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A Microbiological Survey of Minced Beef at Retail in Scotland

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Scotland's Rural College

In collaboration with

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Scottish Microbiological Reference Laboratories

and the

Content

Glossary

ACC	Aerobic colony count, a quantitative estimate of the concentration of (in this work) bacteria in a sample. This count indicates the number of colony forming units (cfu) per g of the sample
ADR	European Agreement of 30 September 1957 concerning the International Carriage of Dangerous Goods by Road
AMR(G)	Antimicrobial resistance (gene)
AST	Antibiotic Sensitivity Testing
BD	Bloody Diarrhoea
BLOD	Below limit of detection
BS	A standard that's released in British English by the British Standards Institute
BPW	Buffered Peptone Water
CC	Clonal Complex - Sequence Types (ST) that are all thought to derive from the same founding genotype
CFU	Colony Forming Unit
C.I.	Confidence interval
CL3	Containment Level 3
Ct	The threshold cycle - this is the cycle number measured at the intersection of the amplification curve of PCR product and threshold of detection
D	Diarrhoea
DLV	Double locus variant
E. coli (O157)	*Escherichia coli* (serogroup O157)
ERU	Epidemiology Research Unit
ESBL	Extended-spectrum β-lactamase
ESS	Edinburgh Scientific Services
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
FSS	Food Standards Scotland
GA	Geographic Area
GBRU	Gastrointestinal Bacteria Reference Unit
generic *E. coli*	generic *Escherichia coli* are bacteria that form part of the microbial flora that are resident in the bovine gastrointestinal tract.
GSS	Glasgow Scientific Services
HPS	Health Protection Scotland
HUS	Haemolytic Uraemic Syndrome
IMS	Immunomagnetic separation
JEMRA	Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment
LIMS	Laboratory Information Management System
Mac	MacConkey agar
MDR	MultiDrug Resistant – generally refers to resistance to 3 or more antibiotic classes
MLST and (cg)-MLST	MultiLocus Sequence Type - a typing approach for assessing the relatedness of strains that uses the sequences of internal fragments of

	seven house-keeping genes. Core-genome (cg)-MLST is a high resolution version that uses a comprehensive set of loci (for *E. coli* n=2513; for *Salmonella*, n = 3002).
Non-O157 STEC	This term describes Shiga toxin-producing *E. coli* (STEC) serogroups other than serogroup O157, for example *E. coli* O26 and *E. coli* O103.
NSF	Non-Sorbitol Fermenter – an *E. coli* strain that does not ferment sorbitol, for example, *E. coli* O157:H7
PCR	Polymerase chain reaction is a widely used molecular biology technique to amplify and detect DNA and RNA sequences.
PHE	Public Health England
rfb$_{O157}$	Gene encoding the O-antigen of *E. coli* O157
SERL	Scottish *E. coli* O157/STEC Reference Laboratory, based at SMiRL (Edinburgh) in NHS Lothian.
Serogroup	This is based on the presence of a specific "O" antigen in the *E. coli* strain under investigation. O157 is an example of a serogroup of *E. coli*; O26 is another serogroup.
Serotype	This is based on the combination of an "O" and "H" antigen. O157:H7 is an *E. coli* serotype; O103:H2 is another serotype.
Serovar	Equivalent of serotype in *Salmonella.* Also determined by combination of "O" antigen and "H" antigen.
SF	Sorbitol Fermenter – an *E. coli* strain that ferments sorbitol. Many *E. coli* strains, other than serotype *E. coli* O157:H7, ferment sorbitol. These include harmless *E. coli* and also some non-O157 STEC strains.
SHPN	Scottish Health Protection Network
SLV	Single locus variant
SMiRL	Scottish Microbiology Reference Laboratories
SNP	Single nucleotide polymorphism
(CT) SMac	Sorbitol MacConkey agar (with cefixime and tellurite)
SRUC	Scotland's Rural College
SS	Scientific Services
SSC	Sample size calculations
SSSCDRL	Scottish *Salmonella, Shigella* and *C. difficile* Reference Laboratory, based at SMiRL (Glasgow) in NHS Greater Glasgow & Clyde.
ST	Sequence Type - is assigned to an organism following 7-gene MLST. No ST is provided when using the cgMLST scheme in BioNumerics.
STEC	Shiga toxin-producing *Escherichia coli*. Previously, Shiga toxin-producing *E. coli* (STEC), were referred to as Verotoxigenic *E. coli* (VTEC), however there has been a widespread move to standardise the nomenclature across Europe to STEC (the preferred term in Northern America). While the two terms are interchangeable, for the purposes of this report, the term STEC will be used. Where referring to historic documentation, the original term will be retained.
Stx	Shiga toxin
stx	Shiga toxin gene
TP	True prevalence
TSS	Tayside Scientific Services
U of A	University of Aberdeen

UA	Unitary Authority
UKAS	United Kingdom Accreditation Service
wgMLST	Whole genome MultiLocus Sequence Typing
WGS	Whole genome sequencing
WHO	World Health Organization

Executive summary

Why we did this study:
The consumption of beef mince that is contaminated with pathogenic bacteria is a known cause of foodborne illness. Despite this association, information about the overall microbiological quality of beef mince on retail sale in Scotland is limited. If it were available, such information could be used to support Scottish businesses in the reduction of the risk of microbiological contamination in the food chain, inform consumers about safe handling, and thus contribute to reducing the likelihood of foodborne illness in the Scottish population.

Inappropriate handling of fresh retail beef mince by consumers after purchase raises the potential risk of becoming unwell, as does insufficient cooking. The growing restaurant trend for rare (less than thoroughly cooked) burgers may encourage consumers to experiment in their own kitchens and cook their own burgers rare, further increasing the potential risk. In order to assess the risk that may be posed to the consumer, an understanding of the baseline microbiological quality of fresh beef mince from retail outlets in Scotland is required. Such information would be useful for risk assessment, risk management and risk communication about the safety of this commodity. It could also improve our understanding of the presence of antimicrobial resistance (AMR) in the beef production chain.

In order to fill this evidence gap, provide underpinning evidence to support management of future incidents, and add to existing surveillance of AMR in Scotland, a survey was designed to characterise the microbiological quality of fresh beef mince sold at retail outlets in Scotland during 2019.

The survey had the following objectives:

- The primary objective was to generate baseline data on the frequency of the three significant microbiological pathogens (*Campylobacter*, *Salmonella* and Shigatoxigenic *E. coli* (STEC), and two process hygiene indicator organisms (counts of generic *E. coli* and Aerobic Colony Count (ACC)) present in fresh minced beef on sale to the consumer in Scotland from retail outlets.
- The second objective was to determine the antimicrobial sensitivity of all the pathogens that were isolated and 100 of the generic *E. coli* that were found. The latter would be a subset of all the generic *E. coli* found.
- The third objective was to analyse any differences, such as geographic or seasonal variations, in microbiological quality, to allow the identification of any factors associated with an increased, or decreased likelihood of microbial contamination.

The regulatory context:

The food safety criteria of Regulation (EC) No. 2073/2005 are used to prevent distribution of foods which are potentially harmful to the health of consumers. Although the sampling for this study could not be done in exactly the same way as set down in the Regulation (e.g. samples were taken at a different point in the production chain), the regulatory limits can provide a useful reference against which to measure the survey findings. For example, Regulation (EC) No. 2073/2005 specifies that *Salmonella* should not be found in fresh minced meat products that are intended to be eaten cooked, when on sale during their shelf lives.

The process hygiene criteria of Regulation (EC) No. 2073/2005 are used to ensure that production processes are operating properly. One of these criteria is the generic *E. coli* count. This is an indicator of faecal contamination. Another of the process hygiene criteria, ACC, is a measure of the background microbiological status of meat. Bacteria that contribute to the ACC can include those from the animals, from the slaughterhouse, and from the meat processing environment. As this measure includes the organisms responsible for spoilage of meat, it will also give an indication of the keeping quality of the meat. Both the generic *E. coli* count and the ACC are used at the end of the production process to assess the hygiene status of the minced meat, produced from red meat, which is intended to be eaten cooked. However, because – as previously stated for food safety criteria - the survey sampling was not exactly the same as set out in the Regulation, the results of the testing provide data for information only. They cannot be used to formally assess compliance with the Regulation.

What we did:

The survey was designed to represent what the "Scottish consumer" typically purchases. A two-stage sampling design was used to reflect both variations in population density across Scotland and the market share of the different categories of retail outlets. The survey was conducted between January and December 2019, during which time, 1009 fresh beef mince samples were purchased from 15 geographical areas of Scotland. The samples that were collected came from the full range of different types of retail outlets in Scotland and included a range of the available product types (such as premium and value products, and variable proportions of fat content). Additional information that was available on the label was collected for use in the analysis.

Objective 1: prevalence

The samples were purchased and kept chilled (3°C ± 2°C) before being delivered to one of the three European Union (EU) Official Food Control Laboratories in the project team. Using UKAS-accredited methods, the samples were examined for the presence of three pathogens and two types of process hygiene indicator organisms. Each sample was tested for the presence of *Campylobacter* and *Salmonella*, and for the genes that indicate the possible presence of either STEC, or O157 *E. coli*. The genes that were looked for to indicate the presence of STEC were *stx*$_1$- all variants, *stx*$_2$ (a-e + g) and *stx*$_2$f. If a sample tested positive for any one of these *stx* genes it was designated as a "presumptive STEC positive" sample.

The gene that was looked for to indicate the presence of O157 *E. coli* was *rfb*$_{O157}$. If any of these STEC or O157 genes were detected, it did not necessarily mean that they came from living bacteria, so further testing would be needed elsewhere. Meanwhile, each sample was also tested for the process hygiene indicators, generic *E. coli* count and Aerobic Colony Count (ACC).

Further testing was carried out at Scotland's Rural College (SRUC) Inverness laboratory. Here, materials from the "presumptive STEC positive" samples and from the samples containing the *rfb*O157 gene were re-tested. They were cultured to see if any STEC or O157 *E. coli* would grow. If they did grow, then bacteria were isolated and tested by polymerase chain reaction (PCR) to confirm whether they were a *stx*-negative *E. coli* O157, or a STEC. There were two groups of STECs; those that were STEC O157 and those that were STEC non-O157s.

Objective 2: antimicrobial resistance (AMR)
All of the pathogens that were isolated, plus 100 of the generic *E. coli*, underwent antibiotic sensitivity testing (AST). One generic *E. coli* had been isolated from each mince sample in which generic *E. coli* could be counted. The 100 that went for AST were a randomly selected subset from all of these. The AST involved testing the pathogens and selected generic *E. coli* bacteria against a panel of antibiotics (active substances) by disc diffusion. This is a phenotypic method where the ability of a micro-organism to grow and multiply is tested in the presence of specified antimicrobials. If they are susceptible to the active substance they do not grow as well as they would if the antibiotic wasn't there. The pathogens and selected generic *E. coli* were also tested for susceptibility to an additional antibiotic, colistin, using a different phenotypic method known as minimum inhibitory concentration. All of these bacteria were also sent to the Scottish Microbiology Reference Laboratories for whole genome sequencing (WGS). This is a technique that determines the complete sequence of an organism's genome. The results from this technique were used to further differentiate and classify the bacteria and to identify if genes that are involved in producing resistance to antimicrobial substances (antimicrobial resistance (AMR) genes) were present.

Objective 3: patterns of variation
Statistical analysis can be used to identify whether observed patterns are due to chance or whether there is evidence for associations with measured factors. If evidence for associations exists then this allows questions, known as hypotheses, to be raised to direct further investigations. Information about when, where and how the samples were collected was combined with a range of information that was obtained about the product. For many samples this information came from the product label. After a range of initial statistical tests, selected factors were included in a type of statistical analysis known as logistic regression. This was used to see if the presence or absence of each of the pathogens, in a sample, was associated with the presence and number of the process hygiene indicator organisms, as well as any of the other selected factors.

What we found:

Objective 1: prevalence of pathogens

The frequency of occurrence (known as the apparent prevalence, i.e. the number of samples testing positive by the diagnostic tests divided by the total number of samples tested without taking the characteristics of the test methods into account) of each of the three significant microbiological pathogens (*Campylobacter*, *Salmonella*, and STEC) was found to be comparable with published results from other countries. The values for *Campylobacter*, *Salmonella* were at the lower end of the range seen in European studies. Direct comparisons are, however, difficult due to many factors such as the different sampling and diagnostic methods used, as well as the fact that studies were done at different time periods.

- The prevalence for *Campylobacter* was 0.1% (95% confidence interval (C.I.) 0 to 0.7%) with only one of the 1009 samples found to be positive.
- The prevalence for *Salmonella* was 0.3% (95% C.I. 0 to 1%) with only three samples that were found to be positive. This is a similar frequency to that of *Campylobacter*.
- The prevalence of confirmed STEC was higher than that of *Campylobacter* and *Salmonella*, at 4% (95% C.I. 2 to 5%), with 35 samples confirmed as STEC positive.

Earlier in the testing process, 226 presumptive STEC samples were identified. At between one in four and one in five of the samples (22% (95% C.I. 20 to 25%)), this frequency was higher than anticipated in the design phase of the survey by the project team, based on published literature and experience. However, it is important to highlight that this test is not equivalent to finding viable STEC bacteria, as demonstrated by the lower number of these samples (35) that were then found to be confirmed STEC positive samples. Of these 35, only 4 were STEC O157, and 31 were STEC non-O157s. From all of the 1009 samples, 13 were found that yielded *stx*-negative *E. coli* O157.

Objective 1: frequency of occurrence of the process hygiene indicator organisms

Most (n=716, 71%, 95% C.I. 66 to 75%) samples had levels of generic *E. coli* that were below the limit of detection. This means that the average (median) generic *E. coli* count for all 1009 samples was below the limit of detection (i.e. <10 colony forming units per gram (cfu/g)) of fresh beef mince). Only a small proportion of all the samples (n= 32, 3%, 95% C.I. 1 to 5%) had an ACC below the limit of detection (<400 cfu/g). The average (median) ACC was $6.4x10^5$ cfu/g of fresh beef mince.

Whole genome sequencing – context for the pathogens

Whole genome sequencing provided additional information about the pathogens that were isolated and how they fit into the overall Scottish context of the occurrence of these pathogens in humans and cattle. The two *Salmonella* serovars that were identified

(Mbandaka (two isolates) and Dublin (one isolate)) are commonly associated with cattle. One of the Mbandaka isolates was very similar to a cluster previously seen in Scottish cattle.

All of the STECs found are thought to have potential to cause clinical illness in humans as, by definition, they contain *stx* genes. However, more than two-thirds (24/35) of the STECs isolated were ranked as having the lowest risk potential for severe disease in humans, according to criteria set by the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA). Eight were in the top two categories of high, or highest risk. Similarities were seen between STEC isolates from the mince samples and those previously identified in Scottish human clinical cases. The sequencing also identified two O157:H7 STEC isolates that matched (100% similarity) those found in a contemporaneous clinical outbreak of foodborne illness in the United Kingdom (UK).

Objective 2: antimicrobial resistance (AMR)
The majority (92%,139/151) of isolates were susceptible to all the antibiotics that they were tested against. Evidence for AMR, as indicated by phenotypic non-susceptibility to one or more of the antibiotic active substances used in the AST, was identified in 12 of the 151 isolates tested. Evidence for AMR was most frequently found to single, commonly used, first-line active substances that have a long history of use in ruminant populations, such as tetracycline and ampicillin. None of the isolates were resistant to any of the critically important antimicrobials that were tested for by disc diffusion. The WGS helped to clarify the situation with regard to an apparent lack of phenotypic susceptibility to colistin for the *Salmonella* Dublin isolate. It was confirmed that this was not a cause for concern as there were no *mcr* genes present and this is a recognised phenomenon for this type of isolate.

Objective 3: patterns of variation
A survey – or cross-sectional study design – such as this one does not enable causal relationships to be established and results of statistical analyses always have to be interpreted with care. That is particularly the case here, given that the analyses were limited by both the sample size and the small number of pathogens identified. This may mean that associations exist but they could not be detected. Because of the limited number of *Campylobacter* and *Salmonella* bacteria found during the survey, no further statistical analyses are reported. The characteristics of the samples in which they were found have been described in the report in detail.

There was evidence that some biologically plausible factors were associated with the distribution of microorganisms in the samples. For example, there was an approximately three times increase in the likelihood (odds) of a sample being presumptive STEC positive when the generic *E. coli* count of a sample was above the limit of detection, rather than below it, having adjusted for other factors. However, after adjusting for other factors, if a sample was purchased during the winter season (January, February & December) then the sample was approximately half as likely to be presumptive STEC positive compared to samples

purchased in the spring (March, April & May). None of the factors recorded were found to be associated with the confirmed STEC status of a sample. However, if a sample was presumptive STEC positive, then it was less likely to be a confirmed STEC positive if the sample had higher generic *E. coli* counts. For each of the categories in which the generic *E. coli* count increased, the likelihood (odds) of a presumptive STEC positive sample being confirmed halved.

Conclusions, outcomes and why the findings matter:
In conclusion, this survey of the microbiological content of Scottish fresh minced beef on retail sale in Scotland during 2019 has:

- provided a baseline measure of the microbiological status of fresh minced beef on retail sale in Scotland.
    - This information fills a gap in our knowledge and evidence base and should help to inform risk assessors and risk managers.
- demonstrated that, while there is always room for improvement, current measures to ensure food safety along the supply chain - from farm to retail sale in Scotland - result in a product in which pathogens occur at a frequency that is comparable to that achieved in other European countries, according to the published literature.
    - The values for *Campylobacter*, *Salmonella* were at the lower end of the range seen in European studies.
- confirmed that because contamination with one of the three significant food-borne pathogens is found to occur at a frequency that is uncommon (*Campylobacter* and *Salmonella,* more than 1 but less than 10 in 1,000) to common (Shigatoxigenic *E. coli*: more than 1 but less than 10 an in 100) it is important to ensure that consumers are aware of the requirements to handle these products appropriately prior to consumption (i.e. hygienically in the kitchen and then thoroughly cooked).
- demonstrated that, at the point of retail sale, the frequency with which an indicator of faecal contamination (generic *E. coli* counts) can be detected in samples is much lower than that in which an indicator of the general background microbiological status of the meat, including spoilage organisms (ACC) can be detected.
    - Fresh beef mince on retail sale in Scotland in 2019 is not a sterile product.
- illustrated the value of WGS in facilitating both the placing of survey findings in the wider context of livestock and public health, and in the provision of finer detail about the bacterial isolates that were detected.

    - This confirms the value of integrating WGS into surveys and surveillance activities

- provided some reassurance that fresh beef mince on retail sale in Scotland is currently unlikely to be a major foodborne route for transmission of AMR to critically important antimicrobials from cattle to humans.

The survey has also produced an archive of the pathogenic isolates and generic *E. coli* that can be used for further study.

Lay summary

Eating raw or undercooked beef mince that has been contaminated with certain types of bacteria may cause humans to become ill. Bacteria that cause disease are known as 'pathogens'. The most common clinical signs of a food-borne illness include loose stools (diarrhoea) and/or vomiting, although there can be other clinical signs too. The degree of severity may vary from extremely mild and almost unnoticeable to a need to be hospitalised and ultimately death.

In 2019, we ran a survey to find out how often three important types of pathogens were found in fresh beef mince on sale from retail outlets in Scotland. The three pathogens were *Campylobacter, Salmonella* and Shiga toxin-producing *E. coli* (STEC). We also looked at two measures that give an indication of the hygiene levels of the meat production process. These measures were the generic *E. coli* count and the aerobic colony count (ACC) for each sample.

We purchased 1009 samples of fresh beef mince from retail outlets in 15 areas of Scotland during 2019. These samples were kept chilled and transported to accredited laboratories to be tested.

We tested each sample for the presence of *Campylobacter* and *Salmonella* bacteria. We found that one of the 1009 mince samples was positive for *Campylobacter* and three were positive for *Salmonella.* These frequencies of occurrence are at the lower end of the range seen in other European studies.

We tested each sample to see if we could detect the presence of STEC genetic material (*stx* genes). This test tells us if genes from STEC bacteria are present in a sample but not whether the sample contains STEC bacteria that are alive and can grow. If this first test was positive the sample was called a 'presumptive STEC positive'. If it was a presumptive STEC positive sample, we then did a second test to see if we could grow STEC bacteria. If we could do this then the sample was a 'confirmed STEC positive.' We found 226 presumptive STEC positive samples. In 35 of these the presence of STEC was confirmed. At 4% this gives us a higher frequency of occurrence for STEC in the 1009 samples than for *Campylobacter* and *Salmonella*. However, this value was in line with the results in other studies.

The number of samples in which the process hygiene indicator organisms was found was higher than for the pathogens. Generic *E. coli* provide an indication of contamination with gut contents (i.e. faecal contamination). These type of bacteria could not be counted in most of the samples (71%) due to being below the limit of detection. Another indicator, aerobic colony count (ACC), is a measure of the background microbiological status of meat. Bacteria that contribute to the ACC can include those from the animals, from the slaughterhouse, from the

meat processing environment and those responsible for spoilage of meat. ACC can, therefore, give an indication of the keeping quality of the meat. The ACC was above the limit of detection in most (97%) of the 1009 samples tested.

