae, M., Ogden, I.D. and Strachan, N.J. (2003) Concentration and prevalence of Escherichia coli O157 in cattle feces at slaughter. *Appl Environ Microbiol* **69**, 2444-7.

Paiba, G.A., Wilesmith, J.W., Evans, S.J., Pascoe, S.J., Smith, R.P., Kidd, S.A., Ryan, J.B., Mclaren, I.M., Chappell, S.A., Willshaw, G.A., Cheasty, T., French, N.P., Jones, T.W., Buchanan, H.F., Challoner, D.J., Colloff, A.D., Cranwell, M.P., Daniel, R.G., Davies, I.H., Duff, J.P., Hogg, R.A., Kirby, F.D., Millar, M.F., Monies, R.J., Nicholls, M.J. and Payne, J.H. (2003) Prevalence of faecal excretion of verocytotoxigenic Escherichia coli O157 in cattle in England and Wales. *Vet Rec* **153**, 347-53.

Paton, A.W. and Paton, J.C. (1998a) Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. *J Clin Microbiol* **36**, 598-602.

Paton, J.C. and Paton, A.W. (1998b) Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. *Clin Microbiol Rev* **11**, 450-79.

Riley, L.W., Remis, R.S., Helgerson, S.D., Mcgee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A. and Cohen, M.L. (1983) Hemorrhagic colitis associated with a rare Escherichia coli serotype. *N Engl J Med* **308**, 681-5.

Skov, M.N., Carstensen, B., Tornoe, N. and Madsen, M. (1999) Evaluation of sampling methods for the detection of Salmonella in broiler flocks. *J Appl Microbiol* **86**, 695-700.

Son, I., Binet, R., Maounounen-Laasri, A., Lin, A., Hammack, T.S. and Kase, J.A. (2014) Detection of five Shiga toxin-producing Escherichia coli genes with multiplex PCR. *Food Microbiol* **40**, 31-40.

Strachan, N.J., Dunn, G.M. and Ogden, I.D. (2002) Quantitative risk assessment of human infection from Escherichia coli O157 associated with recreational use of animal pasture. *Int J Food Microbiol* **75**, 39-51.

Tarr, P.I., Gordon, C.A. and Chandler, W.L. (2005) Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome. *Lancet* **365**, 1073-86.

Tuttle, J., Gomez, T., Doyle, M.P., Wells, J.G., Zhao, T., Tauxe, R.V. and Griffin, P.M. (1999) Lessons from a large outbreak of Escherichia coli O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol Infect* **122**, 185-92.

Visham, B.a.B., L. (2019) Shiga toxin-producing Escherichia coli (STEC) data: 2017. *Public Health England* [Online]. Available: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/774291/STEC_O157_report.pdf.https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/774291/STEC_O157_report.pdf.

FIGURE LEGENDS

Figure 1: Number of positive samples from multiplex PCR for STEC virulence factors. Multiplex PCR was used to screen for the *eae*A, *stx*1, and *stx*2 virulence factors from 720 boot sock samples.

Figure 2: Percentage of boot socks positive for *stx*. A) Percentage of all boot socks positive for *stx* by walk location (n=120 boot socks) and region (n=360). **B)** Percentage of walks with at least one *stx*-positive boot sock by walk location (n=40) and region (n=120).

Figure 3: Seasonality of positive boot socks. Percentage of boot socks positive for *stx* within the two different study regions. Solid lines underneath the dates indicate times when walks were performed weekly. Dashed lines indicate periods of time when walks were undertaken every three weeks. Black line = North West region; Gray line = East Anglia region.