We tested 151 of the bacteria that we grew from the mince samples to see if they were sensitive to a range of antibiotics. If they were not susceptible this was considered to be evidence for antimicrobial resistance (AMR). Fifty-one of these 151 bacteria were pathogens and the other 100 were generic *E. coli*. We found that most (92%,139/151) of the bacteria we tested were susceptible to all of the antibiotics that they were tested against. Of the 12 bacteria in which we found evidence for resistance, more than half (7, 58%) were not susceptible to one antibiotic only. The rest were not susceptible to three antibiotics. The antibiotics to which evidence for resistance was identified were, generally, commonly used, first-line antibiotics that have a long history of use in cattle. We did not find evidence for resistance to high priority, critically important antibiotics.

We also used a process known as whole genome sequencing (WGS) to look in more detail at these 151 bacteria. The sequences showed us that the *Salmonella* that we found were similar to others that have previously been found in Scottish cattle.

All STECs have the potential to cause illness in humans, however, some are more likely to be able to do so than others. We used the sequences and an internationally agreed ranking system (Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment) to classify the STEC bacteria that we found by their potential to cause illness in humans. More than two-thirds (24, 69%) of the 35 confirmed STEC were ranked as having the lowest risk potential for severe disease in humans, while three of the 35 confirmed STEC were in the highest risk category. In addition, we identified two STECs that matched those found in a clinical outbreak of foodborne illness that occurred in humans in the United Kingdom at the same time as this survey. These were a type of STEC known as O157:H7 STEC.

We also looked at the sequences to see if there were any genes present that could indicate if the bacteria was resistant to antibiotics. The results were broadly similar to those we got from the sensitivity testing.

Statistical analyses can be used to see if the presence or absence of a pathogen in a sample is associated with a number of factors. If associations are found, this type of study (a survey, or cross-sectional study) does not enable us to say that there is a cause-effect relationship but it does allow us to generate ideas for further investigation. This type of analysis was not possible for the *Campylobacter* and *Salmonella* positive samples because there were so few of them. We did find that samples in which generic *E. coli* could be detected were more likely to be presumptive STEC positive samples, than samples in which generic *E. coli* could not be detected. In addition, samples purchased during the winter season (January, February & December) were less likely to be presumptive STEC positive compared to samples

purchased in the spring (March, April & May). We did not find any of the factors that we recorded to be associated with the confirmed STEC status of a sample. However, if a sample was a presumptive STEC positive sample, then it was less likely to be confirmed as STEC positive if the sample had higher generic *E. coli* counts, than if it had generic *E. coli* counts that could not be detected. The higher the generic *E. coli* count, the less likely the presumptive STEC sample was to be confirmed. These findings will need further investigation before coming to any conclusion about how they relate to, or affect, the quality and safety of fresh beef mince.

Overall, this survey has provided a baseline picture of the microbiological status of fresh minced beef on retail sale in Scotland. This information fills a gap in our knowledge and evidence base.

Regulations and controls are in place in the production process from farm to retail fridge to ensure the quality and safety of our food. The results of this survey demonstrate that, even with these measures in place, fresh beef mince products can, on occasion, contain organisms that have the potential to cause human illness. It is, therefore, important that they are used as intended i.e. they should be thoroughly cooked before they are eaten. The fact that some of the STEC found in survey samples matched those found in the clinical outbreak of STEC in humans, which occurred during the survey, confirms the importance of following this guidance. Fresh beef mince products should be handled hygienically in the kitchen and thoroughly cooked before being eaten.

A microbiological survey of fresh minced beef on retail sale in Scotland during 2019.

1. General introduction

1.1 Study background

The health and economic burden of foodborne illness is high in the UK, with approximately 2.4 million cases occurring annually (Food Standards Agency, 2020). Eating raw or undercooked mince that has been contaminated with pathogens is known to cause foodborne illness. Although retail beef mince is labelled that it must be fully cooked before consumption, there is still the possibility that a consumer may not fully cook the product or that any foodborne pathogens present may cross-contaminate other foods in the kitchen.

Although direct comparisons between studies are difficult to make due to many factors, a review of the available literature suggests that the prevalence of *Salmonella, Campylobacter* and Shiga toxin-producing *Escherichia coli* (STEC) O157 in fresh minced beef from retail outlets in England and Europe are relatively low. Estimates of the prevalence of *Salmonella* range from 0.1% in the Republic of Ireland (FSAI, 2013) to 1.4% in England (Turnbull and Rose, 1982) and 3.4% in Denmark (EFSA, 2019). Studies into the prevalence of *Campylobacter* in England and Belgium reported estimates of 1.0% and 0% respectively (Turnbull and Rose, 1982; Ghafir *et al*., 2007). While reported estimates in fresh bovine meat (i.e. not specifically minced meat) varied between 5.8% in Spain and 0% in Austria, Germany, Hungary and the Republic of North Macedonia (EFSA, 2019).

STEC are a diverse group of organisms and their presence in a range of matrices can be detected by both traditional culture and molecular techniques. Studies of culturable O157 STEC in minced beef give low prevalence estimates, from 0.1% to 2.9% (Vernozy-Rozand *et al*., 2002, Cagney *et al*., 2004, FSAI, 2013). Prevalence estimates for cultured STEC of all serogroups (i.e. not just O157) in fresh minced beef on retail sale ranged from 0% to 5.8% in European countries, and was reported to be 10% in Chile. However, the sample sizes of these studies varied between 7 and 787 (FSAI, 2013; EFSA, 2019; Toro *et al*., 2018).

An understanding of the baseline microbiological quality of fresh beef mince from retail outlets in Scotland would be useful for risk assessment, risk management and risk communication on the safety of this commodity. It could also improve our understanding of the presence of antimicrobial resistance (AMR) in the beef production chain. In order to fill this evidence gap, a survey was commissioned to characterise the microbiological quality of fresh beef mince sold at retail in Scotland.

1.2 Study objectives

This study had three objectives:

- To generate baseline data on the significant microbiological pathogens (*Salmonella*, *Campylobacter* and STEC) and process hygiene indicator organisms present in fresh minced beef on sale to the consumer in Scotland from retail outlets.
- To determine the antimicrobial sensitivity of any pathogens that were isolated and of a random subset of 100 of the generic *E. coli* that were found.
- To analyse any patterns of variation, such as geographic or seasonal, in order to identify any potential risk factors that may be associated with microbial contamination.

2. Methods

2.1 Sample size

The minimum sample size was pre-determined by Food Standards Scotland (FSS) as 1000 samples[1]. Resource constraints meant that this was the target number for acquisition for the survey. Given these limitations and assuming that true prevalence of the pathogens of interest would be low, some preliminary calculations were conducted to demonstrate the effect of sample size (see Appendix 1).

Based on the literature and the general experience of the project partners, the working assumptions were that, of 1000 samples, approx. 0.5% (n=5) would be positive for either *Salmonella* or *Campylobacter*. It was assumed that 4% (n=40) of samples would be positive for *stx* genes by an initial PCR test. Of these, on subsequent culture, only a proportion would be expected to yield STEC colonies. This led to an assumption that approx. 25 mince samples would result in confirmed STEC isolates, which would require whole genome sequencing (WGS).

2.2 Sampling method

A simple random sample of 1000 portions of fresh beef mince purchased from retail outlets in Scotland was not logistically feasible because a comprehensive list of every sampling unit (i.e. every package of fresh mince beef on retail sale in Scotland during the survey time period, 2019) was not available.

In order to devise a sampling strategy that was logistically feasible, optimised for cost-effectiveness and that captured the requirements of the objectives, a number of assumptions were made. These were as follows:

[1] This was based on prevalence estimates from "comparable European studies" and was determined by Food Standards Scotland (FSS) after consultation with Biomathematics & Statistics Scotland (BioSS).

- A sample of fresh beef mince at retail is either a pre-packaged quantity of fresh beef mince of approx. 500g, or a sufficient quantity (approx. 500g) of 'loose'/unprepackaged fresh beef mince sold through a retail outlet.

- While a calendar year is usually assumed to consist of 52 weeks, it would not be possible to acquire samples and process them in a timely manner in every week of the year. It was therefore assumed that sampling would be planned to be distributed across 45 weeks. Sampling weeks would occur in every month of the year and there would be no more than two consecutive weeks in which sampling did not occur. There were to be three Rounds of 15 weeks of sampling to capture any temporal variation (Rounds 1-3, 45 weeks in total).

- If 1000 samples were to be evenly distributed across 45 operational sampling weeks either 22, or 23, samples would need to to be acquired in each operating week i.e. (35 weeks of 22 and 10 weeks with 23 samples)

- As the primary aim was to achieve a distribution of samples that would represent the pattern of consumer purchasing, it was necessary to account for the fact that consumer purchasing behaviour will be influenced by, and thus reflected in, the range of products available from the retailers operating in their local area. The proportional distribution of market share (Kantar Worldpanel beef mince sales by retail outlet in the 52 weeks to 20/05/2018, pers. com. A. Trail) was used to classify the retail outlets that sell beef mince into five categories (ranging from the largest retailers to butchers and small independent shops). Each category contributed between 12% and 27% of sales by volume in that period. These categories have been assigned numbers due to considerations of commercial confidentiality and assurances that were provided regarding anonymity. An approximation to this distribution was used to determine the number of samples that would need to be purchased from each Retail Category in each operating week (Table 2.2.1)

Table 2.2.1 Percentage market share of beef mince by retail outlet category and the proportional distribution of fresh beef retail mince samples to be obtained during an operating week

Retail Category for beef mince sold in Scotland	Percentage of total beef mince sales volume in Scotland	Proportional distribution of an operating week's fresh beef retail mince samples Proportion (Number)
1	24%	0.25 (6)
2	27%	0.25 (6)
3	20%	0.20 (4)
4	12%	0.10 (2) In 10 weeks where n=23 this will be (3)
5	17%	0.20 (4)
Total	**100%**	**1.00 (990 + 10 = 1000)**

It was also assumed that as a purchase may be consumed by more than one person, a household would be a better indication of a 'purchasing consumer'. Therefore, an indication of the number of households[2] was used to define geographic areas in which to sample.

The range of the number of households per local authority (unitary) area was large (10.4k-291k). To improve the feasibility of sampling in sparsely populated areas, neighbouring local authorities were combined into Geographic Areas (GAs), each with at least 41.5k households. This was based on examination of the number of households per local authority and their geography relative to one another (Appendix 2, App Table 2.1, App Figure 2.1).

A two-stage sampling strategy was used (Table 2.2.2). The first stage was the selection of fifteen GAs randomly selected proportional to their size (number of households). The second stage of sampling was 'Retail Category' with a defined number of samples to be acquired per week (Table 2.2.1). The 15 selected GAs were assigned in order of selection to a sampling week in a Round (Week 1-15). The same GAs were to be sampled, in the same order, in each of the three Rounds.

The ten weeks in which the additional sample from Retail Category 4 needed to be purchased were determined by randomly[3] assigning one extra sample from Retail Category 4

[2] https://www.nrscotland.gov.uk/files//statistics/household-estimates/2017/house-est-17-all-tabs.xlsx

[3] R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

to 10 of the sampling weeks. This random selection ensured that no GA was required to provide an extra sample in more than one week (Table 2.2.2; Appendix 2, App Table 2.2).

Table 2.2.2 – Sampling plan for 1000 fresh beef mince samples from retail outlets in Scotland during 2019; total number of samples by Geographic Area and Retail Category

Geographic Area	Retail Category					Total
	1	2	3	4	5	
Fife	18	18	12	6	12	**66**
City of Edinburgh	18	18	12	7	12	**67**
Aberdeen City	18	18	12	7	12	**67**
South Ayrshire	18	18	12	7	12	**67**
Glasgow City	18	18	12	7	12	**67**
Aberdeenshire	18	18	12	7	12	**67**
Moray	18	18	12	6	12	**66**
Dundee City	18	18	12	7	12	**67**
Mid and East Lothian	18	18	12	7	12	**67**
Falkirk	18	18	12	7	12	**67**
Clackmannanshire & Stirling	18	18	12	6	12	**66**
Highland	18	18	12	6	12	**66**
Scottish Borders	18	18	12	6	12	**66**
Dumfries and Galloway	18	18	12	7	12	**67**
East Renfrewshire & East Ayrshire	18	18	12	7	12	**67**
Total	**270**	**270**	**180**	**100**	**180**	**1000**

For ease of logistics and to capture the element ‘product type’, retail premises were randomly selected within each Retail Category to be visited in the selected GA. Retail premises were initially identified from information provided by FSS. This listed retailers by local authority location, name, trading name and address. However, there was little information on the types of outlets that were in one of the Retail Categories and there was no information for the GA of East Ayrshire. The following approach was used to make sure the information was as complete as possible.

Members of the project team initially entered the geographic area and Retail Category descriptors into Google. The results were entered into one Excel spreadsheet per GA. The company name, trading name, street address and postcode were entered onto the spreadsheet and the information obtained was compared with that supplied by FSS. Deletions were made for duplicate entries, retailers that did not obviously sell fresh produce and those not in the correct GA. The GA was confirmed by checking the postcode location[4].

These master lists of retailers in each Retail Category, by GA, were used to randomly[5] select the visit list of retail premises that was supplied to the samplers for that operating week. A specific seed (starting point) was used to allow replicability, the number of premises selected for each Retail Category aimed to be sufficient to allow for some redundancy. For instance, if the premises were no longer in business, or they did not sell the requisite range of product on the day of the visit to obtain sufficient numbers of samples. A small market research pilot in Inverness (July 2018) indicated that the number of product ranges per retailer varied (1-5), with butchery outlets often only having one range. In addition, origin of product types varied by retailer. This supports the more comprehensive findings of an earlier regional market research exploration of the distribution of declaration of origin on retail red meat products in Scottish supermarkets (Denvir, 2013 for Scotland Food and Drink).

In each operating week (and thus in the designated GA) for each Retail Category, the sampling staff were instructed to visit the first listed premises for that category and purchase an appropriate sample from the product ranges of fresh beef mince that were available, up to the required number of samples for that category for that week (between two and six). If the total number of samples required (for that category, for that week) exceeded the number of different product types, and therefore samples that could be purchased, the sampler was to then visit the next premises on the list and repeat, until the required number of samples for that category for that week had been purchased. If the number of product types available exceeded the total required for the Retail Category that week, then only sufficient samples to meet the total required were purchased. In these cases selection of the samples was in the order of discovery.

The weekly sampling plans were distributed to the project team members at the centres responsible for sample acquisition (Scientific Services: Edinburgh, Glasgow and Tayside), on a rolling basis for that Round. Each centre was responsible for sampling in specific GAs. Where, on the occasion that it was not logistically possible for a centre to sample in a GA in a designated operating week, this was discussed with the project team and the order of the GAs was amended. Training was cascaded from the centre leads with detailed instructions provided to the samplers (Appendix 6). Samplers were asked to report back any difficulties such as closed businesses, insufficient premises, or product types in a Retail Category to the

[4] https://geopunk.co.uk/councils.php#.W-VyQ_5LFMx
[5] R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

project team so that the master lists could be updated prior to selection of premises for the following Rounds. This also allowed missing samples to be acquired in subsequent rounds in the correct Retail Category-GA-week combination. Where this was not possible due to the nature of the deficit, a number of additional samples from specific Retail Category-GA combinations were purchased during the weeks after the end of Round 2 and before Round 3. These numbers were designed to ensure that by the end of Round 3 the original proposed sampling plan for the 1000 fresh beef mince samples from retail outlets in Scotland (Table 2.2.2) was achieved. It was recognised that this might lead to a small number of 'spare' samples, if planned deficits did not occur in Round 3.

2.3 Ethics and awareness

No social or animal ethics committee approval was required for this study.

Prior to the start date, the FSS project team informed the major retailers that the survey would be run in 2019. An explanatory leaflet (Appendix 5) was produced for the samplers to give to smaller retailers and independent retail outlets at their sample purchase visit.

2.4 Scientific services

Three Scientific Services laboratories took part in this project. These were: Edinburgh Scientific Services operated by the City of Edinburgh Council, Glasgow Scientific Services operated by Glasgow City Council and Tayside Scientific Services operated by Dundee City Council. All three are designated as EU Official Food Control Laboratories and are accredited by UKAS for the examination of food[6]. The laboratories used their Food Examiner and other in-house laboratory staff to provide full coverage of the sample locations using detailed local authority sampling knowledge and robust, tried and trusted procedures.

For each week of each Round the retailers within each category were visited to purchase fresh beef mince samples as per the sampling plan described in Section 2.2 – Sampling method. The minimum weight of produce to be bought was 500g. An agreed SOP was followed for sample collection and submission to the laboratory for testing. Samples were maintained at chill temperatures[7] in a cool box and delivered to the testing laboratory within 12 hours of collection. The cool box temperatures were monitored using either calibrated dataloggers, calibrated thermometers, or calibrated temperature probes, as per the usual standardised methods used by the laboratories. Each sample was individually bagged with a unique identifying number. The standard protocol for labelling and transporting samples was followed. Packaging and bagging of each sample ensured that any leakage remained with the originating sample and prevented external contaminants reaching the mince samples.

[6] https://www.food.gov.uk/about-us/official-feed-and-food-control-laboratories
[7] Standard Operating Procedure aim is 3°C ± 2°C

Sampling time frames were in accordance with the plan agreed with FSS. Processing was begun immediately on arrival at the laboratory. On receipt, the product packaging was labelled with the unique identifying number and photographed with the information on the packet. A sample submission form was completed for each sample collected. Each sample was subdivided for processing and to retain a portion for archiving. Details of all samples were entered into the SS LIMS system, allowing traceability of each sample.

Examination of the samples for the process hygiene indicators and for the two pathogens *Campylobacter* and *Salmonella* was performed using UKAS accredited methods at Tayside and Glasgow, whilst PCR examination for STEC and O157 was performed in Edinburgh. Transfer between laboratories was achieved using a dedicated driver based centrally at Edinburgh. This was achieved without delay, with maintenance of the chill transport conditions as previously described. It took approximately two hours from the original receiving laboratory to the secondary laboratory, depending on traffic conditions.

2.4.1 Process hygiene indicator organisms

The Aerobic Colony Count (ACC) was based on BS ISO 4833-2:2013. Dilutions of the minced beef sample were prepared by aseptically weighing 10g of the minced beef into a sterile bag (stomacher bag) followed by the addition of 90ml of maximum recovery diluent (MRD) to prepare the first dilution (10^{-1}). Further dilutions were prepared by adding 1ml of the initial dilution into 9ml of MRD to produce a further dilution(10^{-2}) of the initial suspension. This process was repeated to produce the final dilution of the sample (10^{-3}). Both the 10^{-1} and 10^{-3} dilutions of the sample were inoculated onto Plate Count Agar using a spiral plater. Inoculated plates were incubated aerobically at 30°C for 72 hours ± 4 hours. Colony counts were calculated using a weighted mean from two plates to derive the final aerobic colony count per gram of sample.

The generic *E. coli* quantification was based on BS ISO 16649-2:2001. The first dilution of the minced beef sample was prepared in the same way (10g of minced beef with 90ml of diluent) as described above for ACC. Decimal dilutions of 0.5ml of sample were then spread onto the selective agar and incubated, initially at 37°C, followed by 44°C for a maximum of 24 hours. Typical *E. coli* colonies were counted from plates with less than 150 and 300 typical and atypical colonies respectively. Up to ten randomly selected colonies were biochemically confirmed as *E. coli* from each positive sample.

One generic *E. coli* isolate per positive mince sample was sent to SRUC Inverness for archiving and possible further study.

2.4.2 Campylobacter culture

The method for *Campylobacter* isolation was based on BS EN ISO 10272-1:2017. It involved inoculation of 25 g of sample into Bolton Broth which was incubated microaerophilically at 37°C for four hours followed by further incubation at 41.5°C for 44 hours. The incubated broth

was sub-cultured to each of two selective media, *Campylobacter* Blood Free Agar and Biorad Rapid *Campylobacter* Agar. These plates were incubated microaerphilically at 41.5°C for 48 hours and examined for typical colonies. Confirmation procedures were carried out on suspect colonies found on the plates. Confirmed isolates were sent to SRUC Inverness for archiving, further study and subsequent submission to the Scottish Microbiology Reference Laboratories (SMiRL) (see Figure 2.4.2 for sample flow and testing procedure).

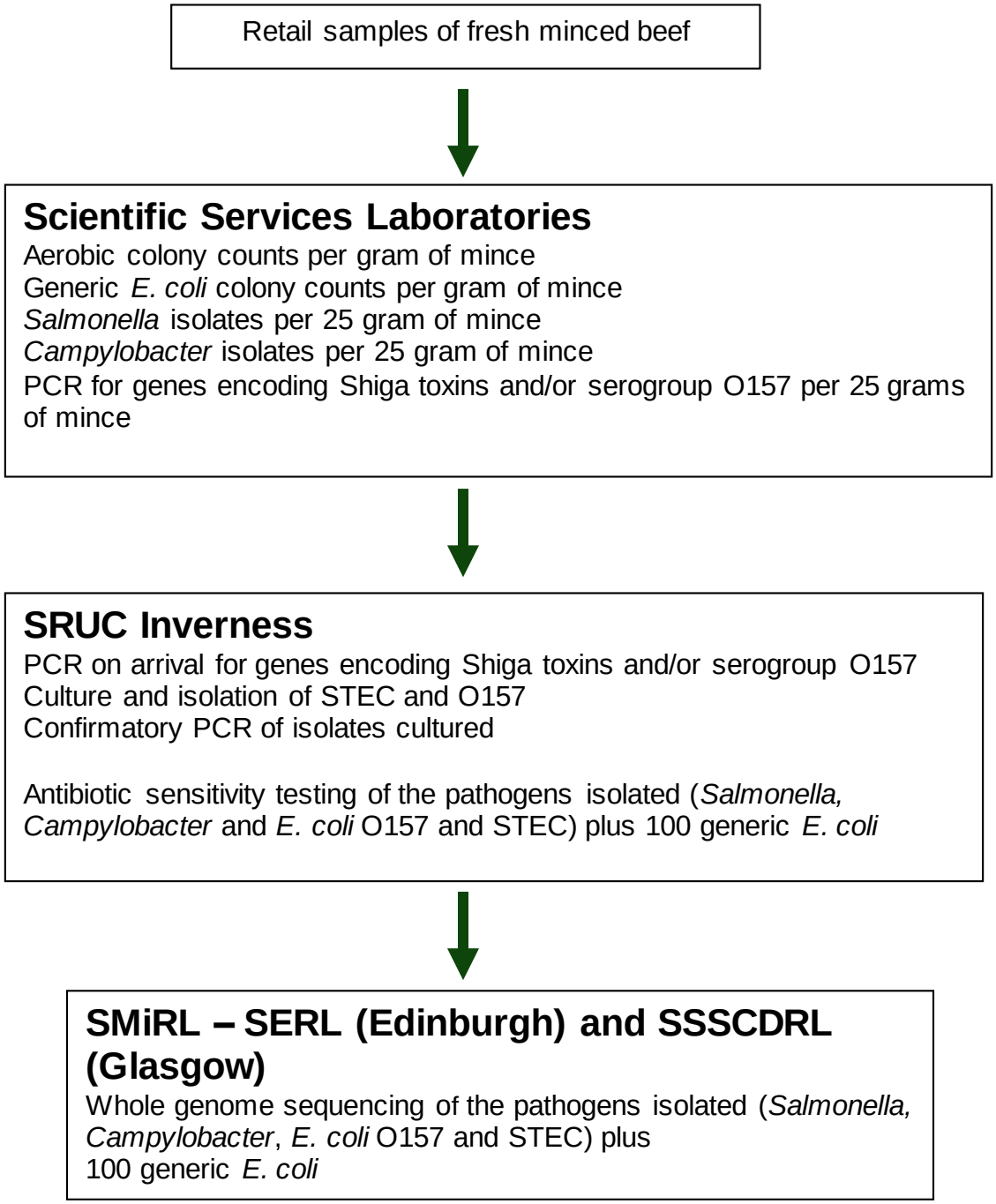
2.4.3 Salmonella culture

The method used for *Salmonella* isolation was based on BS EN ISO 6579:2002. It involved pre-enrichment of the sample in buffered peptone water (BPW) for 18 hours ± 2 hours (h) at 37°C ± 1°C, then inoculation of the enrichment broth on to two separate selective media, Rappaport-Vassiliadis medium with soya (RVS broth) and Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn broth). The RVS broth was incubated at 41.5°C ± 1 °C for 24 h ± 3 h, and the MKTTn broth at 37°C ± 1°C for 24 h ± 3 h. Two selective solid media were inoculated from each of the incubated selective media, xylose lysine deoxycholate agar (XLD agar) and Brilliant Green Agar (BGA). The XLD agar plate was incubated at 37°C ± 1°C and examined for colony growth after 24 h ± 3 h. The BGA plate was incubated at 35°C ± 1°C for up to 48 hours. The identity of presumptive *Salmonella* were confirmed by biochemical and serological tests. Confirmed isolates were sent to SRUC Inverness for archiving, further study and subsequent submission to the SMiRL.

2.4.4 STEC PCR

An enrichment broth was prepared using 25g of mince in 225ml of OXOID ISO BPW. Genomic DNA was extracted using BioPharm's SureFood Prep *E. coli* extraction kit, specifically used for the isolation of *E. coli* DNA from enrichment cultures and food matrices. The extracted DNA was analysed with TAQ-MAN® based primer/probes to allow the amplification of target genes. The target genes were *stx*$_1$ (Shiga toxin 1 all variants), *stx*$_2$ (Shiga toxin 2 a-e + g), *stx*$_2$f, and *rfb*$_{O157}$ (*E. coli* O157 toxin gene). The method was based on ISO/TS 13136:2012. "Microbiology of food and animal feed - Real-time polymerase chain reaction (PCR) -based method for the detection of foodborne pathogens - Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups." If one or more of the target *stx* genes were detected, then the mince sample was designated as a "presumptive STEC positive". Frozen enrichment cultures from presumptive STEC positive samples and STEC-negative *rfb*$_{O157}$-positive samples were submitted to SRUC Inverness for further evaluation. These consisted of a sub-aliquot of 1ml from the original broth. This was spun down in to a pellet, using a centrifuge (12,000g x 5 minutes). The supernatant was removed and the aliquot was then securely packaged in a cold storage box and sent to SRUC via a specialised courier.

Figure 2.4.2. Schema for the testing of the fresh beef mince samples and isolates purchased for the survey from retail outlets in Scotland during 2019



2.5 SRUC Inverness laboratory methods

2.5.1 Isolation of E. coli O157 and non-O157 Shiga toxin-producing E. coli (STEC)

Frozen enrichment cultures from mince samples that had given a positive signal to any genes encoding Shiga toxins or *rfb*$_{O157}$ on testing by PCR were sent to SRUC Inverness by overnight courier from Edinburgh Scientific Services. On receipt, the PCR was repeated on a boiled portion of the enrichment broth using the same primers, probes and conditions as above. These PCR results informed subsequent treatment of these samples.

Enrichment broths testing positive for *rfb*$_{O157}$ were cultured according to ISO 16654:2001 for isolation of *E. coli* O157. In brief, 20µl of serogroup O157 IMS beads (ThermoFisher) and 1ml of BPW were concentrated and washed three times in PBS –Tween 20 (0.005% v/v). The washed beads were cultured on Sorbitol MacConkey agar (SMAC) supplemented with cefixime (0.05mg/L) and tellurite (2.5 mg/L, CT-SMac), and Mac agars (ThermoFisher) and incubated at 37°C overnight. Non-sorbitol fermenting colonies from the CT-SMac and representative colonies from the Mac agars were subcultured to Chromocult (Merck) agar and incubated at 37°C overnight. Colonies were confirmed as *E. coli* O157 by latex agglutination (ThermoFisher) and PCR (for *rfb*$_{O157}$).

Enrichment broths testing positive for *stx* but with no *rfb*$_{O157}$ present, indicating the potential presence of non-O157 STEC, were cultured on MacConkey agar only.

Bacteria cultured by both of these methods were used to identify PCR positive colonies. The PCR method used, based on ISO/TS 13136:2012, was common to bacteria cultured from both *rfb*$_{O157}$ positive and negative samples. In order to isolate a PCR positive organism, 14 pools of five colonies were prepared from each enrichment culture and tested by PCR for *stx* and *rfb*$_{O157}$ genes. If a pool was positive to these PCR tests then individual constituent colonies from the positive pools were re-tested to identify those colonies that gave positive signals on repeat testing. The joint microbiological expertise within the project team identified that a reasonable cut off for searching for isolates from the PCR positive samples within this survey was 70 colonies per sample. i.e. if an STEC was not detected in any of 70 colonies (14 pools of five), then PCR testing was stopped. This method was approved by the FSS project officers as the most appropriate balance of available resources and detection effort.

Pure PCR positive colonies were stored on microbank beads at -80°C for further testing and subsequent supply to the SMiRL (Edinburgh).

2.5.2 Antimicrobial sensitivity testing (AST)

Disc diffusion testing, using the antimicrobials listed (Table 2.5.2), was carried out on all *Salmonella*, STEC, O157 and 100 generic *E. coli* isolates according to EUCAST guidelines and based on ISO 20776 – 1:2006, part 1 using commercially available discs and 4mm Mueller Hinton agar (Thermofisher).

The 100 generic *E. coli* were randomly selected, within each round, for further study from those sent to SRUC Inverness for archiving. The 100 were equally distributed across the rounds with 33, 34 and 33 selected from Rounds 1, 2 and 3 samples respectively.

Disc diffusion relies on the establishment of an antimicrobial concentration gradient within agar upon which the test bacteria have been spread. Zones of inhibition are measured around each disc to determine sensitivity to the agent. This method is unreliable for colistin. Instead, the Micronaut broth microdilution system (Bioconnections, UK) was used to establish the minimum inhibitory concentration (MIC) for colistin. Cut-off values for both methods were as described by EUCAST (EUCAST, 2016). The single *Campylobacter* isolate failed to grow on subculture and was therefore not available for AST.

All of the isolates that were subject to AST were transported by road courier, in three batches (May, September and December 2019) to SMiRL (Edinburgh) for distribution for WGS.

Table 2.5.2 – The panel of antimicrobials used for phenotypic antibiotic sensitivity testing of the selected isolates.

Antibiotic/active substance(s)	Antibiotic Group
Ampicillin 10µg	β-Lactam 1st generation cephalosporin
Cefotaxime 5 µg	β-Lactam 3rd generation cephalosporin
Ceftazidime 10µg	β-Lactam 3rd generation cephalosporin
Ertapenem 10µg	β-Lactam carbapenem
Amoxicillin + Clavulanic acid 30µg	β-Lactam and β-lactamase inhibitor
Piperacillin + Tazobactam 30µg	β-Lactam and β-lactamase inhibitor
Chloramphenicol 30µg	Chloramphenicol
Tetracycline 10µg	Tetracycline
Gentamicin 10µg	Aminoglycoside
Ciprofloxacin 5µg	Quinolone
Trimethoprim 5µg	Folate pathway inhibitor
Sulphamethoxazole + Trimethoprim 25µg	Sulphonamide and trimethoprim synergism
Colistin (MIC)	Polymyxin

2.6 SMiRL methods

2.6.1 DNA extraction, library preparation and sequencing

After receipt from SRUC Inverness at SMiRL (Edinburgh), all *Salmonella* and *Campylobacter* isolates were forwarded to SMiRL (Glasgow) along with a percentage of the 100 generic *E. coli* strains, thus distributing the WGS workload between the two laboratories. All STEC, O157 *E. coli* and the remaining generic *E. coli* strains were retained at SMiRL (Edinburgh).

At SMiRL (Edinburgh), all isolates were cultured on SMAC. Submitted O157 *E. coli* were confirmed by O157 *E. coli* latex agglutination and real-time PCR for the detection of *rfb*O157 and Stx genes (SERL PCR) (Holmes *et al.,* 2018). Submitted STEC were confirmed using SERL PCR. Isolates were grown overnight in Tryptone Soya broth (E&O Labs). Genomic DNA was extracted manually using the DNeasy Blood and Tissue Kit (Qiagen) following a pre-lysis step as recommended by the manufacturer. DNA was quantified via the Qubit Fluorimeter 3.0 (ThermoFisher Scientific) used with the dsDNA Assay HS Kit (ThermoFisher Scientific). DNA libraries were prepared using the Nextera XT DNA Preparation Kit following the manufacturer's instructions (Illumina, Cambridge, UK). Paired-end sequencing was performed on the Illumina MiSeq (Illumina, CA, USA) using 500 cycle v2 reagent kits to produce 2 x 250bp reads.

The isolates received at SMiRL (Glasgow) from SMiRL (Edinburgh) were subcultured (Maconkey agar (Oxoid, P00148A) for purity. For WGS, a single colony from the purity plate was cultured overnight in Brain Heart Infusion (BHI) broth (Oxoid, BO1230D). Genomic DNA was extracted using a Qiagen Symphony automated extraction platform with the QIASymphony DSP Mini Kit (Qiagen) following a pre-lysis step as recommended by the manufacturer. DNA was quantified as described above. DNA libraries were prepared using the Nextera Flex DNA Preparation Kit following the manufacturer's instructions (Illumina, Cambridge, UK). Paired-end sequencing was performed on the Illumina MiSeq (Illumina, CA, USA) using 600 cycle v3 reagent kits to produce 2 x 300bp reads.

2.6.2 Data analysis

WGS data were analysed using highly similar UKAS accredited bioinformatics workflows at SMiRL (Edinburgh and Glasgow). Both laboratories use the Scottish Microbiology Reference Laboratory WGS Bioinformatics Pipeline (SMiRLWBP) which incorporates applications and tools developed at PHE and elsewhere (Chattaway *et al.,* 2016; Chattaway *et al.,* 2017; Dallman *et al.,* 2018). In addition, the SMiRL Edinburgh workflow employs the wgMLST and *E. coli* genotyping plug-in tools within BioNumerics v7.6 (Holmes *et al.,* 2018), while SMiRL Glasgow uses the MLST, cgMLST and serotype prediction functions for both *Salmonella* and *E. coli* in Enterobase v4.1. Enterobase v4.1 was also used to provide new designations for novel Sequence Types (ST).

A detailed description of the workflow used at SMIRL Edinburgh can be found in a recent FSS report (Food Standards Scotland, 2020) and in Holmes *et al.* (2018).

The *Salmonella* workflow consisted of the same software applications to process, quality control and analyse the raw sequence data as the *E. coli* workflow. Using the GeneFinder tool, FASTQ reads were mapped to a panel of serotype and AMR genes (including chromosomal mutations) using Bowtie 2 (Langmead *et al.,* 2009). The best match to each target was reported with metrics including coverage, depth, mixture and nucleotide similarity in XML format for quality assessment. Only *in silico* predictions of serotype and AMR that match a gene determinant at >80% nucleotide identity and over >80% target gene lengths were accepted. MLST alleles of seven housekeeping genes (*aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A, t*hr*A) were determined using software called Metric-Oriented Sequence Typer (MOST, Tewolde *et al.,* 2016).

A summary of the outputs from the SMiRL bioinformatics workflows are shown in Table 2.6.2.

In addition to AMR prediction from the SMiRLWBP, assembled genomes were analysed for antibiotic resistance prediction using ResFinder v2.1 through the online server access for the Center for Genomic Epidemiology (DTU, Lyngby, Denmark)[8]. Detection parameters and target gene database details were as described earlier.

[8] https://cge.cbs.dtu.dk/services/

Table 2.6.2 - Outputs from the SMiRL bioinformatics workflows

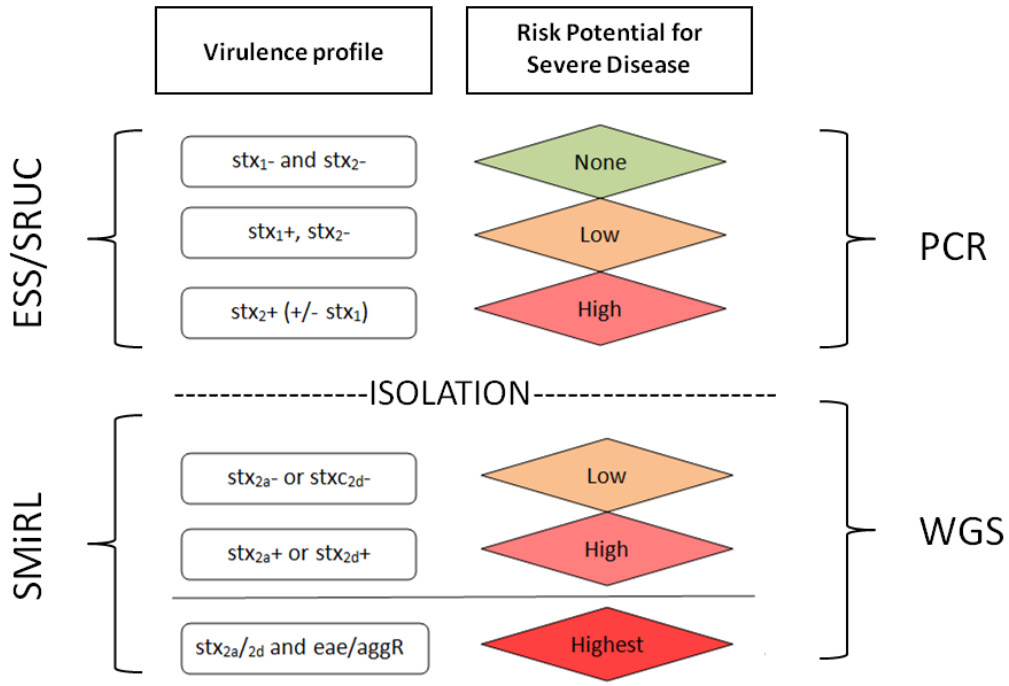
	SMIRL Edinburgh		SMIRL Glasgow	
	SMiRLWBP Pipeline	BioNumerics v7.6	SMiRLWBP Pipeline	Enterobase v4.1 (*Salmonella*)
Species ID	Yes	Yes	Yes	Yes
Serotype	Yes	Yes	Yes	Yes
7-gene MLST	Yes	Yes	Yes	Yes
Sequence Type (ST)[a]	Yes	No	Yes	Yes
cgMLST	No	Yes	No	Yes
eae	Yes	Yes	No	No
stx subtype	Yes	Yes	No	No
Additional virulence genes	Yes[b]	Yes (Virulence Finder database)	No	No
AMR	Yes	Yes (ResFinder Database[c])	Yes	Yes (ResFinder Database and Gastro-Resistance Finder[c])

[a] ST is based on the seven house-keeping genes: *adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A, *rec*A *for E. coli; and aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A, *thr*A for *Salmonella*.
[b] *bfpA, aggR, ipaH_type, aaiC, ltcA, sta1, stb.*
[c] *Contains genes associated with* resistance to aminoglycosides, sulphonamides, β-lactams, colistin, tetracyclines, macrolides, trimethoprim, phenicols, quinolones, and lincosamides.

2.6.3 Potential to cause clinical disease – JEMRA level assignment

In 2018, a report was published by the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (thereafter referred to as the JEMRA Report, (JEMRA. 2018)). This report suggested a testing strategy to assess the level of potential risk and severity of infections associated with exposure to STEC present in food. It suggested a molecular risk assessment approach based on the presence of certain virulence genes and adherence factors and ranked risk potential for severe disease as “highest”, “high”, “low” and “none”, where “none” represents the absence of *stx* genes (Figure 2.6.3). This testing approach was used in this study.

Figure 2.6.3: Testing strategy for STEC to assess health risk based on virulence genes. Adapted from JEMRA report, 2018.



The virulence profiles of the STEC identified in this study were categorised according to virulence gene combination and assigned a JEMRA level, ranging from level 1 (the potential for that strain to cause D/BD/HUS) to level 5 (potential to cause diarrhoea). The strains were also assigned a risk potential to cause severe disease.

2.7 Statistical analysis

Statistical analysis of the laboratory results and associated sample data was executed in STATA 13.0[9]. The initial dataset was cleaned, the samples collected by GA and Retail Category were compared to the sampling plan and the dependent and independent variables described.

2.7.1 Case Definitions for statistical analysis

[9] StataCorp LLC, Texas, USA, https://www.stata.com/

Throughout the statistical analysis, the following case definitions were used for the process hygiene indicators and pathogens, as agreed with FSS.

Two bacterial measures were used as process hygiene indicators – ACC and generic *E. coli*. Both were measured as continuous variables, although they were categorised later for use in the analysis.

A positive enrichment culture result for *Campylobacter* and for *Salmonella* was defined by the ISO standards BS EN ISO 6579:2002 and ISO 10272-2:2017 respectively.

Due to the multiple methodologies and confirmatory stages in testing for Shiga toxin-producing *E. coli* (STEC), several definitions were developed and used throughout the analysis. These included:

- presumptive STEC: the extracted genomic DNA had at least one of the genes (*stx*$_1$, *stx*$_2$) identified using RT-PCR by Scientific Services.
    - presumptive STEC O157: the extracted genomic DNA had at least one of the STEC genes - *stx*$_1$ or *stx*$_2$ - and the *rfb*$_{O157}$ gene identified using RT-PCR by Scientific Services.
    - presumptive non-O157 STEC: the extracted genomic DNA had at least one of the STEC genes - *stx*$_1$ or *stx*$_2$ – but not the *rfb*$_{O157}$ gene identified using RT-PCR by Scientific Services.
- confirmed STEC positive: samples which were presumptive STECs from which STEC O157 and/or non-O157 STEC were isolated and cultured at SRUC Inverness.

2.7.2 Prevalence calculations

To account for the clustered sampling method, the prevalence, standard error and 95% confidence intervals of the process hygiene indicators, pathogens and antimicrobial sensitivity were calculated using Ratio Estimates (Frerichs, 2004). The prevalence estimated was the 'apparent' prevalence. It was based on the assumption that the sensitivity and specificity of the diagnostic testing protocol used were 100%.

2.7.3 Risk factor analysis for pathogens in minced beef

The aim of the risk factor analysis was to determine whether the process hygiene indicator organisms were associated with the presence of each of the pathogens in the survey – *Campylobacter*, *Salmonella* and presumptive and confirmed STEC. The null hypothesis was that the process hygiene indicator organisms (ACC and generic *E. coli*) were not associated with the presence of each individual pathogen in minced beef on retail sale in Scotland.

The outcome variables: there were two separate outcome variables. Each one was the binary variable - presence or absence of the each of the individual pathogen definitions (presumptive and confirmed STEC). Risk factor analyses for *Salmonella* and *Campylobacter* positive samples were not pursued, due to the small number of positive samples that were obtained.

The exposure (i.e. independent) variables: an overview of the exposure variables, how they were recorded and how they were included in one of the final logistic regression models can be found in Table 2.7.3.