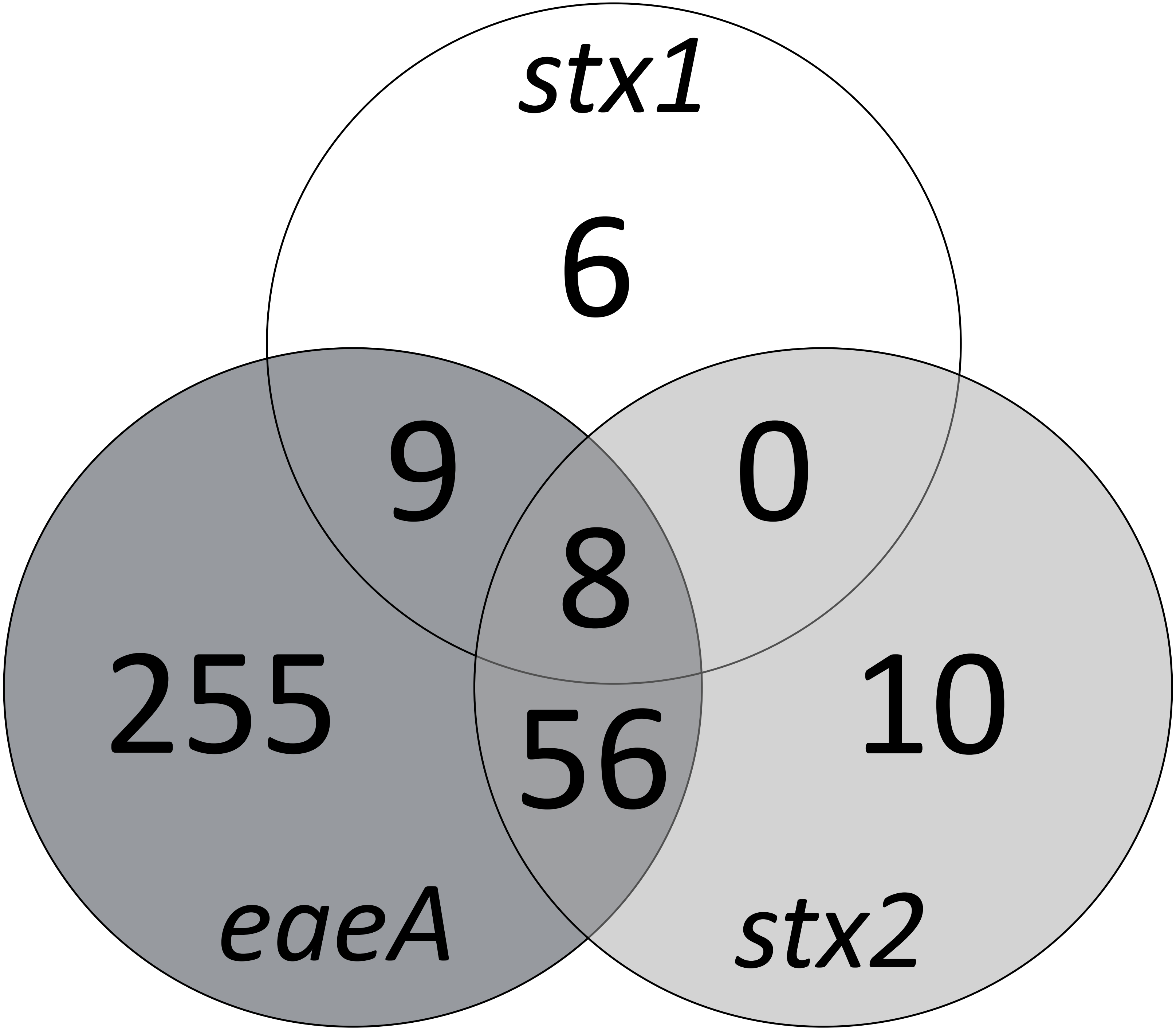
Figure 4: Internal consistency in number of positive boot socks in individual walks. Since three boot socks were collected on each walk, the internal consistency based on the number of boot socks positive for *stx* was determined. Total walk number = 240.