The process hygiene indicators: ACC and generic *E. coli* were categorised as follows:

The first category cut-off was the limit of detection. This was <400 cfu/g for ACC, with subsequent cut points at 5 x 10^5 and 5 x 10^6 cfu/g, giving four categories. For generic *E. coli*, the limit of detection was 10 cfu/g, with subsequent cut points at 50 and 500 cfu/g. This again led to four categories. These cut points were based on the thresholds for process hygiene indicator criteria in Regulation EC No. 2073/2005 on microbiological criteria for foodstuffs[10]. However, the different sample collection strategy means that the survey results cannot be compared directly with the statutory interpretations for these values.

To determine the median, the values were ranked in increasing order with all those that were below the limit of detection first. The value for the 505th sample was taken as the median. For each outcome, the variables were tabulated by category against the status of the sample and compared using the Wald Chi-Squared test to determine if there was an association between the categorised exposure variable and outcome.

Nested logistic regression models were then used to determine whether the categorised process hygiene indicator variables should be included as nominal (categories with no specified order) or ordinal (categories ranked in order), i.e. which of these options best explains variation in the outcome variable. This was achieved by using the likelihood ratio test to compare nested logistic regression models with the process hygiene indicators included as either nominal or ordinal variables. If the p-value provided no evidence against the null hypothesis (at the 5% level), the process hygiene indicators were included in the logistic regression models as ordinal (rather than nominal) variables.

Treatment of other categorical independent variables: the other categorical independent variables (Geographic Area, Retail Category, the country of origin (as stated on the packaging under ORIGIN) of the minced beef, the month the sample was purchased and the product type e.g. finest range, or value range) were tabulated and compared using the Wald Chi-Squared test for each of the outcomes.

[10] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02005R2073-20200308

Some categories of an independent variable had no positive samples. Where the categories could logically be combined, new categories were constructed. For example: the months the sample was purchased could be categorised into seasons. The variable Season was defined as Spring (March to May, inclusive), Summer (June to August, inclusive), Autumn (September to November, inclusive) and Winter (December to February, inclusive).

If it was not logical to condense categories, or if collapsing the categories still resulted in groups with no positives, the independent variable was excluded from the analysis.

Treatment of discrete and continuous independent variables: discrete and continuous independent variables (sample weight, cool box temperature, fat percentage and the number of days between purchase and use-by-date) were categorised into multiple or binary categories based on sample distribution or biological plausibility and tabulated. They were compared for each of the outcomes, with similar treatment (condensed categories, exclusion if no positives), as above.

As per the process hygiene indicators, and where appropriate, the categorised continuous independent variables were included in logistic regression models for each outcome as either a nominal variable, or as an ordinal variable. The two models were compared using the likelihood ratio test. If the p-value provided no evidence against the null hypothesis (at the 5% level), the independent variable was included as an ordinal variable in the logistic regression models (rather than nominal).

Univariable Analysis: the crude odds ratios (OR) and their 95% confidence intervals for the association between each independent variable and each pathogen were calculated using univariate logistic regression models, with the addition of Geographic Area included as a random effect to account for clustering. The null hypothesis was tested using the Wald Chi-Squared test.

Investigating Confounding Factors: the potential for confounding to exist between the process hygiene indicators and other independent variables (Retail Category, country of origin, month of sample purchase, fat percentage, sample weight, cool-box temperature and number of days between purchase and use-by-date) was explored using a data-driven approach. Counts and percentages to look for trends were tabulated for multiple combinations. Independent variables considered potential confounders, due to their association with the pathogens, process hygiene indicator organisms and absence from the causal pathway, were incorporated into nested logistic regression models with and without interaction with the process hygiene indicators. The models were compared using likelihood ratio tests. If the p-value provided no evidence against the null hypothesis (at the 5% level), the independent variable was incorporated without interaction in the logistic regression model.

Multivariable Analysis: independent variables with evidence of an association with the pathogens, based on their crude OR and Wald Chi-Squared statistic, were incorporated in a

forward stepwise manner into a multivariable logistic regression[11] model with Geographic Area modelled as a random effect. Independent variables with evidence of confounding were incorporated first, followed by those with the strongest association with the pathogens. As each independent variable was added, nested logistic regression models were compared using the likelihood ratio test to determine whether the addition of the independent variable improved model fit. If there was good evidence (using 5% as a cut off) that the independent variable improved fit, it was kept in the model. If there was weak or no evidence that it improved the fit, it was removed. The adjusted OR and their 95% confidence intervals were reported for the variables in the final logistic regression model.

Testing model robustness: every logistic regression model was checked for stability. They were considered stable if the final iteration on the STATA output reported that the model had converged and the quadratic check produced relative differences of less than 0.01. If the logistic regression model was unstable, the approach described above was repeated using a Poisson regression model. If the Poisson model also did not converge or there was only a small number of positive samples, then Fischer's Exact test was used to explore univariable associations between the independent variables and the pathogens.

[11] Using a binomial distribution with the logit link function.

Table 2.7.3 – A list of the exposure and independent variables with a description of their original recorded format and the format that they were converted to for the initial exploration and descriptive summaries.

Variable Name	Original format as recorded by samplers in Excel	Converted format for initial data exploration
Geographic Area	Categorical	No change made
Generic *E. coli*	Continuous	Categorical
ACC	Continuous	Categorical
Country of Origin	Categorical	No change made
Retail Category	Categorical	No change made
Fat Percentage	Continuous	Categorical
Product Range	Categorical	No change made
Season of sampling	Date	Categorical
Number of days between purchased date & use-by-date	Purchase date and Use-by-date	Binary[12]
Type of Packaging	Categorical	No change made
Packaging Atmosphere	Categorical	No change made
Weight of Sample	Continuous	Categorical[13]
Coolbox Temperature	Continuous	Categorical[14]

[12] Initial categories were <=0 day, >0 days
[13] Initial cut-offs were <=500g, 501-750g, >750g
[14] Initial cut-offs were <4˚C, 4-7˚C, >7˚C

3. Results

3.1 The sampling procedure

From January to December 2019, 1009 samples of minced beef were collected by 23 individuals from 15 Geographic Areas in Scotland (Table 3.1).

Table 3.1 – Distribution of the 1009 fresh beef mince samples collected from retail outlets in Scotland during 2019, by area & Retail Category, compared to planned sampling by Geographic Area

Geographic Area	Retail Category					Total	Planned
	1	2	3	4	5		
Fife	18	18	12	5	13	66	66
Edinburgh City	18	18	12	7	12	67	67
Aberdeen City	18	18	12	7	12	67	67
South Ayrshire	19	18	12	6	12	67	67
Glasgow City	18	18	12	7	12	67	67
Aberdeenshire	18	18	12	7	12	67	67
Moray	18	18	12	6	12	66	66
Dundee City	18	18	12	7	12	67	67
Mid & East Lothian	19	18	12	8	12	69	67
Falkirk	20	17	12	11	12	72	67
Clackmannanshire & Stirling	18	18	12	7	12	67	66
Highlands	18	18	12	6	12	66	66
Scottish Borders	18	18	12	6	12	66	66
Dumfries and Galloway	18	18	12	5	14	67	67
East Renfrewshire & East Ayrshire	18	18	12	6	14	68	67
Total	**274**	**269**	**180**	**101**	**185**	**1009**	**1000**

Nine hundred and seventy-four samples were collected in accordance with the original sampling schedule i.e. from the nominated type of retailer, in the GA designated to be sampled that week in that Round. For a variety of logistical reasons, both under and over sampling occurred in Retail Category/Round/GA combinations (For details see Appendix 3,

App Table 3.1 to App Table 3.6). Thirteen samples were obtained during the additional 'catch-up' sampling period between Rounds 2 & 3, in an attempt to resolve previous 'missing' samples and pre-empt anticipated issues, such as retail outlets not being in business or having insufficient product types available to obtain the requisite number of samples (see Methods). Overall, 1009 samples were purchased.

The closest fit to the original planned distribution of samples, across both GAs and types of retailer, could be achieved using 1004 of the 1009 samples. Five of the 1009 samples could therefore have been excluded from the statistical analysis to achieve the optimum balance. A description of how this could be achieved can be found in Appendix 4. The statistical analysis was re-run on the chosen 'best fit' 1004 samples. There were minor changes in confidence intervals around some prevalence estimates but the statistically significant potential risk factors were unchanged. These results have, therefore, not be included in this report.

3.2 Description of the samples

The statistical analysis in subsequent sections of the report is based on all 1009 samples.

The median sample weight was 500g (min 250g[15], max 1kg) and the fat percentage varied from 1 to 25%. The distribution of fat percentage in the minced beef was bimodal with peaks at 5% and 20%. Data on fat percentage was not available for 268 samples.

Minced beef was collected from premium (n=150, 15%), organic (n=4, 0.5%), standard (n=469, 46%), discount (n=7, 1%) and other ranges (n=379, 38%). Information regarding the product range was not available for 31 samples.

The majority (n=939, 93%) of samples were pre-packed. Of these, 98% (n=920) had a modified atmosphere. All 70 loose samples had an unmodified atmosphere.

There were 33 (3.3%) samples where the country of origin of the minced beef was unknown. Of the remaining samples, all except three were labelled as originating within the UK and Ireland.

Two samples were purchased one day after their use-by-date, 48 (5%) were purchased on their use-by-date and the remainder had a median of four days until their use-by-date expired.

[15] The minimum weight of 250g occurred because although the minimum weight of a specific product to be purchased to constitute a sample was specified as 500g, on occasion this size of product type was not available. In this case it was agreed that the samplers would purchase 2 x 250g from the same batch. One of these 250g packs would be sufficient for the laboratory testing that was required for the survey i.e. for immediate survey purposes 250g could have been set as the minimum weight. The 500g had been set due to the request for an immediate project-lifetime duration archive, in case further investigations were needed

3.3 Process Hygiene Indicators

3.3.1 Aerobic Colony Count

The median ACC was 6.4 x 10^5 cfu per gram of minced beef (IQR: 6.9 x 10^4 to 9.6 x 10^6). Only a small proportion (3%) of samples had ACCs below the limit of detection. (Table 3.3.1).

Table 3.3.1 – Distribution of the Aerobic Colony Counts (ACC) of 1009 fresh beef mince samples, purchased from retail outlets in Scotland during 2019, by categories defined as per Methods section 2.7.3.

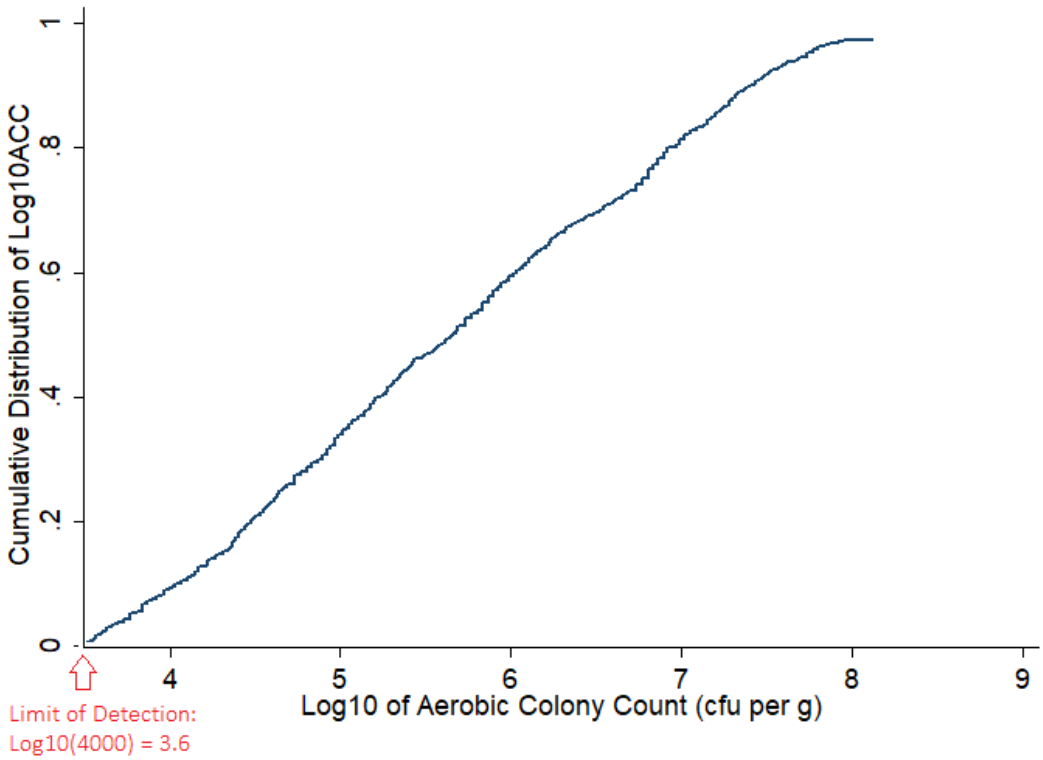
Aerobic Colony Counts (cfu per gram minced beef)	Number of samples within specified range	Proportion of samples (95% confidence interval)
< 4 x 10^3*	32	0.03 (0.01 to 0.05)
4 x 10^3 ≤ cfu < 5 x 10^5	447	0.44 (0.40 to 0.49)
5 x 10^5 ≤ cfu < 5 x 10^6	233	0.23 (0.21 to 0.26)
≥ 5 x 10^6	297	0.29 (0.26 to 0.33)

* limit of detection (LOD) for ACC

The cumulative distribution curve[16] of the ACC values that were observed in the mince samples (Figure 3.3.1) shows an approximately straight line relationship. This indicates a uniform distribution of ACC values .

[16] the value of the curve on the y-axis indicates the proportion of samples that had values less than or equal to the corresponding value on the x-axis. Note that the x-axis value is on the log scale.

Figure 3.3.1 – Empirical Cumulative Distribution Curve of Log_{10} ACC in 1009 fresh beef mince samples purchased from retail outlets in Scotland during 2019.



3.3.2 Generic E. coli count

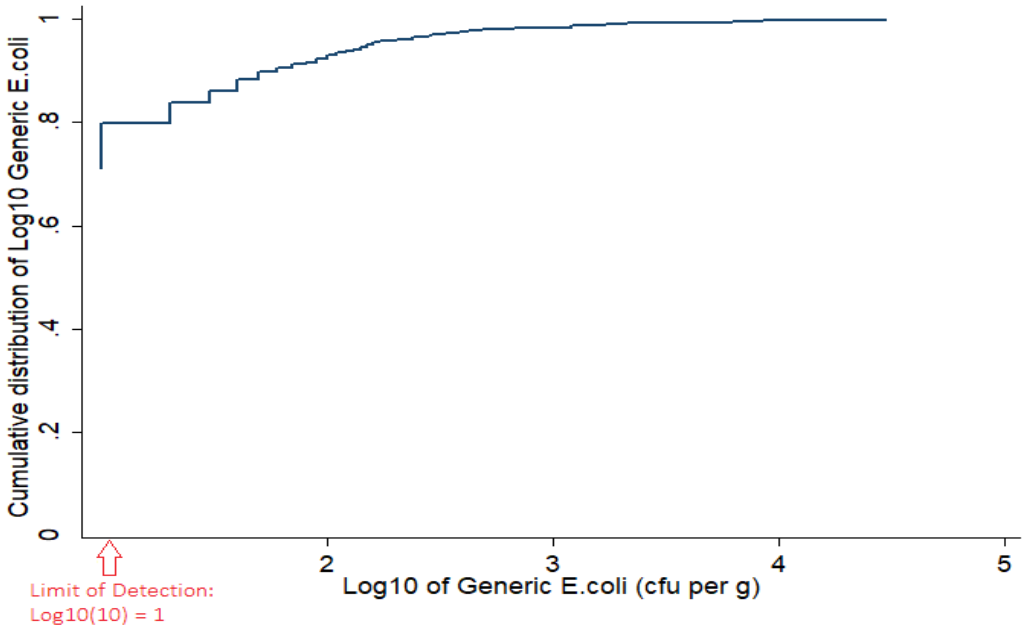
The median generic *E. coli* count was <10 cfu per gram of minced beef (IQR: <10 to 10). The majority (71%) of the samples had a generic *E. coli* count below the limit of detection. (Table 3.3.2 and Figure 3.3.2).

Table 3.3.2 – Generic E. coli counts of 1009 fresh beef mince samples, purchased from retail outlets in Scotland during 2019, by categories defined as per Methods section 2.7.3.

Generic *E. coli* (cfu per gram minced beef)	Number of samples within specified range	Proportion of samples (95% confidence interval)
cfu < 10*	716	0.71 (0.66 to 0.75)
10 ≤ cfu < 50	174	0.17 (0.14 to 0.21)
50 ≤ cfu <500	98	0.10 (0.08 to 0.12)
cfu ≥ 500	21	0.02 (0.01 to 0.03)

* limit of detection for generic *E. coli*

Figure 3.3.2 – Empirical Cumulative Distribution Curve[17] of Log_{10} Generic E. coli in 1009 fresh beef mince samples purchased from retail outlets in Scotland during 2019



[17] the value of the curve on the y-axis indicates the proportion of samples that had values less than or equal to the corresponding value on the x-axis. Note that the x-axis value is on the log scale.

3.4 Pathogens

Campylobacter was isolated from only one sample using the UKAS accredited method based on BS EN ISO 10272-1:2017. Although the identity of this isolate was confirmed at this point as *Campylobacter* spp., the *Campylobacter* isolate subsequently proved to be unrecoverable (see section 3.6.2).

Salmonella spp. was isolated from three samples (Table 3.4)

Presumptive STEC (O157 and non-O157) were found in over a fifth of samples and, of these, almost one in five were confirmed through colony isolation on subsequent culture (Table 3.4).

Table 3.4 – Prevalence of pathogens in 1009 fresh beef mince samples purchased from retail outlets in Scotland during 2019, (as a proportion)

Pathogen	Number of positive samples	Proportion of all 1009 samples (95% confidence interval)
Campylobacter	1	0.001 (0 to 0.007)
Salmonella	3	0.003 (0 to 0.01)
Presumptive STEC (O157 & non-O157)	226	0.22 (0.20 to 0.25)
Presumptive non-O157 STEC	193	0.19 (0.17 to 0.22)
Presumptive O157 STEC	33	0.03 (0.02 to 0.04)
Confirmed STEC (O157 & non-O157)	35	0.035 (0.02 to 0.05)
Confirmed non-O157 STEC	31	0.03 (0.02 to 0.05)
Confirmed O157 STEC	4	0.005 (0.002 to 0.02)
Indicative O157 (non-STEC)	20	0.02 (0.013 to 0.27)

There were 226 presumptive STEC (O157 and non-O157), four samples that would have been classified as STEC negative by the initial PCR due to late positive signals[18], and 20 samples in which the *rfb*$_{O157}$ gene but no *stx* genes were detected by PCR at Edinburgh Scientific Services. All of these 250 samples went forward for culture and isolation.

Of the 226 presumptive STEC positive samples (frozen enrichment broth sub-aliquots) that were received by the SRUC Inverness laboratory, 203 were positive and 23 were negative when retested at the SRUC Inverness laboratory using PCR.

All of the 35 confirmed STEC samples were from samples that had the *stx* gene detected by PCR at both Edinburgh Scientific Services (ESS) and SRUC Inverness. Thirty-one colonies possessed *stx* genes but not *rfb*$_{O157}$ genes; four possessed both *stx* and *rfb*$_{O157}$ genes (Table 3.4).

There were 13 colonies that possessed only the *rfb*$_{O157}$ but not *stx* genes. Six of these were cultured from presumptive STEC samples. The other seven were derived from the 20 samples in which the *rfb*$_{O157}$ gene but no *stx* genes were detected by PCR (at ESS). i.e. 13 of these non-STEC *rfb*$_{O157}$ did not get confirmed as O157-positive by culture and isolation.

In total, 48 STEC and/or stx-negative O157 colonies were isolated from 47 samples. because one presumptive STEC O157 sample yielded two different colonies; a stx negative *E. coli* O157 and a STEC non-O157 colony.

3.5 Antimicrobial sensitivity testing (AST)

Of the 1009 samples, 51 samples of fresh beef mince were positive for one or more of the pathogens (*Campylobacter, Salmonella*, STEC or *E. coli* O157). As stated in the section above, one sample yielded two isolates; a STEC colony and a non-STEC O157 colony, resulting in 52 isolates. However, the sample that was *Campylobacter* positive could not be tested for AST as the *Campylobacter* isolate could not be recovered on subculture. Hence, with the addition of the 100 randomly chosen generic *E. coli*, a total of 151 bacterial isolates underwent AST.

Of the 151 AST tests, 12 had non-susceptible phenotypic patterns indicative of antimicrobial resistance. For each of the antibiotics/active substances to which a lack of susceptibility was detected, the prevalence of non-susceptible phenotypes in the population of 151 isolates was one in 20, or less (Table 3.5.1). Of the 12 patterns observed, seven had non-susceptible phenotypic patterns to just one antibiotic and five had phenotypic patterns of non-susceptibility to three antibiotics (Table 3.5.2).

[18] The additional four samples were ones that gave a positive signal after the accepted PCR Ct value positive cut-off at 35 cycles i.e. they would have been called as negative

Table 3.5.1 – Prevalence of non-susceptible phenotypes in the 151 isolates tested that were obtained from fresh beef mince samples purchased from retail outlets in Scotland during 2019, as a proportion.