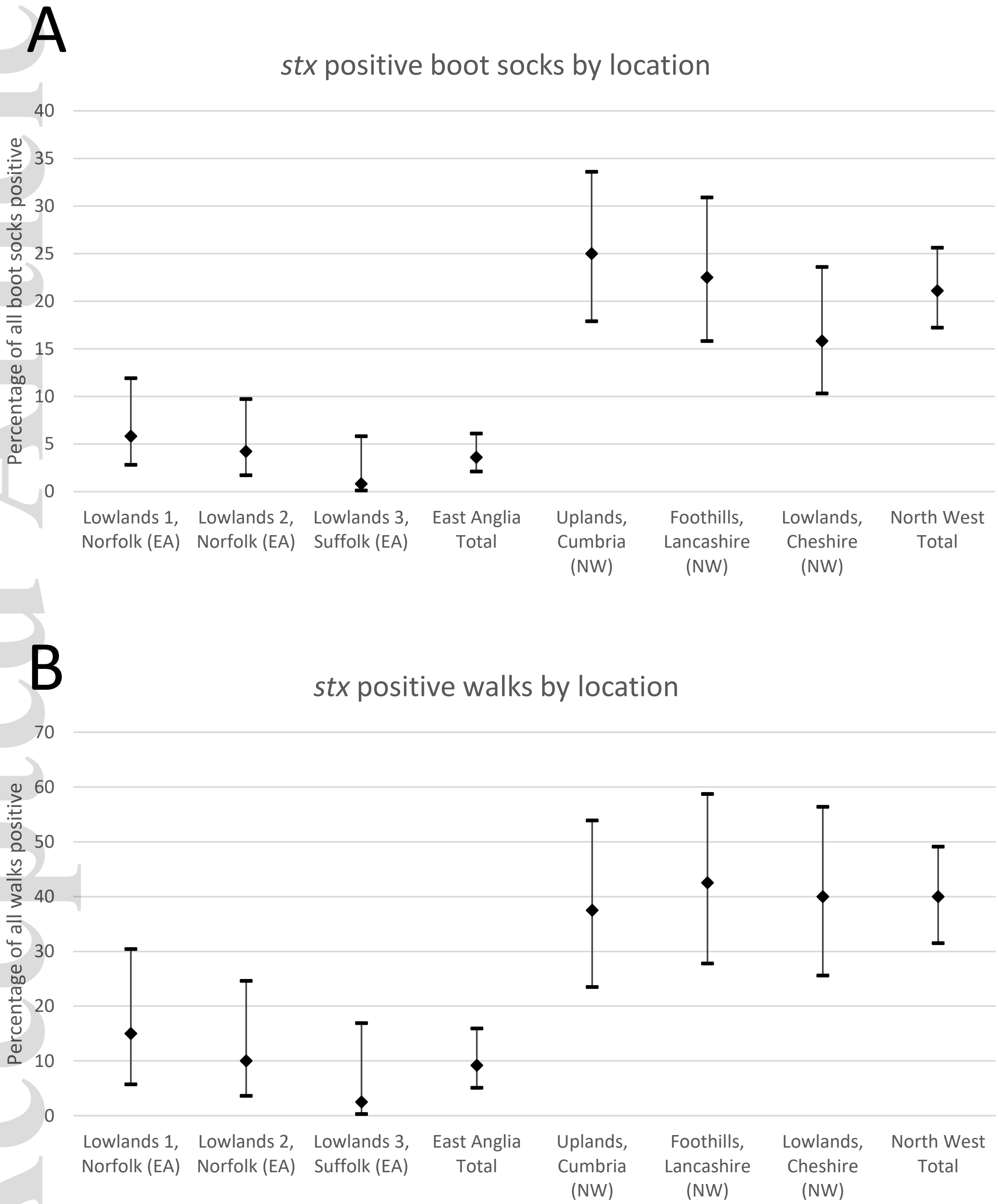
TABLES

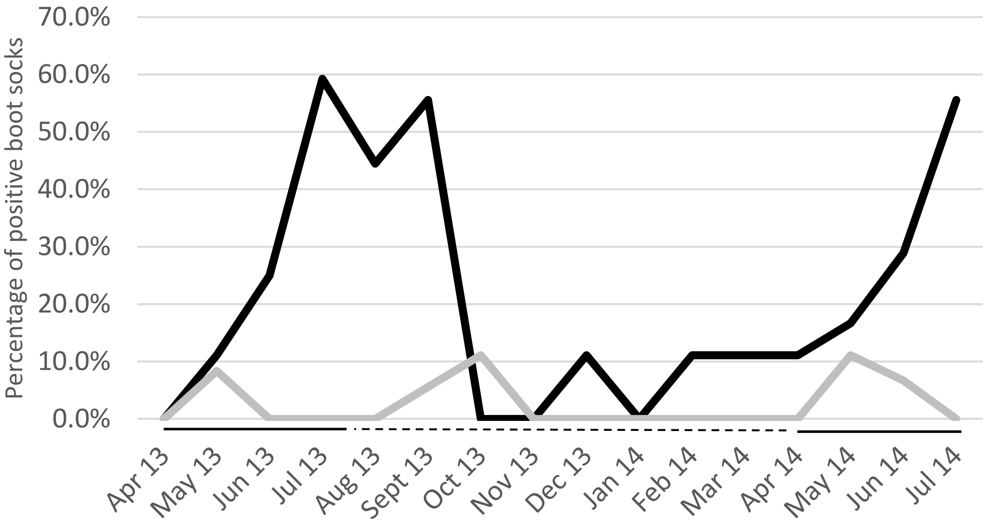
Table 1: Results of negative binomial regression of number of boot socks per walk positive for *stx.*