Antibiotic/active substance(s)	Number* of samples with phenotypic resistance	Proportion (95% confidence interval)
Ampicillin	4	0.03 (0.01 to 0.05)
Trimethoprim	3	0.02 (0 to 0.04)
Sulphamethoxazole/ Trimethoprrim	3	0.02 (0.002 to 0.04)
Tetracycline	7	0.05 (0.01 to 0.08)
Chloramphenicol	4	0.03 (0.002 to 0.05)
Colistin**	1	0.01 (0 to 0.02)

***** The total number of non-susceptible phenotypes is higher than the number of non- susceptible isolates as some isolates were non-susceptible to more than one antibiotic/active substance combination (see Table 3.5.2)

****** This result is not classed as a cause for concern. It is based on phenotype and is a recognised phenomenon amongst certain *Salmonella* isolates (Agers *et al.,* 2012; Ricci *et al.,* 2020). Analysis of the whole genome sequence of this organism found no evidence of *mcr* genes.

Table 3.5.2 – AST phenotypic patterns of the 100 generic E. coli, 48 STEC and/or O157 and three Salmonella isolates obtained from fresh beef mince samples purchased from retail outlets in Scotland during 2019.

	Number of isolates with each phenotypic pattern						
	Susceptible to	Not susceptible to					
Type of isolate	AST: disc diffusion panel and colistin MIC	Ampicillin	Tetracycline	Colistin	Ampicillin, Tetracycline, Chloramphenicol	Ampicillin, Trimethoprim, Sulphamethoxazole plus trimethoprim	Chloramphenicol, Trimethoprim, Sulphamethoxazole plus trimethoprim
Generic *E. coli*	93	1	3	0	2	1	0
Salmonella	2	0	0	1*	0	0	0
O157 STEC	4	0	0	0	0	0	0
Non-O157 STEC	28	0	1	0	0	0	2
Non-STEC O157	12	0	1	0	0	0	0

* This result is not classed as a cause for concern. It is based on phenotype and is a recognised phenomenon amongst certain *Salmonella* isolates (Agers *et al.* 2012, Ricci *et al.* 2020). Analysis of the whole genome sequence of this organism found no evidence of *mcr* genes.

3.6 Whole genome sequencing

3.6.1 Generic E. coli

Of the 100 generic *E. coli*, there were 59 different serotypes for which the O and H serogroups were identified by WGS. There were 14 isolates where the O group was not identified, and these have been designated O unidentifiable (O-). There were five isolates where novel sequence types (ST) were assigned (Table 3.6.1.1). There were 2 OB serogroup designations (Boyd antigens). One generic *E. coli* carried the *eae* gene.

Table 3.6.1.1 - Characteristics of the 100 generic E. coli isolates by WGS

Serotype	No. Isolates	ST (s)	*eae* No. positive	AMR Genotype (No. positive)
O-:H10	2	441, 4086	0	-
O-:H11	1	4628	0	-
O-:H12	1	88	0	*aph*(3')-Ia, *str*A, *str*B, *sul*2, *bla*TEM-1B, *gyr*A[83:Ser-Leu]
O-:H14	1	1081	0	-
O-:H16	2	295	0	-
O-:H2	1	1665	0	-
O-:H21	3	10, 56, 58	0	-
O-:H27	1	Novel ST. Closest ST215 (SLV) [8677]	0	-
O-:H28	1	942	0	-
O-:H38	1	1616	0	-
O-:H39	1	10	0	-
O-:H56	1	7416	0	-
O-:H6	1	Novel ST. Closest ST115 (SLV) [8828]	0	-
O-:H9	1	472	0	-
O5:H11	2	155	0	-
O5:H16	1	5745	0	-

Serotype	No. Isolates	ST (s)	*eae* No. positive	AMR Genotype (No. positive)
O6:H14	3	1434	0	-
O6:H39	2	1830	0	-
O6:H49	2	1079	0	*tet*(C) (n=2)
O7:H4	1	216	0	-
O8:H16	1	2602	0	-
O8:H21	1	3202	0	-
O8:H8	1	109	0	*tet*(A)
O8:H9	1	410	0	-
O9:H30	2	540	0	-
O10:H25	1	635	0	-
O10:H42	3	164	0	-
O13:H10	1	1434	0	-
O15:H12	5	399	0	-
O15:H6	1	69	0	*bla*TEM-1B
O18:H14	2	1434	0	-
O18:H7	1	1463	0	-
O21:H21	1	101	0	*str*A, *str*B, *sul*2, *tet*B
O22:H21	2	58	0	*str*A, *str*B-like, *sul*2-like, *bla*TEM-1B, *tet*A, *flo*R-like (n=1)
O23:H11	1	3168	0	-
O28/O42:H21	1	278	0	-
O29:H10	1	1122	0	-
O32:H34	1	109	0	-
O36:H9	1	401	0	-

Serotype	No. Isolates	ST (s)	*eae* No. positive	AMR Genotype (No. positive)
O39:H19	3	399	0	-
O50/O2:H6	1	141	0	-
O54:H28	1	5552	0	-
O74:H39	1	548	0	-
O75:H38	1	Novel ST. Closest ST4198 (SLV) [10786]	0	-
O80:H42	1	3779	0	-
O91:H7	1	1304	0	-
O93:H28	1	4038	0	-
O93:H30	1	685	0	-
O103:H21	1	2354	0	-
O107:H54	1	10	0	-
O109:H27	1	5334	0	-
O110:H2	1	187	0	-
O112:H40	1	154	0	-
O113:H21	1	56	0	-
O116:H48	2	3519	0	-
O116:H8	1	7040	0	-
O117:H25	1	635	0	-
O126:H30	1	399	0	-
O134:H21	2	345	0	-
O136:H20	1	Novel ST. Closest ST1252 (SLV) [10668]	0	-
O138:H48	1	1400	0	-
O140:H21	1	1423	0	-

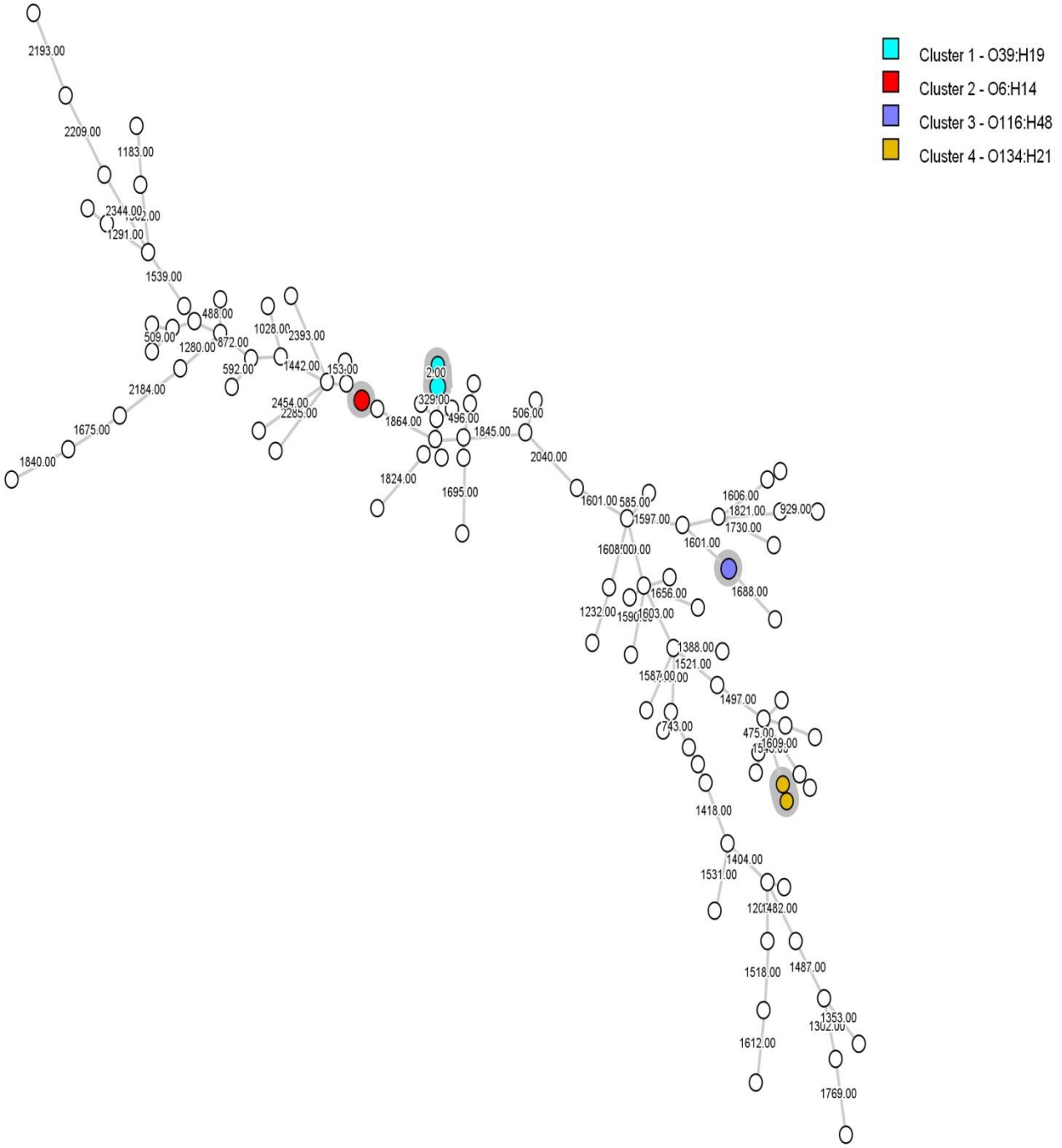
Serotype	No. Isolates	ST (s)	*eae* No. positive	AMR Genotype (No. positive)
O146:H19	1	1623	0	-
O146:H21	2	442	0	-
O148:H32	1	Novel ST Closest ST10 (SLV) [10609]	0	-
O150:H8	2	906	0	-
O154:H9	2	536	0	-
O156:H8	1	327	1	-
O170:H8	1	2111	0	-
O174:H7	1	278	0	-
O175:H16	1	603	0	*str*A, *str*B, *sul*2,*bla*TEM-1B, *tet*A, *flo*R
O178:H7	1	278	0	-
O179:H8	1	297	0	-
OB17:H39	1	10	0	-
OB18:H3	1	10	0	-

Ninety-seven different sequence types were identified by cgMLST analysis of the 100 generic *E. coli*. The mean number of allelic differences between isolates was 994 indicating a highly diverse collection of strains (Figure 3.6.1). Four small clusters (three clusters of two isolates, one cluster of three isolates) were detected where the isolates were linked at <10 allelic differences.

Table 3.6.1.2 - Characteristics of four cgMLST clusters of generic E. coli isolates

cgMLST Cluster	ERU number	Serotype	Source - Retail Category (RC) and Geographic Area (GA)	Sample Date	cgMLST distance
Cluster 1	2019-0268	O39:H19	RC4, GA4	05/08/2019	0-2 alleles
	2019-0766	O39:H19	RC4, GA8	21/10/2019	
	2019-0524	O39:H19	RC2, GA3	16/09/2019	
Cluster 2	2019-0013	O6:H14	RC3, GA1	14/01/2019	0 alleles
	2019-0033	O6:H14	RC3, GA4	04/02/2019	
Cluster 3	2019-0143	O116:H48	RC1, GA3	20/05/2019	0 alleles
	2019-0145	O116:H48	RC1, GA3	20/05/2019	
Cluster 4	2019-0247	O134:H21	RC4, GA 2	15/07/2019	4 alleles
	2019-1055	O134:H21	RC5, GA13	03/12/2019	

Figure 3.6.1 - Minimum spanning tree derived from cgMLST profiles (Enterobase v1, 2513 alleles) of 100 generic E. coli isolates from minced meat in Scotland in 2019.
Partitioning (shaded) indicates isolates clustering at ≤10 allelic differences. All branch distances are allelic differences.



3.6.2 Campylobacter

One sample was identified as *Campylobacter* positive at Scientific Services. However, the isolate did not behave as expected on subculture following receipt at SMiRL (Glasgow). Following further characterisation by WGS and MALDI-TOFF, it was identified as a strain of *Ochrobactrum* spp. (most likely *O. anthropi* or *O. intermedium*). Substantial follow-up was conducted and no *Campylobacter* was recoverable from any point throughout the laboratory chain. It is believed that the original *Campylobacter* was lost through competition by the *Ochrobactrum* during transport. This bacteria has previously been reported from cattle at slaughter (Alonso *et al.*, 2017) but little is known about competition during transport.

3.6.3 Salmonella

Of the three *Salmonella* isolates, two different serotypes were identified.

Table 3.6.3 - Characteristics of the three Salmonella isolates by WGS

Serotype	No. Isolates	ST (s)	AMR Genotype
Mbandaka (O 6,7:z10:e,n,z15)	2	413	none detected
Dublin (O 9,12:g,p:-)	1	10	none detected

Salmonella phylogenetic overview: *Salmonella* serovar Mbandaka isolates make up approximately 2% of all Salmonellae submitted to SMiRL (Glasgow) each year from all sources. It is commonly associated with cattle, accounting for 56 of 503 (11.1%) bovine submissions since the beginning of 2017 (UK VARSS, 2019). Both isolates from this study clustered relatively closely to other *S.* Mbandaka isolates from Scotland, in particular those of bovine origin.

One study isolate (2019-0469) fell within 10 cgMLST allelic differences of a recognised Scottish bovine cluster (VetWGS_2018_034_Mbandaka). This cluster includes isolates from at least as far back as early 2017 to the present, probably reflecting an endemic clone within the Scottish national cattle herd. cgMLST comparison with the Enterobase dataset (accessed 20 May 2020) revealed that 36 of the bovine isolates from Scotland were clustered in HC20:28252 (hierarchical cluster with 20 allelic differences) with only seven other isolates in the same cluster being from human and unspecified food sources in the UK, submitted by Public Health England (PHE) between 2014 and 2018. It should be noted that only certain centres routinely upload sequence data to Enterobase. While regular submissions are made from major agencies such as PHE in England, Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) in the United States, and several European reference laboratories, the dataset is not fully comprehensive.

Salmonella serovar Dublin is rarely isolated from human infection in Scotland with only six human cases in 2019. This serovar, however, is more commonly associated with

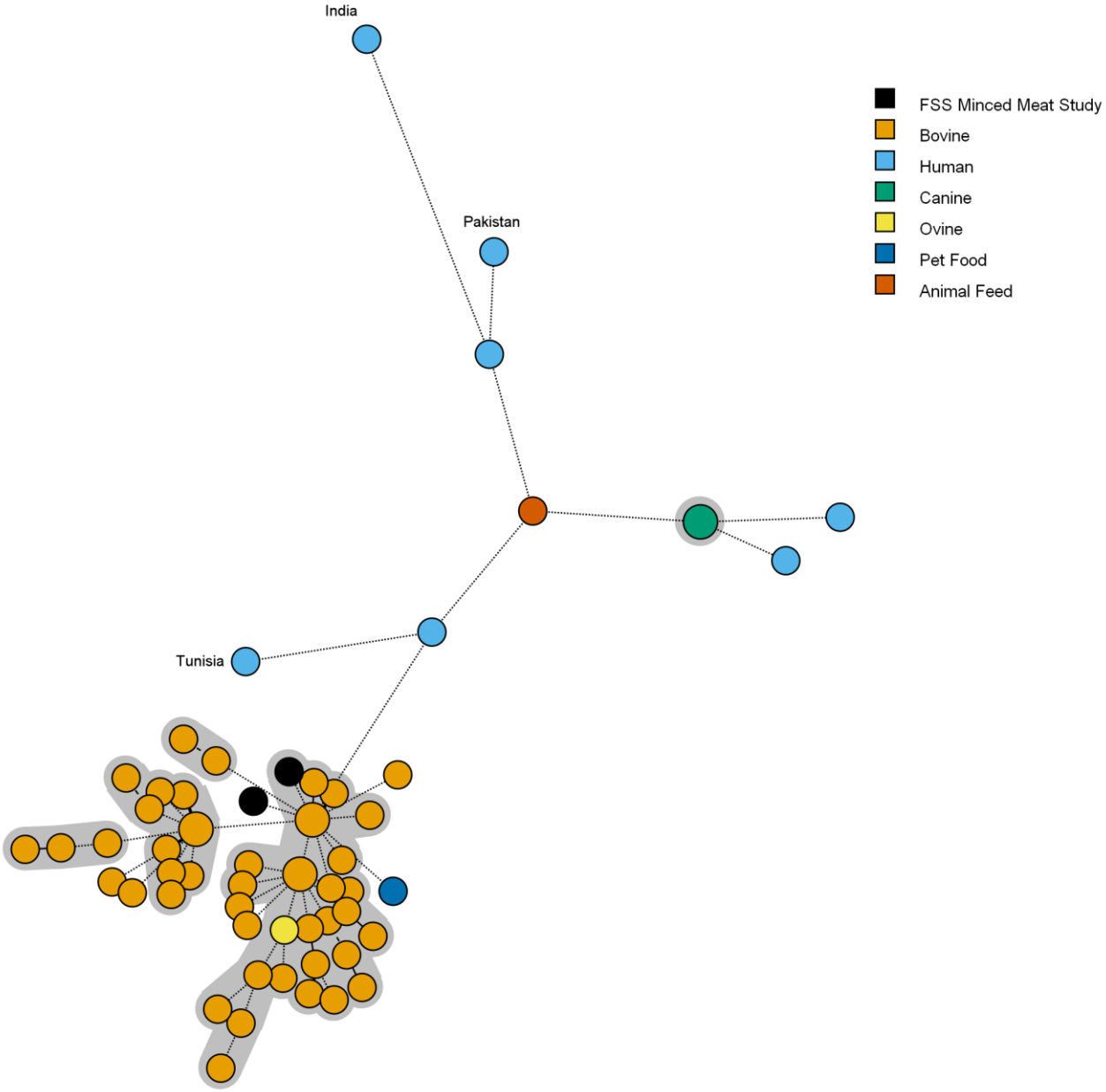
extraintestinal infections than most other non-typhoidal serovars and is therefore a significant public health concern (Harvey *et al*., 2017, Kudirkiene *et al*., 2020).

In 2019, 108 isolates of serovar Dublin were submitted to the Scottish Microbiology Reference Laboratories from veterinary sources in Scotland, 98 of which were bovine in origin, (SMiRL, unpublished data).

The isolate recovered during this study was not identified as belonging to any existing cgMLST cluster, being 18 allelic differences distant. cgMLST comparison with the Enterobase dataset (accessed 20 May 2020) did not reveal any more closely related isolates submitted by other laboratories. As above, it should be noted that only certain centres routinely upload sequence data to Enterobase and therefore the dataset is not comprehensive.

Figure 3.6.3 - Minimum spanning tree derived from cgMLST profiles (Enterobase v.2, 3002 alleles) of Salmonella Mbandaka isolates submitted to SMiRL (Glasgow) between January 2018 and end April 2020.

Partitioning (grey background shade) indicates isolates clustering at ≤10 allelic differences. Nodes highlighted in orange indicate clustering in the same 25-SNP SnapperDB cluster (1.1.19.54.%). Where human isolates are associated with foreign travel, the country has been stated.



3.6.4 Shiga toxin-producing E. coli

Twenty two different serotypes were identified among the 35 STEC study isolates; O113:H4 *E. coli* (n=4) and O84:H20 *E. coli* (n=4) were the most common (Table 3.6.4.1). Twenty different STs were detected among the serotypes: ST10 was shared by three seroytpes; O113:H4, O38:H26 and O50/O2:H27, while ST58 was shared by O117:H9 and O88:H25. In one case (O113:H21, 2019-000) a novel ST was detected. Six isolates carried *eae*, and these belonged to serotypes O157:H7, O26:H11 and O182:H25.

Only three Shiga toxin-producing O157:H7 *E. coli* strains were identified and one O157:H38 *E. coli* strain.

Two of the 35 (5.7%) study isolates carried AMR genes, which is a considerably lower frequency than that detected among isolates associated with clinical infection in Scotland (~14-18%, Holmes *et al.,* 2015, FSS, 2020)

Table 3.6.4.1 - Characteristics of the 35 STEC isolate serotypes by WGS

Serotype	No. Isolates	ST (s)	*eae* No. (%) positive	*Stx* Subtype	AMR Genotype (No. positive)
O26:H11	2	21	2 (100%)	*stx*1a, *stx*2a (n=1) *stx*1a (n=1)	-
O38:H26	2	10	0	*stx*1c (n=2)	-
O50/O2:H27	1	10	0	*stx*2a (n=1)	-
O51:H1	1	706	0	*stx*2b (n=1)	-
O8:H28	1	4496	0	*stx*2a (n=1)	-
O8:H30	1	88	0	*stx*2a (n=1)	-
O84:H20	4	4444	0	*stx*1d (n=4)	-
O88:H25	1	58	0	*stx*1d (n=1)	-
O109:H5	1	647	0	*stx*1a (n=1)	-

Serotype	No. Isolates	ST (s)	*eae* No. (%) positive	*Stx* Subtype	AMR Genotype (No. positive)
O113:H21	2	223 & 11060*	0	*stx*2a (n=1) *stx*2d (n=1)	-
O113:H4	4	10	0	*stx*2d (n=4)	*sul*2, *flo*R (n=2)
O117:H9	2	58	0	*stx*1d (n=2)	-
O146:H21	1	442	0	*stx*2b (n=1)	-
O149:H1	2	132	0	*stx*1d (n=2)	-
O149:H8	1	344	0	*stx*2a, *stx*2g (n=1)	-
O153/O178:H19	1	443	0	*stx*1a (n=1)	-
O157:H38	1	1113	0	*stx*1d (n=1)	-
O157:H7	3	11	3 (100%)	*stx*2a, *stx*2c (n=2) *stx*2c (n=1)	-
O163:H19	1	679	0	*stx*2a, *stx*2d (n=1)	-
O168:H8	1	718	0	*stx*1a (n=1)	*sul*2, *tet*B, *str*A, *str*B (n=1)
O171:H2	1	332	0	*stx*2a (n=1)	-
O182:H25	1	300	1 (100%)	*stx*1a (n=1)	-

*NOVEL allele. Closest ST: 56 (Single Locus Variant)

Table 3.6.4.2 – The frequency (number) of Shiga toxin gene profiles detected among the 35 STEC isolates

stx subtype profile	No. of isolates
*stx*1a	5
*stx*1c	2
*stx*1d	10
*stx*2a	5
*stx*2b	2
*stx*2c	1
*stx*2d	5
*stx*1a *stx*2a	1
*stx*2a *stx*2c	2
*stx*2a *stx*2d	1
*stx*2a *stx*2g	1

A total of 11 different *stx* subtype profiles were detected, with most known *stx* subtypes identified, apart from *stx*2e and *stx*2f. The most common subtype profile observed was *stx*1d. It was harboured by five different serotypes including O84:H20 (n=4) and O157:H38 (n=1). This *stx* subtype is not commonly observed in clinical cases of infection (only observed in 3/522 Scottish clinical cases of infection (FSS, 2020)).

STEC phylogenetic overview: the 35 STEC study isolates were analysed alongside sequences in the SERL genomic database. This mostly contains strains from clinical cases (~n=1439 including all non-O157 received by SERL since 2002 and O157 STEC since August 2017) but also small numbers from animal, food or environmental sources. A cgMLST comparison was used to select sequences from the database that clustered most closely, but within ≤ 200 allelic differences, of the study isolates for further analysis. These included 20 sequences from human clinical cases, four from cattle and one from deer.

Phylogenetic analysis of the 60 isolates (Figure 3.6.4) revealed two of the O157:H7 STEC study isolates shared 100% cgMLST similarity, and matched three Scottish clinical strains from a contemporaneous UK outbreak of O157 *E. coli* phage type (PT) 21/28 (highlighted in grey in Figure 3.6.4). A further clinical isolate also clustered with these strains, however it was isolated four months earlier. With permission from the group, sequence data from the mince samples were sent to PHE for analysis. It was confirmed that the SNP address of the mince isolates matched that from the clinical isolates. The Outbreak Control Team (OCT) involved in the investigation concluded that the source of clinical infection was most likely Scottish cattle (manuscript in preparation). The other O157:H7 STEC study isolate clustered most closely with a PT34 isolated from a clinical case in 2016, differing by nine alleles. One O157:H38

E. coli was already present in the SERL database. It had been isolated from cattle faeces. This had the same sequence type (ST1113) as the study isolate but did not carry any *stx* genes and was genetically distant, differing by 120 alleles.

Of the 31 non-O157 STEC study isolates, twelve isolates (belonging to 11 serotypes) did not cluster within ≤200 alleles of any isolates in the SERL database. The serotypes of the strains were O8:H28, O171:H2, O80:H30, O50/O2:H27, O163:H19, O117:H9, O51:H1, O153/O178:H19, O109:H5, O88:H25 and O113:H21 (ST11060).

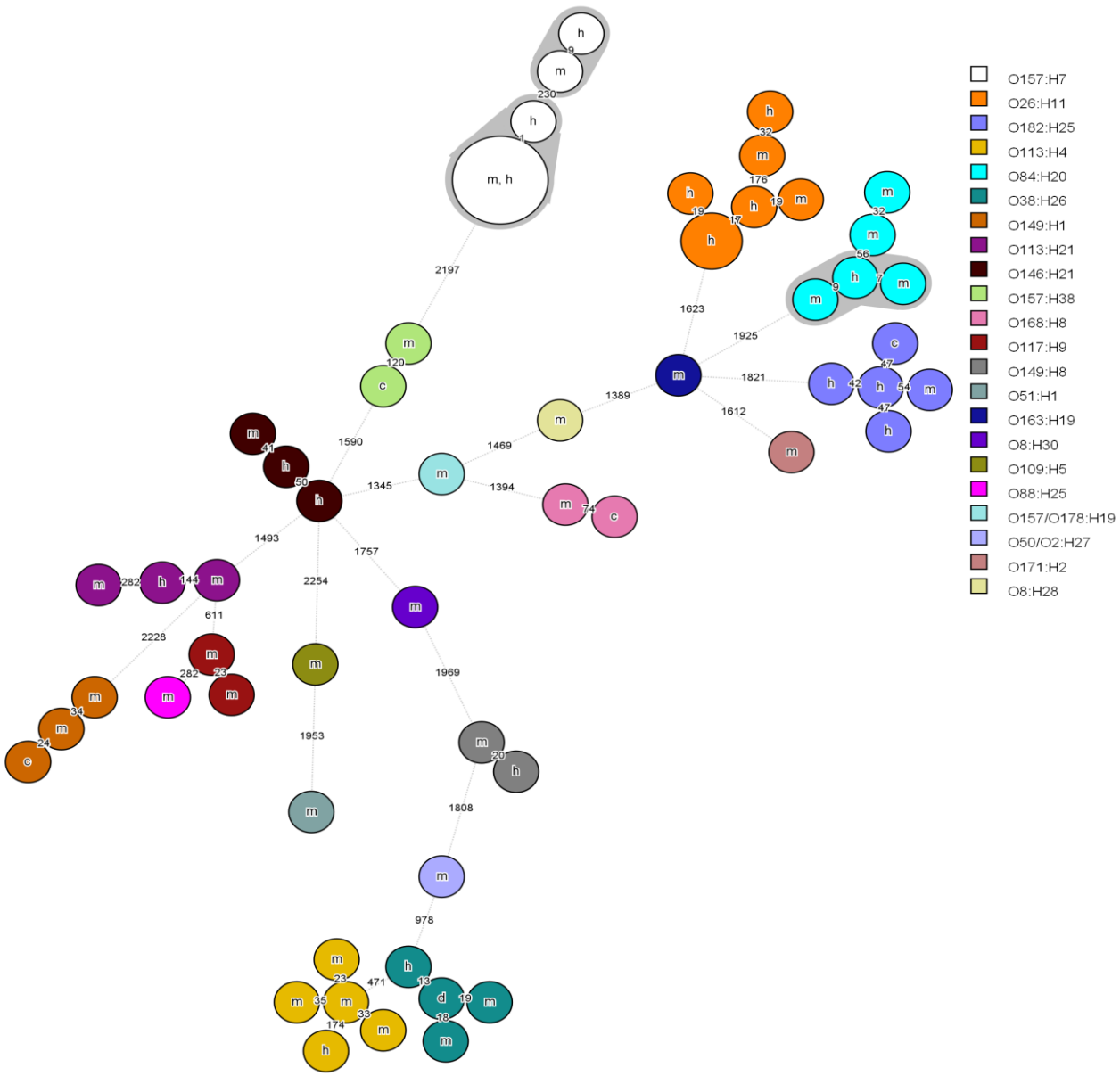
The remaining 19 non-O157 STEC clustered with isolates from human, cattle and/or deer origin (Figure 3.6.4). O26:H11 *E. coli* is the second most common non-O157 *E. coli* associated with clinical infections in Scotland, and worldwide. The two O26:H11 study isolates shared the same ST but carried different *stx* genes and clustered most closely with human isolates belong to ST21 sub-lineages, 21C1a (2019-0053) and 21C1b (2019-0817) respectively; ST21C1b is the most common sublineage in Scotland. The next two most common non-O157 STEC isolated from clinical cases in Scotland, O103:H2 and O145:H28 were not isolated from any mince samples, neither were isolates belonging to serotypes O111:H8, O121:H19 and O45:H2 which along with O26:H11, O103:H2 and O145:H28 make up the 'Big Six" serotypes identified as adulterants on raw, non-intact beef in the USA (USDA FSIS, 2012).

Of the two most common serotypes isolated from the mince, O113:H4 has been isolated from a small number (n=12) of clinical cases in Scotland between 2006 and 2020. Only one of these shared the same *stx* profile (stx_{2d}) as the O113:H4 mince study isolates, but was distantly related differing by ~174 alleles. One *E. coli* O84:H20 was already present in the SERL database. It had been isolated from a clinical case in 2018. This clinical isolate shared the same ST and *stx* profile as the O84:H20 study isolates. The study isolate that most closely matched it differed by by only seven alleles. The study isolate belonging to serotype O182:H25 fell into a cluster (~54 allelic differences) with three clinical isolates, from 2006 (n=1) and 2018 (n=2), and an isolate from cattle faeces (unknown isolation date), all of which carried *eae* and were stx_{1a} positive.

Study isolates with serotypes O146:H21, O149:H8 and O113:H21 clustered most closely with clinical sources, while those belonging to O168:H8 and O149:H1 only clustered with isolates from cattle (unknown isolation dates). The same AMR profile (*sul2*, *tetB*, *strA*, *strB*) was shared by the study and cattle O168:H8 isolates, although they differed by 74 alleles. The two O38:H26 study isolates fell into a small cluster with a clinical case from 2010 and an isolate from a deer (unknown isolation date).

Figure 3.6.4. Minimum spanning tree derived from cgMLST profiles (BioNumerics v7.6, 2315 alleles) of the 35 study STEC isolates (m) and 25 of the most closely matching E. coli isolates (h, c & d) from the SERL genomic database.

Partitioning (grey) indicates isolates clustering at ≤10 allelic differences. cgMLST profiles are represented by circles, and the size of the circle is proportional to the number of isolates that share the same cgMLST profile. Each circle is colour coded by serotype and isolates of human (h), cattle (c), deer (d) and mince (m) origin are denoted. The number of allelic differences between isolates is shown.



Potential to cause clinical disease – JEMRA level assignment: of the 35 confirmed STEC isolates in this study, eight (22.9%) were ranked as having a "High" or "Highest" risk potential to cause severe disease, due to their virulence profile (Table 3.6.4.3). These comprised strains of O157:H7 *E. coli*, O26:H11 *E. coli*, O113:H4 *E. coli* and O113:H21 *E. coli*. Of the remaining twenty-seven strains, all were designated low risk potential to cause severe disease, although three (8.6%) were assigned level 3 or 4 with potential to cause diarrhoea or bloody diarrhoea and 24 (68.6%) were assigned to level 5 with potential to cause diarrhoea.

Table 3.6.4.3 - JEMRA Level Assignment of the 35 STEC isolates

JEMRA Risk Potential for Severe Disease	JEMRA Level[1]	Subtype combination[2]	No. of strains	Serotype
Highest	1 (D/BD/HUS)	*stx*$_{2a}$, *eae* (with *stx*$_{1a}$)	1	O26:H11
		stx$_{2a}$, *eae* (with *stx*$_{2c}$)	2	O157:H7
High	2 (D/BD/HUS[3])	*stx*$_{2d}$	5	O113:H4, O113:H21
Low	3 (D/BD)[4]	*stx*$_{2c}$, *eae*	1	O157:H7
Low	4 (D/BD)[4]	*stx*$_{1a}$, *eae*	2	O26:H11, O182:H25
Low	5 (D)	other *stx* subtypes	24	O8:H28, O8:H30, O38:H26, O50/O2:H27, O51:H1, O84:H20, O88:H25, O109:H5, O113:H21, O117:H9, O146:H21, O149:H1, O149:H8, O153/O178:H19, O157:H38, O163:H19, O168:H8, O171:H2

[1] Potential to cause illness in parenthesis. This will also be dependent on host susceptibility and other factors such as antibiotic treatment.
[2] Extra genes present in parenthesis
[3] Association with HUS dependent on *stx*$_{2d}$ variant and strain background
[4] Some subtypes have been reported to cause BD, and rarely HUS

Shiga Toxin Negative *E. coli* O157: thirteen *stx* negative O157 *E. coli* isolates were submitted to SMiRL. These were identified as *E. coli* O157:non-H7 serotypes, with O157:H29 predominating (n=10) (Table 3.6.4.4).

Table 3.6.4.4 - Characteristics of the 13 Shiga toxin negative E. coli O157

Serotype	No. isolates	ST(s)	eae No. positive	Mean Number of virulence genes
O157:H12	2	10	0	2
O157:H29	10	7009	0	6
O157:H42	1	3744	0	3

Only nine different virulence genes were associated with these strains and none possessed *eae*. O157:H7 typically carry >20 virulence genes. There was a high degree of clustering between the O157:H29 isolates with nine out of 10 strains differed by fewer than three alleles and these shared similar virulence profiles.

3.6.5 Antimicrobial sensitivity testing and resistance genes overview

Table 3.6.5 summarises the antimicrobial resistance gene profiles found and places them in the context of the results from the phenotypic AST.

Table 3.6.5 – The frequency (number) of AMR genes identified by WGS in 151 isolates from fresh beef mince samples purchased from retail outlets in Scotland during 2019.

Gene	Expected Phenotype	Number of isolates with gene detected by WGS	Number of isolates with expected phenotype
TEM-1B	ampicillin	4	4
*flo*R	chloramphenicol	4	4
*tet*A	tetracycline	2	2
*tet*B	tetracycline	3	3(1)[a]
*tet*C	tetracycline	2	1[b]
*sul*2	sulphonamides	7	Not tested
*dfr*A5	trimethoprim	1	1
*dfr*A36	trimethoprim	2	2
*sul2/dfr*A5 or *sul2/dfr*A36	co-trimoxazole	3	3
aph(3')-Ia	kanamycin	1	Not tested
*str*A	streptomycin	5	Not tested
*str*B	streptomycin	5	Not tested
*gyr*A_EC2[83:S-L] mutation	reduced ciprofloxacin[c]	1	0

[a] One isolate showed phenotypic resistance to tetracycline, however no corresponding resistance gene could be detected.
[b] One isolate possessed a *tet*C but did not demonstrate the associated tetracycline resistance.
[c] Reduced susceptibility to ciprofloxacin in accordance with EUCAST guidelines.

3.7 Risk Factor Analysis

3.7.1 Campylobacter

The single *Campylobacter* positive minced beef sample was 750g, pre-packed in a modified atmosphere. The packet was labelled with the UK as the country of origin and the sample was purchased from Retail Category 5, from the Glasgow GA, in October. The date of purchase was six days before the use-by-date and the cool box temperature was recorded as 4.5˚C.

Both ACC and generic *E. coli* count were below the limit of detection for this sample.

3.7.2 Salmonella

The three *Salmonella* positive minced beef samples were 500g, pre-packed in a modified atmosphere and the country of origin, according to the package labelling, was the UK.

Two of the samples were purchased on the same date in September, in the Edinburgh GA, on the same day as their use-by date – one each from Retail Category 1 and 5. The cool box temperature was recorded as 2.6˚C. Of these two samples, one had an ACC below the limit of detection and a generic *E. coli* count of 30 cfu/g and the other had an ACC 2.1 x 10^7 cfu/g and generic *E. coli* count of 8.4 x 10^3 cfu/g.

The third sample was purchased in January from a Retail Category 3 outlet in the Aberdeen GA, five days before the use-by date and the cool box temperature was recorded as 2˚C. The sample had an ACC of 4.6 x 10^5 and a generic *E. coli* count below the limit of detection.

3.7.3 Presumptive STEC (stx positive by PCR)

The independent variable, country of origin, was dropped from the analysis because only three samples originated from outside the UK and none of these samples were presumptive STEC positive.

There was no evidence that using ACC as a nominal variable, as opposed to an ordinal variable, improved model fit (p=0.861). Therefore, ACC was modelled as an ordinal exposure variable, i.e. ranked. There was strong evidence (p=0.001) that generic *E. coli* modelled as a nominal variable produced a better fit. Therefore, generic *E. coli* was modelled as a nominal variable (no specified order).

Univariable analysis: univariable analysis (Table 3.7.3.1) showed strong evidence (p<0.001) of an association between each of the process hygiene indicator organisms - ACC and generic *E. coli* count – and presumptive STEC status of the sample. In addition, three other independent variables – the Retail Category, the Season the sample was collected, and the packaging atmosphere - showed evidence of an association with presumptive STEC status with GA incorporated as a random effect.

Investigating confounding in the relationship between ACC & presumptive STEC: there was evidence for an association between ACC and each of the following variables: generic *E. coli* count, the Retail Category, and the packaging atmosphere. However, packaging atmosphere and generic *E. coli* count were considered as potentially part of the causal pathway between ACC and presumptive STEC status. No evidence of confounding by Retail Category (p=0.781) was found using the likelihood ratio test.

Multivariable analysis of the relationship between ACC & presumptive STEC: after adjusting for the effect of generic *E. coli* count and the Retail Category, there was no evidence for an

association between either ACC (p=0.119) or the type of packaging atmosphere (p=0.159) and presumptive STEC status.

Investigating confounding variables in the relationship between generic *E. coli* & presumptive STEC: the generic *E. coli* count was associated individually with both the Retail Category and the Season in which the sample was collected. However, there was very weak or no evidence of confounding by Retail Category (p=0.097) and Season (p=0.437).

Table 3.7.3.1 – Univariable analysis of factors associated with the presumptive STEC status of 1009 fresh beef mince samples purchased from retail outlets in Scotland during 2019

Risk Factor	Total[a]	Number of positives	Crude OR[b] (95% confidence interval)	P value[c]	Rho: within cluster variation (95% confidence interval)	P value for Rho statistic[c]
ACC category[19]	1009	226	1.44 (1.22 to 1.70)	<0.001	0.004 (0 to 0.55)	0.356
Generic *E. coli*[20]						
cfu <10	716	104	Base	<0.001	0.004 (0 to 0.47)	0.342
10 ≤ cfu <50	174	65	3.55 (2.44 to 5.18)			
50 ≤ cfu <500	98	49	5.90 (3.77 to 9.25)			
cfu ≥500	21	8	3.65 (1.47 to 9.06)			
Retail Category[20]						
1	274	37	Base	<0.001	0.004 (0 to 0.52)	0.350
2	269	51	1.50 (0.94 to 2.37)			
3	180	78	4.92 (3.12 to 7.75)			
4	101	18	1.39 (0.75 to 2.57)			
5	185	42	1.88 (1.16 to 3.07)			
Fat Percentage[21]	1009	226	1.21 (0.88 to 1.67)	0.238	0.01 (0 to 0.31)	0.291
Product Range[20]						
Premium	150	36	Base	0.180	0.01 (0 to 0.19)	0.258
Standard	469	81	0.65 (0.41 to 1.01)			
Discount	7	1	0.54 (0.06 to 4.61)			
Organic	4	1	1.07 (0.11 to 10.67)			
Other	379	107	1.24 (0.80 to 1.93)			

[19] Categories as per Methods: the cut offs were below the limit of detection (BLOD <4000 cfu) ,≥ 4 x 10^3, 5 x 10^5 and 5 x 10^6 cfu/g.
Interpretation (ordinal): For each categorical increase in ACC i.e. from category 1 (<4000) to category 2 (≥ 4 x 10^3 to 5 x 10^5), there was a 1.44 increase in the odds of a sample of minced beef being presumptive STEC positive; likewise from category 3 to 4

[20] The odds ratio for each category is compared to the baseline category.

[21] Categories were ≤10%, 11 to 20% and >20%. Interpretation (ordinal): similar to footnote 19; for each categorical increase in fat percentage of the minced beef i.e. from category 2 (11 to 20%) to category 3 (>20%), there was a 1.21 increase in the odds of a minced beef sample being presumptive STEC positive

Risk Factor	Total[a]	Number of positives	Crude OR[b] (95% confidence interval)	P value[c]	Rho: within cluster variation (95% confidence interval)	P value for Rho statistic[c]
Season of sampling[20]						0.356
Spring	281	63	Base	0.029	0.003 (0 to 0.55)	
Summer	234	66	1.37 (0.91 to 2.06)			
Autumn	289	64	0.99 (0.66 to 1.47)			
Winter	205	33	0.66 (0.41 to 1.06)			
Number of days between sampling & use-by-date						
On or after use by date	50	16	Base	0.098	0.002 (0 to 0.93)	0.405
Before use by date	959	210	0.59 (0.32 to 1.10)			
Type of packaging						
Loose	70	11	Base	0.169	0.001 (0 to 0.99)	0.442
Prepacked	939	215	1.59 (0.82 to 3.08)			
Packaging atmosphere						
Unmodified	88	11	Base	0.022	0.0006 (0 to 1)	0.477
Modified	920	215	2.13 (1.11 to 4.09)			
Weight of sample (g)[22]	1009	226	0.85 (0.58 to 1.26)	0.416	0.001 (0 to 0.99)	0.450
Coolbox temperature (°C)[23]	1009	226	1.06 (0.84 to 1.35)	0.445	0.001 (0 to 0.99)	0.445

a: totals may not equal 1009 if there were missing data. Please see text for details.
b: crude OR calculated using logistic regression with Geographic Area (the cluster variable) as a random effect
c: calculated using the Wald Chi-Squared statistic; c: all p values for the rho statistic are >0.2. Therefore, there is no evidence of within cluster correlation.