		Univariate Analysis			
Variable		IRR	L95%CI	U95%CI	p
Ground	Dry	1			0.012
	Wet	0.845	0.493	1.449	
	Boggy	0.349	0.173	0.703	
Weather	Dry	1			0.099

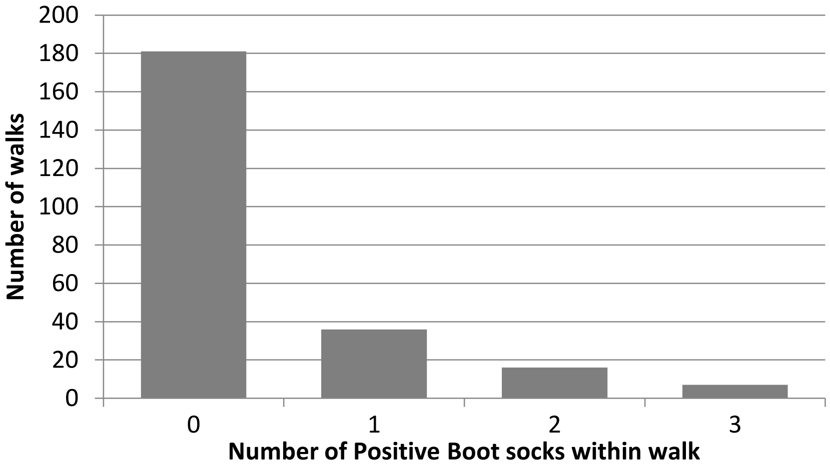
	Rain	0.527	0.246	1.128	
People	0	1			0.29
	1-10	1.132	0.583	2.198	
	>10	1.802	0.776	4.188	
Sheep	0	1			0.098
	1-10	2.606	0.595	11.409	
	>10	4.008	1.134	14.166	
Cows	0	1			0.408
	1-10	1.778	0.766	4.127	
	>10	1.141	0.467	2.786	
Horses	0	1			0.117
	1-10	1.752	0.78	3.939	
	>10	3.583	1.07	12.001	
Muck	N	1			0.368
	Y	1.393	0.677	2.868	
Mean daily precipitation in previous 7 d	mm	0.961	0.869	1.062	0.434
Mean river flow in previous 7 d	M^3/s	0.764	0.632	0.924	0.005
Mean daily temperature in previous 7 d	°C	1.215	1.141	1.294	<0.0001


stx1
6
9
0
8
255
56
10
eaeA
stx2


A
stx positive boot socks by location
Percentage of all boot socks positive
0
5
10
15
20
25
30
35
40
Lowlands 1, Norfolk (EA)
Lowlands 2, Norfolk (EA)
Lowlands 3, Suffolk (EA)
East Anglia Total
Uplands, Cumbria (NW)
Foothills, Lancashire (NW)
Lowlands, Cheshire (NW)
North West Total
B
stx positive walks by location
Percentage of all walks positive
0
10
20
30
40
50
60
70
Lowlands 1, Norfolk (EA)
Lowlands 2, Norfolk (EA)
Lowlands 3, Suffolk (EA)
East Anglia Total
Uplands, Cumbria (NW)
Foothills, Lancashire (NW)
Lowlands, Cheshire (NW)
North West Total



Percentage of positive boot socks
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
Apr 13
May 13
Jun 13
Jul 13
Aug 13
Sept 13
Oct 13
Nov 13
Dec 13
Jan 14
Feb 14
Mar 14
Apr 14
May 14
Jun 14
Jul 14


200
180
160
140
120
100
80
60
40
20
0
Number of walks
0
1
2
3
Number of Positive Boot socks within walk