[22] Categories were <= 500g, 501 to 750 g, > 750g –continuous variable used as ordinal categorical variable – interpretation as for footnote 19 and 21.
[23] Categories were <4˚C, 4-7˚C, >7˚C - continuous variable used as ordinal categorical variable – interpretation as for footnote 19 and 21.

Multivariable Analysis of the relationship between generic *E. coli* & presumptive STEC: After adjusting for the Retail Category and the Season, there was no evidence (p=0.119) of an association between the ACC and presumptive STEC positive samples so ACC was removed from the model. However, after adjusting for the Retail Category and the Season, there remained strong evidence (p<0.001) that the generic *E. coli* count was associated with beef mince being presumptive STEC positive, with Geographic Area as a random effect (Table 3.7.3.2). This was accepted as the final multivariable logistic regression model.

For each category of generic *E. coli* count, the odds of the sample being a presumptive STEC positive was increased compared to the baseline category of below the limit of detection (BLOD). There were only small numbers of records in the final category (> 500 cfu/g). This may partially explain why this variable fitted better when categorised with no specific order (nominal) than as an ordinal variable. Although the 95% C.I.s overlapped, except for this category there was an apparent 'dose-response' of the point adjusted OR estimates for the first two categories above the baseline.

Table 3.7.3.2 – Final multivariable logistic regression model for the association between generic E. coli count and presumptive STEC status based on 1009 fresh beef mince samples purchased from retail outlets in Scotland during 2019.

Risk Factor	Total[a]	Number of positives	Adjusted OR[b] (95% confidence intervals)	P value[c]
Generic *E. coli*				
BLOD cfu <10	716	104	Base	<0.001
10 ≤ cfu <50	174	65	3.04 (2.06 to 4.49)	
50 ≤ cfu <500	98	49	4.53 (2.80 to 7.33)	
cfu ≥500	21	8	2.70 (1.04 to 6.70)	
Retail Category				
1	274	37	Base	<0.001
2	269	51	1.31 (0.82 to 2.12)	
3	180	78	3.13 (1.92 to 5.12)	
4	101	18	1.08 (0.56 to 2.06)	
5	185	42	1.64 (0.99 to 2.72)	
Season of sampling				
Spring	281	63	Base	0.015
Summer	234	66	1.29 (0.83 to 2.00)	
Autumn	289	64	0.92 (0.60 to 1.41)	
Winter	205	33	0.57 (0.34 to 0.94)	

a: totals may not equal 1009 if there were missing data. Please see text for details.
b: adjusted OR calculated using logistic regression with Geographic Area as a random effect. The model constant is 0.13 (95% C.I.: 0.08 to 0.20). Sigma, the measure of between cluster variation, is 0.14 (95% C.I.: 0.02 to 1.15) and rho, the measure of within cluster variation, is 0.01 (95% CI: 0 to 0.29). The likelihood ratio test provided no evidence (p=0.297) of within cluster correlation.
c: calculated using the likelihood ratio test.

3.7.4 Confirmed STEC positive relative to all samples tested (n=1009)

Thirty-five samples had STEC colonies isolated (O157 and non-O157).

The country of origin was removed from the analysis for the same reasons as given for the previous analyses (i.e. only three samples originated outside of the UK). Due to model instability, the crude odds ratio and associated values cannot be reported for the variable 'product range'.

None of the other independent variables had evidence of an association with confirmed STEC status of a sample (Table 3.7.4.1). The categorised continuous variables ACC, generic *E. coli*, and coolbox temperature all fit better as ordinal categorised variables (rather than nominal categorised variables).

Table 3.7.4.1 – Univariable analysis of factors associated with confirmed STEC status for 1009 fresh beef mince samples purchased from retail outlets in Scotland during 2019.

Risk Factor		Total[a]	Number of positives	Crude OR[b] (95% confidence interval)	P value[c]	Rho: within cluster variation (95% confidence interval)	P value for Rho statistic
ACC category[24]		1009	35	0.94 (0.65 to 1.37)	0.760	0.01 ($1.22e^{-6}$ to 1)	0.410
Generic *E. coli*[24]		1009	35	0.99 (0.63 to 1.56)	0.978	0.01 ($9.64e^{-7}$ to 1)	0.412
Retail Category							
	1	274	11	Baseline	0.699	0.01 ($7.24e^{-7}$ to 1)	0.415
	2	269	10	0.92 (0.39 to 2.21)			
	3	180	7	0.97 (0.37 to 2.55)			
	4	101	4	0.98 (0.31 to 3.16)			
	5	185	3	0.39 (0.11 to 1.43)			
Fat Percentage							
	≤ 10%	257	6	Baseline	0.140	0.01 ($1.40e^{-10}$ to 1)	0.455
	> 10%	484	22	1.99 (0.80 to 4.99)			
Season							
	Spring	281	9	Baseline	0.948	0.01 ($4.85e^{-8}$ to 1)	0.948
	Summer	234	9	1.20 (0.46 to 3.10)			
	Autumn	289	9	0.97 (0.38 to 2.48)			
	Winter	205	8	1.22 (0.46 to 3.24)			

[24] continuous variable used as ordinal categorical variable – interpretation: for each categorical increase in the independent variable (i.e. ACC or generic *E. coli*), the odds of a mince beef sample being confirmed STEC positive increases by the value of the crude OR.

Risk Factor	Total[a]	Number of positives	Crude OR[b] (95% confidence interval)	P value[c]	Rho: within cluster variation (95% confidence interval)	P value for Rho statistic
Number of days between sampling & use-by-date						
≤ 0 days	50	2	Baseline	0.806	0.01 ($3.30e^{-6}$ to 0.98)	0.400
> 0 days	959	33	0.83 (0.19 to 3.68)			
Type of Packaging						
Loose	70	1	Baseline	0.354	0.01 ($1.31e^{-7}$ to 1)	0.427
Pre-Packed	939	34	2.58 (0.35 to 19.17)			
Packaging atmosphere						
Unmodified	88	1	Baseline	0.240	0.01 ($2.31e^{-8}$ to 1)	0.436
Modified	920	34	3.32 (0.45 to 24.60)			
Weight (g)						
≤ 500g	904	33	Baseline	0.369	0.01 ($1.05e^{-8}$ to 1)	0.440
> 500g	105	2	0.52 (0.12 to 2.19)			
Cool box temperature (°C)[24]	1009	35	1.09 (0.65 to 1.81)	0.753	0.001 ($1.9e^{-31}$ to 1)	0.488

a: totals may not equal 1009 if there were missing data. Please see text for details.
b: crude OR calculated using logistic regression with Geographic Area (cluster variable) as a random effect
c: calculated using the Wald Chi-Squared statistic.

3.7.5 Factors potentially associated with the confirmation (by culture and isolation of a STEC positive colony) of presumptive STEC positive minced beef samples

There were 226 presumptive STEC positive minced beef samples. Of these, 35 were confirmed STEC positive by culture and isolation.

The country of origin was removed from the analysis as there were no confirmed STEC samples for which this was outside of the UK. The univariable analysis for the product range was unstable so has not been reported.

Univariable analysis: there was weak evidence (p=0.057) that the ACC (as an ordinal variable) of a sample was associated with the odds of a presumptive STEC sample being confirmed positive on culture, with GA included as a random effect (Table 3.7.5.1).

There was strong evidence (p=0.004) that, with GA included as a random effect, the generic *E. coli* count (as an ordinal variable) of minced beef was associated with the odds of a presumptive STEC sample being confirmed positive on culture (Table 3.7.5.1).

There was significant evidence (p=0.028) that the Retail Category that the minced beef was purchased from was associated with the odds of a presumptive STEC sample being confirmed positive on culture and isolation. Retail Categories 3 & 5, each compared to Category 1, had a significantly lower odds of presumptive STEC samples being confirmed positive (Table 3.7.5.1).

Multivariable analysis of the relationship between ACC & confirmed STEC: after adjusting for the Retail Category, there was no evidence (p=0.184) of an association between the ACC and presumptive STEC samples being confirmed positive on culture (adjusted OR 0.73, 95% C.I.: 0.45 to 1.16).

Multivariable analysis of the relationship between generic *E. coli* & confirmed STEC: after adjusting for the Retail Category, there remained significant evidence (p=0.016) of an association between the generic *E. coli* and presumptive STEC samples being confirmed positive on culture (adjusted OR 0.50, 95% C.I.: 0.29 to 0.88). However, after adjusting for generic *E. coli,* the evidence for an association between Retail Category and presumptive STEC samples being confirmed positive on culture and isolation was reduced (p=0.064). The adjusted odds ratios only changed slightly.

The accepted final model for the odds of a presumptive STEC sample being confirmed positive on culture therefore included an association with the generic *E. coli* count (as an ordinal categorised variable), and GA as a random effect. For every categorical increase in generic *E. coli*, the odds of a presumptive STEC positive minced beef sample being confirmed positive roughly halved (Table 3.7.5.1)

Table 3.7.5.1 – Univariable analysis of factors potentially associated with the confirmation (by culture and isolation of an STEC positive colony) of presumptive STEC positive minced beef samples in Scotland, in 2019 (n=226)

Risk Factor	Total[a]	Number of positives	Crude OR [a] (95% confidence interval)	P value[b]	Rho: within cluster variation (95% confidence interval)	P value for Rho statistic
ACC category[25]	226	35	0.67 (0.45 to 1.01)	0.057	0.04 (0.001 to 0.66)	0.276
Generic *E. coli* category[25]	226	35	0.45 (0.26 to 0.78)	0.004	0.08 (0.007 to 0.48)	0.142
Retail Category						
1	37	11	Baseline	0.028	0.08 (0.01 to 0.50)	0.149
2	51	10	0.59 (0.21 to 1.64)			
3	78	7	0.22 (0.07 to 0.65)			
4	18	4	0.66 (0.16 to 2.61)			
5	42	3	0.16 (0.04 to 0.82)			
Season of sampling						
Spring	63	9	Baseline	0.551	0.04 (0.001 to 0. 66)	0.272
Summer	66	9	0.93 (0.33 to 2.59)			
Autumn	64	9	1.02 (0.37 to 2.83)			
Winter	33	8	1.94 (0.65 to 5.82)			
Fat Percentage						
≤ 10%	55	6	Baseline	0.211	0.01 ($1.84e^{-10}$ to 1)	0.454
>10%	119	22	1.86 (0.70 to 4.90)			
Number of days between sampling & use-by-date	16	2				

[25] continuous variable used as ordinal categorical variable –interpretation: For each categorical increase in the independent variable (i.e. ACC or generic *E. coli*), the odds of a presumptive STEC positive mince beef sample being confirmed on colony isolation increases by the value of the crude OR.

Risk Factor	Total[a]	Number of positives	Crude OR [a] (95% confidence interval)	P value[b]	Rho: within cluster variation (95% confidence interval)	P value for Rho statistic
≤ 0 days	210	33				
> 0 days			Baseline	0.775	0.04 (0.001 to 0.63)	0.264
			1.26 (0.26 to 6.19)			
Packaging atmosphere						
Unmodified	11	1	Baseline	0.583	0.04 (0.001 to 0.64)	0.270
Modified	215	34	1.81 (0.22 to 14.91)			
Type of packaging						
Loose	11	1	Baseline	0.563	0.02 (0.001 to 0.64)	0.270
Prepacked	215	34	1.81 (0.22 to 14.91)			
Weight of sample (g)						
≤ 500	203	33	Baseline	0.404	0.03 (0 to 0.80)	0.325
>500	23	2	0.52 (0.11 to 2.41)			
Coolbox temperature (˚C)25	226	35	1.23 (0.68 to 2.23)	0.487	0.04 (0.001 to 0.59)	0.244

a: totals may not equal 1009 if there were missing data. Please see text for details.
b: crude OR calculated using logistic regression with Geographic Area (cluster variable) as a random effect
c : calculated using the Wald Chi-Squared statistic.

4. Discussion

In 2019 the project team completed the first microbiological survey of fresh minced beef on retail sale in Scotland. From this survey, baseline data have been generated about significant microbiological pathogens (*Salmonella*, *Campylobacter* and STEC) and process hygiene indicator organisms present in fresh minced beef on sale to the consumer in Scotland. Initial estimates of their frequency of occurrence (prevalence) have been obtained. The antimicrobial resistance patterns of the pathogens and a selection of the generic *E. coli* isolates were elucidated. Patterns of variation have been analysed to identify any potential risk factors associated with microbial contamination.

4.1 Methodology – the sampling plan

The aim was to ensure that the survey design was statistically robust enough to address the primary requirement of being representative of the consumer purchasing pattern, within the constraints of available resources and logistics. A two-stage sampling strategy was used to incorporate both the geographical location and the market share of the different categories of retail outlets. Secondary requirements, such as temporal aspects, and product origin and type, were also captured within the limits of the sample size.

As a balance has to be struck between theory and what happens in practice, it is not entirely surprising that there were minor deviations from the sampling plan. These occurred due to a number of factors. There was no available complete up-to-date list of all retail outlets currently operating within Scotland. This meant substantial time and effort was required to generate such information through publicly available resources. The main factor that led to deviations from the sampling plan was occasional difficulties in obtaining the requisite number of samples, for a week, in a specific GA. This occurred for two reasons, firstly, insufficient retail outlets in a Retail Category and secondly, insufficient product types in the retail outlets in a Retail Category. In some cases this was because retail outlets selected from the master list for a visit were found to be no longer in business. The sampler needed to move on to the next listed retail outlet in the list. However, if there were insufficient retail outlets on the list for the GA, in that Retail Category, and only a small range of product types available then it was not possible to purchase sufficient samples. In some GAs this could be addressed after discovery by removing the 'no longer in business' premises from the master list, increasing the number of retail outlets selected for the next visit list and requesting the collection of additional samples in a later round. However, this was not possible where a GA had only a limited number of retail premises within a Retail Category and insufficient product types. A short 'catch-up' round between round 2 and 3 was introduced to try and remedy any remaining shortfall in round 1 and 2, and pre-empt any under-sampling in round 3. It was understood that this might lead to additional samples being purchased over and above those that were required to complete the original sampling plan.

The other factor that contributed to deviations from the sampling plan is human fallibility. In order to achieve the countrywide purchase of samples required there were two main options. Firstly, a small dedicated project team of purchasers who would travel to the areas each

operating week and purchase the required samples before delivering them to the appropriate Scientific Services laboratories. This would have entailed a substantial budget for travel and subsistence. The alternative was to use, as implemented, an existing network of local personnel already conversant with and experienced in the transport and submission methods used by Scientific Services, thus providing sustainability over the duration of the survey. Even so, in some cases, considerable distances needed to be travelled to achieve the required coverage. The disadvantage of this approach was multiple samplers, cascaded training and the potential for increased variation in interpretation and application of the sampling plan instructions.

There was a difference between the planned and achieved sampling, with the closest fit to the originally planned distribution of samples, across both GAs and Retail Category, achieved using 1004 of the 1009 purchased samples. It is highly unlikely that this has had a significant impact on the findings.

4.2 Microbiological baselines

4.2.1 Process Hygiene Indicator Organisms

In order to establish a meaningful baseline for the process hygiene indicators, the category thresholds for these organisms were based on Regulation EC No. 2073/2005[26]. However, there was a difference between sampling strategy in this study and the sampling described in EC No 2073/2005[17]. Firstly, the samples in this study were collected from retail outlets rather than at the end of manufacture. Secondly, a single sample was collected rather than five as prescribed by the regulation. Therefore, the results in this study are not directly comparable with findings from investigations in accordance with EC No 2073/2005[17] and the experience of the project team lies with ready to eat products. This makes comparisons difficult, especially given the lack of any other publicly available information.

The purpose of testing process criteria is not usually to assess the fitness of an individual carcase or sample but to provide an indication of performance and control of the whole process from slaughter to point of sampling, at the time of sampling. Combining this with the fact that the samples were not taken in accordance with the regulation means that the results should not be used to label individual samples as 'unsatisfactory' or 'satisfactory'. The criteria in the regulation can, and did, provide useful limits for categorising these variables.

With those caveats for interpretation in place, only a very small proportion of samples had ACCs below the limit of detection of the method used, with almost one in three samples being in the highest category with ≥ 5 x 10^6 cfu ACC/g minced beef. ACC is a general measure of the background microbiological status of the meat. The bacteria counted in ACCs

[26] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R2073&from=EN

include those arising from animals, from the slaughterhouse and from the subsequent meat processing environment. They include bacteria responsible for spoilage and so can provide an indication of the keeping quality of the meat. This may be a factor that contributes to the univariable associations seen with this variable.

Generic *E. coli* are bacteria that live in the intestines of animals (and man). They are shed in faeces. Thus, generic *E. coli* count is an indicator of faecal contamination that – in this case – has survived throughout the production process. As this count can be used as a proxy for potential risk from faecally transmitted pathogens, it is therefore good to find that more than two-thirds of the samples had generic *E. coli* counts below the limit of detection. To the project team's knowledge, the WGS serotype results from the 100 generic *E. coli* is the first available data about the diversity of serotypes found. The randomised selection of the 100 generic *E. coli* for WGS should have ensured that those selected were representative of all isolates. The isolate archive contains a single generic *E. coli* isolate from each of the samples from which generic *E. coli* were grown. They can be made available for further study if required.

4.2.2 Campylobacter

The baseline estimate of the prevalence of *Campylobacter* in fresh minced beef from retail outlets in Scotland during 2019 of 0.1% (95% C.I.: 0 to 0.7%) that was estimated from this survey falls within, and towards the lower end of, the range reported in the European literature. It should be noted that the diagnostic and study methodologies vary across all these studies and so while not directly comparable, they provide an indicative range and context within which to place the results of the Scottish 2019 fresh beef mince samples from retail outlets.

Turnbull and Rose (1982) reported a prevalence of 1.0% in minced beef at retail and other outlets in England based on 2015 samples. In Belguim, a *Campylobacter* prevalence of 0.6% was reported from a survey of 110 abattoirs, from which 300 minced beef samples were collected every year between 2000 and 2003 (Ghafir *et al.,* 2007). It is lower than a study in the UK from 2003 to 2005 that estimated the prevalence of *Campylobacter* in red meat to be 4.9% (Little *et al.,* 2008), although it is unclear whether minced beef was included in the sampling for that study and how retail outlets were selected. In a more recent 12-month survey of butchers and large supermarkets in the Republic of Ireland, mince beef was specifically collected for PCR and a *Campylobacter* prevalence of 36% (Lynch *et al.,* 2011) was demonstrated.

International retail outlet studies in a similar time frame have also yielded a range of estimates of the prevalence of *Campylobacter* in minced beef products: these include 3.5% in New Zealand 2003-2004 (Wong *et al.,* 2007); 7.35% in the US in 2010 (Vipham *et al.,* 2012) and two Canadian studies, one in Alberta in 2013 and another in Saskatchewan in 2011-12, reported a Campylobacter prevalence of 0% and 16.2% respectively (Narvaez-Bravo *et al.,* 2017 and Tronkhymchuk *et al.*, 2014).

The baseline estimate for *Campylobacter* in fresh minced beef from this 2019 Scottish survey is also lower than those obtained from meat on retail sale in the UK that comes from other species. The prevalence of *Campylobacter* in lamb was estimated as 7.4% in the UK, with pork a little lower at 5% (Little *et al,.* 2008). A higher value (22%) was reported for pork in Northern Ireland (Lynch *et al.,* 2011), and *Campylobacter* most frequently occurs in retail chicken at 22% in Northern Ireland (Lynch *et al.,* 2011) and 60.9% in the UK (Little *et al.,* 2008).

Given the higher *Campylobacter* prevalence estimates at the retail point of sale for other species, it is reassuring that the 2019 survey has resulted in a low estimate for fresh beef mince on retail sale in Scotland. This is especially the case as, although there are no contemporaneous, methodologically similar, prevalence estimates for *Campylobacter* at the beginning of the food chain i.e. on the farm, or at the slaughterhouse, previous studies have reported much higher frequencies of occurrence at both farm and animal level. In 2005 to 2006, a survey of 63 farms in North East and 71 farms in South West of Scotland found that 22% of cattle and 50% of farms were *Campylobacter* positive (Rotariu *et al.,* 2009). In Northern Ireland, faecal samples from 220 cattle in seven abattoirs over nine months found 24.8% of cattle to be positive for *Campylobacter* (Madden *et al.,* 2006). Previously, in 2003, in a 12-month survey of 93 abattoirs in Great Britain, 7703 faecal samples were collected and 54.6% were positive for *Campylobacter (*Milnes *et al.,* 2007. According to the authors of that study, their methods were comparable to an earlier study in 1999-2000 and showed no significant change in the prevalence of *Campylobacter* in cattle.

As only one *Campylobacter* isolate was obtained in this study, it was disappointing that no viable organism was available for further study (speciation, AST or WGS), despite many attempts at recovery. It is likely that the original *Campylobacter* was lost during transport through competition with the *Ochrobactrum* that was recovered from the transport swab and medium. The genus *Ochrobactrum* is ubiquitous across ecological niches and has previously been reported from cattle at slaughter (Alonso *et al.*,2017). The Gastrointestinal Bacteria Reference Unit (GBRU) at Public Health England, report *Campylobacter* losses of around 5% (pers. comm.) due to die off or contamination.

4.2.3 Salmonella

The baseline prevalence of *Salmonella* established in this survey of fresh minced beef from retail outlets in Scotland during 2019 was estimated to be low at 0.3% (95% C.I. 0% to 0.7%). As with *Campylobacter,* this falls within and towards the lower end of the range reported in the European literature. It should be noted that the diagnostic and study methodologies vary across all these studies and so while not directly comparable, they provide an indicative range and context within which to place the results of the Scottish 2019 fresh beef mince samples from retail outlets.

In Europe, the prevalence estimated in different countries varies, from 0.1% in The Republic of Ireland (FSAI, 2013) to 1.4% in England (Turnbull and Rose, 1982) and 3.4% in Denmark

(EFSA 2019). A previous study in the UK during 2003 to 2005 (Little *et al.,* 2008) assessed the prevalence of *Salmonella* in raw red meat and offal samples at point of sale. It is unclear whether minced beef was included in the sampling and how retail outlets were selected. However, of the 1563 beef samples, 1.3% were positive for *Salmonella,* with a significantly higher prevalence in offal than muscle (6.1%% compared to 1.1%) (Little *et al.,* 2008). More recently (July 2007 to June 2009), from a small study of 100 minced beef samples collected from retail outlets in the Dublin region of the Republic of Ireland, 3% were culture positive for *Salmonella* (Khen *et al.,* 2014). In two Belgian surveys, 1997-99 and 2000-03, the prevalence of *Salmonella,* based on culture and isolation, in minced beef was 4.2% (n=120) and 3.5% (n=488) respectively (Ghafir *et al.,* 2005). In Denmark in 2001 and 2002, 1.5% of 2747 minced beef samples collected from butchers and supermarkets were positive for *Salmonella* (Hansen *et al.,* 2016).

Internationally, prevalence estimates for *Salmonella* at the retail level in a variety of comparable beef products over the last 20 years, also lie in the 0-5% range. In the United States in 2010, a total of 2,885 samples (2199 minced beef and 686 whole muscle cuts) were purchased from 38 cities. Overall, *Salmonella* was detected in 0.55 and 1.02% of ground beef and whole muscle respectively (Vipham *et al.,* 2012). Another US study from 2005 to 2007, collected 4136 ground beef samples from seven regions and reported a *Salmonella* prevalence of 4.2%. Two Canadian studies did not find *Salmonella* in ground beef in 100 samples collected from four supermarket chains in Alberta (Bohaychuk *et al.*, 2006) and 134 samples from three supermarkets and one independent butchers in Alberta in 2007-08 (Aslam *et al.*, 2012). From 2003 to 2005 in New Zealand, 232 samples of minced, diced and strips of beef from supermarkets and butchers in five cities on the North and South Island were collected and the prevalence of *Salmonella* was 0.4% (Wong *et al.,* 2007).

The baseline estimate for *Salmonella* in fresh minced beef from this 2019 Scottish survey is also lower than estimates obtained for meat from other species on retail sale in the UK. The prevalence of *Salmonella* in lamb was 1.7%, 1.9% in pork, and 5.6% in chicken (Little *et al.,* 2008).

The low prevalence observed provides reassurance that existing controls, along the chain from farm to retail point of sale, are contributing to managing the potential risk to the consumer. Absence of *Salmonella* in minced meat intended to be cooked is an established food safety criterion. Compliance is demonstrated by absence from a specified number of samples per number of samples examined as specified in sub-sections 1.4 – 9, Annex 1 of Regulation EC No. 2073/2005[27]. This requires there to be no *Salmonella* positive samples in five 10 gram samples of products, placed on the market during their shelf-life, when tested using the analytical reference method EN/ISO 6579. For cattle carcases at the slaughterhouse, as a process hygiene criterion, this requires one sponge sample to be taken from each of five carcases per sampling session with two or less samples being positive over

[27] http://data.europa.eu/eli/reg/2005/2073/oj

10 consecutive sampling sessions (i.e. from 50 samples). Unsatisfactory results from minced meat sampling require the product to be removed from the market; unsatisfactory results at the slaughterhouse lead to a review of the production processes to improve process hygiene.

There are no contemporaneous estimates of the prevalence of *Salmonella* in cattle available from UK slaughterhouses, or from UK farms and at animal-level. However, historical UK animal-level prevalence studies of *Salmonella* based on faecal samples at the slaughterhouse provided estimates from 0.2% to 1.4% (Davies *et al.,* 2004, Milnes *et al.,* 2007 respectively. The former was derived from 891 cattle faecal samples from 117 red meat abattoirs, in Northern Ireland in 1999 (Davies *et al.,* 2004). The latter was from 7703 faecal samples from 93 slaughterhouses across the UK in 2003. These faecal samples were cultured for *Salmonella* and a regional variation in prevalence was observed, from 0.3% in the North West to 3.1% in the South West (Milnes *et al.,* 2007). In addition, 3% of cattle faecal samples (n=220) from seven abattoirs in Northern Ireland were found to be *Salmonella* positive (Madden *et al.,* 2006). While faecal samples provide an indication of the carriage of *Salmonella* in cattle presented for slaughter and thus the potential for exposure risk, swabbing of carcases provides an indication of contamination. Again, published UK information is limited. In the South West of England, 12.7% of 330 steer and heifer carcases, 0% of 330 cull cow and bull carcases and 20% of 80 calf carcases younger than 14 days of age, from five red meat abattoirs were positive for *Salmonella* (Small *et al.,* 2006). In 1997 in Northern Ireland, of the 200 carcases swabbed, three were PCR positive for *Salmonella* (Madden *et al*., 1998).

It had been anticipated in the planning stages for this 2019 Scottish survey that there would be approximately five isolates of *Salmonella* and *Campylobacter*, so the fact that a total of four (*Salmonella* = 3, *Campylobacter* =1) were identified meets with expectations.

The three *Salmonella* isolates all came from different retail category types. The fact that two of the same serovar (Mbandaka) were identified on the same sampling date in one Geographic Area could lead to a hypothesis of a similar supply source and/or cross-contamination. The WGS phylogenetics placed both isolates relatively closely to contemporaneous *S.* Mbandaka isolates of bovine origin from Scotland. However, the isolates were not the same. These observations suggest that the isolates were derived from the predominant clonal group endemic within the cattle population in Scotland at the time. *S.* Mbandaka is a generalist *Salmonella* and may be introduced to farms through purchased feeds such as soya bean meal or rapeseed products[28]. After which, it can be spread through the faecal/oral route between cows. It is usually diagnosed by SRUC Veterinary Services[29] in cattle that have concurrent disease, are stressed, or immunocompromised. Diagnoses have increased significantly from SW Scotland and NW England over the last five years. Environmental sampling on some affected farms has shown that the organism was

[28] https://www.sruc.ac.uk/info/120144/farm_animal_diagnostics/2028/salmonella_mbandaka
[29] SRUC Veterinary Services, ON THE HOOF, South West Veterinary Services - Bitesize local updates, 06/11/2020

widespread in the cows' environment and feed stores. It could, therefore, be expected that cattle exposure on affected farms was widespread.

4.2.4 STEC

The baseline prevalence of presumptive STEC generated in this survey of fresh minced beef from retail outlets in Scotland during 2019 was 22% (95% C.I.: 20% to 25%). This was much higher than the anticipated prevalence, which was 4%. The figure for the anticipated prevalence had been reached in discussion with Scientific Services and Gastrointestinal Bacteria Reference Unit, Public Health England (GBRU, PHE).

The baseline prevalence estimate of confirmed STEC was slighter higher than anticipated at 4% (95% C.I.: 3% to 5%), rather than the anticipated 2.5%. The majority of these confirmed isolates were non-O157 STEC, with very few STEC-O157 confirmed by culture. The planning estimates for STEC prevalence were based on the expected recovery rate and culturable STEC O157 in previous work in England and more widely across Europe, which ranged from 0.1 – 2.9% (Vernozy-Rozand *et al.,* 2002, Cagney *et al.,* 2004).

The PCRs used for the initial identification and assignation of presumptive STEC and/or O157 status can detect as few as five copies of a given target. They demonstrate that the target nucleic acids are present. Culture and isolation demonstrate that viable bacteria are present. A difference between the number of enrichment broths found to be positive by PCR and the number of isolated STEC or *E. coli* O157 has been noted before in other studies. The rate of STEC PCR positive samples found in enriched ground beef culture ranged from 8 to 78%, while rates for isolation of STEC from the same samples were between 5 and 27%. These studies occurred in Australia, Asia, North and South America and South Africa (Barlow *et al.,* 2006; Ju *et al.*, 2012; Brusa *et al.*, 2013; Hoang Minh *et al.*, 2015; Toro *et al.*, 2018 and Onyeka *et al.*, 2020).

In America, 4133 samples of minced beef were collected from 18 commercial producers over 24 months. Of these samples, 24.3% were PCR positive and 7.3% of samples were confirmed on isolation (Bosilevac *et al.,* 2011). From 1998 to 1999 in Australia, 285 minced beef samples from 31 butchers were collected and 26% were PCR positive for STEC genes and 16% had STEC isolated (Barlow *et al.,* 2006).

A similar survey that targeted *E. coli* O157, not STEC, had positive rates of 33% and 10% for PCR and culture respectively (Chapman *et al.*, 2001). So, while the overall recovery rate in the 2019 Scottish survey at just over one in five is low, it is not exceptional.

In addition to the aforementioned fact that a PCR test only demonstrates that the target nucleic acids, not viable organisms, are present, there are a number of other factors that may contribute to culture negative results from positive PCR enrichments. These include, in this study, that the testing could not be completed on identical material. It involved a sub-aliquot of the initial enrichment broth. This may contribute to explaining the difference in the PCR

results from Scientific Services and from SRUC Inverness. In addition, loss of the *stx* prophage during subculture (Senthakumaran *et al.*, 2018) and the possibility of (sub)lethal injury to target organisms during either meat processing, or the freezing and thawing of the enrichment culture, may also contribute to the observed results. It is also possible that pathogenic *E. coli* may have been outcompeted, or their presence masked, by other bacteria either in the enrichment broth or on solid culture i.e. when there are a lot of *E. coli* bacteria present in a sample, if one or a few are STECs then the initial PCR may be sensitive enough to detect the stx genes; however, this may not result in culture of a viable colony, by which to confirm the STEC status of the sample. The fact that the accepted final model for the odds of a presumptive STEC sample being confirmed positive on culture included an association with the generic *E. coli* count in which, for every categorical increase in the generic *E. coli* count, the odds of a presumptive STEC positive minced beef sample being confirmed positive roughly halved (Table 3.7.5) lends support to this theory.

Isolation of PCR positive organisms are further hampered because there are no obvious markers that distinguish sorbitol fermenting STEC/O157 from other *E. coli*. A compromise has to be achieved between the resources available and the degree of effort expended to track down the potential source of a PCR positive result, for example the number of colonies tested per PCR positive broth. The joint microbiological expertise within the project team identified that a reasonable cut off for searching for isolates from the PCR positive samples within this survey was 70 colonies per sample. It is possible that with additional resources, the culture recovery rate would have been higher. This highlights the need to define what is 'fit for purpose' and to be careful about making direct comparisons with other studies, or surveys, without a close examination of not only the design and sampling strategies but the details of the diagnostic methods used. It highlights the need to consider carefully what the purpose is within the context and resources available. It may not be necessary, or possible, in a survey or surveillance program that is designed to estimate prevalence within specified bounds to go to the same effort and endpoint that would be required and expected for detection and identification of cases in an outbreak scenario. The apparent prevalence estimates for the 2019 Scottish retail fresh beef mince survey have been reported here i.e. without any adjustment for test sensitivity and specificity. Their relatively narrow 95% C.I.s demonstrate that the sample size of the study was sufficient to produce robust baseline estimates, given the resources available. The testing protocol used was according to regularly used standardised protocols. An advantage of using BPW as a growth buffer is that it is less harmful to non-O157s, as well as *E. coli* O157s; the disadvantage is that it will not have discouraged the growth of background flora. There are specialised media available that could help to identify different strains of STEC and non STEC[30]. However, they also have their advantages and disadvantages. One alternative is to modify the Ct value used as a cut-off for

[30] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4483743/

the designation of a PCR positive, another is to explore additional methods, such as acid pre-treatment to optimise recovery.

In 2020, the EFSA BIOHAZ panel published a Scientific Opinion[31] in which the public health risk posed by contamination of food with STEC was reviewed. In it they described the various methods used to provide evidence of the presence of STEC. They highlighted that "*Methods based on PCR are the most appropriate approaches to detect STEC in complex matrices*" with the detection of the *stx* gene(s) being the only true discriminant between STEC and other *E. coli.* However, "*Currently, if one or more stx genes are detected in foods during routine testing, it does not provide sufficient evidence that viable STEC capable of causing human disease is present in the matrix. Since Stx phages can be present in foods, these may result in false-positive findings*.", thus confirmatory isolation of STEC from all samples where *stx* genes are isolated is required. Both of the methods used in this 2019 Scottish retail beef mince survey (CEN ISO/TS 13136:2012 & ISO 16654:2001) were noted to be standard methods. The latter is the first standard for the detection of STEC in food, although it is focused on detection of O157 *E. coli*, and the former is under revision at the time of writing this report.

With the caveats on detection and methods mentioned above in mind, previous published estimates of the prevalence of STEC in a range of beef products at the retail level vary. In the UK, between 1996 to 1997, 1.1% of 2075 samples of minced beef from 81 butchers in Yorkshire were O157 *E. coli* positive with one or more STEC genes (Chapman *et al.,* 2000). This is substantially lower than the 22% presumptive STEC prevalence estimate obtained here; however, not only has more than two decades passed between the two studies, but there is also the possible effect of geographical representation (or lack of it) to consider. A survey of raw beef in the UK reported a STEC prevalence by "DNA probes" of 17% (Willshaw *et al*., 1993).

A Scientific Report published by the European Food Safety Authority reported an overall European prevalence of STEC in bovine meat samples of 0.3% to 2.3% from 2007 to 2009 with 0.1% to 0.7% being O157 STEC. The proportion of positive samples varied between the individual Member States ranging from 0% to 14.9% (EFSA, 2011).

From Europe, in a survey of mince beef on retail shelves between 1995 and 2003, in Spain, 12% of samples were PCR positive for STEC. Similar to the 2019 Scottish survey, the majority (11%) were non-O157 STEC, only 1% were STEC O157:H7 (Mora *et al.,* 2007), although it is a slightly lower proportion of STEC O157 (0.08 c.f. 0.14). Lower levels of PCR STEC positive minced beef samples were reported from small butchers in Switzerland during 2000 (2.3%, Fantelli *et al.,* 2001) than from the 2019 Scottish survey. Additionally, a lower prevalence of PCR STEC O157 positive minced beef samples was reported from retail outlets in Italy during 2000-2001 (0.43% Conedera *et al.* 2004); in the Republic of Ireland,

[31] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2020.5967

2.8% of 457 mince beef samples were O157:H7 positive with 20 of the 43 isolates having one or more of the STEC genes (Cagney *et al.,* 2004).

In the UK, cattle alongside other ruminants are considered as one of the major reservoirs of infection for human clinical cases of STEC, particularly O157 STEC. While the role of environmental reservoirs is recognised, source attribution has not been fully elucidated, and both beef and lamb have been implicated as foodborne sources of human infection (Wilson *et al.,* 2018). In the EU (2007-2009), the prevalence of STEC in sheep meat samples ranged from 0 to 10.5%, while that in cattle varied widely between the Member States from 0% to 48.5% (EFSA, 2011). In the most recent (2014-2015) British countrywide study of *E. coli* O157 in cattle herds, between a fifth and a quarter of farms were found to be positive for *E. coli* O157 on culture and isolation from faecal pat samples (23.6% (16.6–32.5) of Scottish farms and 21.3% (15.6%–28.3%) of English and Welsh farms (Henry *et al.,* 2019)), with the presence of *stx* genes confirmed by PCR in almost all isolates. In 2002 and 2004, when cattle from 34 farms were followed to 12 slaughterhouses in Scotland, 55% of 222 hides tested positive (after immunomagnetic separation and culture) for O157 STEC, (Mather *et al.,* 2007). With such a high frequency of occurrence at farm and slaughterhouse level, and given the ubiquitous nature of these organisms, it is perhaps not surprising to detect so many presumptive PCR positive samples in fresh beef mince samples. This is especially so given that mince is often an economical product, often containing surface parts of the carcase and it may consist of meat from multiple carcases. The production process can distribute organisms throughout the product (Meat Industry, 2019).

A diverse range of serotypes were identified from the 35 confirmed STEC isolates, with three O157:H7 STEC isolates. Two of these shared 100% similarity, and matched Scottish clinical strains from a contemporaneous UK outbreak of O157 *E. coli* phage type (PT) 21/28. The third also clustered closely to a human clinical case from 2016, highlighting the power of WGS to identify isolates from common or similar origins.

The other mince isolates (serotypes O26:H11, O113:H4 and O113:H21, with their associated virulence combinations) that were ranked as having “high/highest” risk potential for severe disease have also been observed in Scottish clinical cases. However, not all serotype/virulence combinations detected in this mince study reflect what has been observed in Scottish clinical cases. There could be a number of reasons for this. The Scottish diagnostic laboratory screening algorithm, like many other countries, is weighted towards the detection of O157 *E. coli*. Non-O157 STEC are only detected and isolated from patients with more severe disease (as explained in more detail in the following section). Therefore, it is possible that the other strains isolated from mince may only be associated with milder disease and these would not be detected as clinical cases of infection. JEMRA classification backs up this theory, with 69% (24/35) of the mince strains assigned to Level 5 - low risk potential to cause diarrhoea (not bloody diarrhoea or HUS).

4.3 Antimicrobial sensitivity

The overall prevalence of phenotypic non-susceptibility was in line with expectations from surveys of generic *E. coli* from faecal samples reported in the 2020 Scottish One Health Antimicrobial Use and Antimicrobial Resistance (SONAAR) report[32]. Samples were taken from healthy cattle at the slaughterhouse in Scotland in 2017, 2018 and 2019. The antimicrobials selected for the phenotypic panel in this 2019 Scottish fresh beef mince survey were chosen to be comparable with the panel used in the slaughterhouse surveys. Most of the phenotypic non-susceptibility profiles detected in the isolates from the fresh mince samples were in generic *E. coli*. They were non-susceptible to single, commonly used, first-line active substances that have a long history of use in ruminant populations (e.g. tetracycline, ampicillin). No lack of phenotypic susceptibility was identified to any of the critically important antimicrobials that were tested for by disc diffusion. Overall, the evidence for resistance from both the phenotypic and genotypic methods are broadly comparable, although it was not considered appropriate, due to the small numbers of isolates, to formally test for concordance. Comparability between methods has been observed in several studies including those focused on foodborne pathogens and Enterobacteriaceae. High concordance (>96%) between the presence of known AMR genes or mutations and Minimum Inhibitory Concentration (MIC) of several antimicrobials at or above the epidemiological cut-off value or clinical breakpoint for resistance has been seen (see Hendriksen *et al.,* 2019 for a summary).

Although all the isolates were phenotypically susceptible to the critically important antimicrobials that were tested for by disc diffusion, a single isolate of O-:H12 *E. coli* was found to possess a single point mutation resulting in a single amino acid change (Serine to Leucine at position 83) in the chromosomal DNA gyrase gene (*gyr*A). This spontaneous mutation is not unusual in Enterobacterales and is known to be sufficient to generate reduced susceptibility to fluoroquinolones. However, further accumulation of amino acid changes in *gyr*A and the simultaneous presence of *par*C alterations are required for the development of high level resistance (Bansal and Tandon, 2011); these were not seen. Low level fluoroquinolone resistance is of concern in *Salmonella* for which the EUCAST clinical breakpoint has been reduced to 0.06mg/L[33], in light of evidence of treatment failures in the medical field. However, for the other Enterobacterales, the breakpoint remains at 0.25mg/L. The percentage of human *E. coli* bacteraemia isolates in Scotland which were non-susceptible to ciprofloxacin in 2018 ranged from 15.5% in the community, to 26.2% in healthcare associated infections[34]. In the same report, 1.4% of *E. coli* isolates from healthy cattle sampled at Scottish slaughterhouse sampling in 2017 were non-susceptible to ciprofloxacin. This was not seen in the fresh beef mince samples.

[32] https://hpspubsrepo.blob.core.windows.net/hps-website/nss/3133/documents/2_2020-11-17-sonaar-2019-report.pdf

[33] https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf

[34] https://www.hps.scot.nhs.uk/publications/hps-weekly-report/volume-53/issue-45/hps-publishes-sonaar-annual-report/

The lack of phenotypic susceptibility that was seen to colistin is a recognised phenomenon amongst certain *Salmonella* isolates (Agers *et al.,* 2012; Ricci *et al.,* 2020). Analysis of the whole genome sequence of this organism found no evidence of *mcr* genes (i.e. no evidence of recognised colistin resistance genes conferred by mobile genetic elements). This finding was therefore not a cause for concern.

The frequency of AMR gene carriage in the confirmed STEC isolates from the survey (only two of the 35 (5.7%) study STEC isolates) is considerably lower than that detected among STEC isolates associated with clinical infection in Scotland (~14-17%). The frequency of one or more AMR genes detected in non-O157 STEC from human cases in England from 2014-2016 was also substantially higher at 27.3% (Gentle *et al.*, 2020) than detected in this 2019 survey of fresh beef mince on retail sale in Scotland. Two-thirds of their resistant isolates were resistant to three or more classes of antibiotic, with good overall concordance (97.7%) demonstrated between the phenotypic and genotypic methods (Gentle *et al.*, 2020). As previously stated, concordance has not been estimated for the survey due to the small numbers of isolates with evidence for AMR. The difference noted above between the pattern in human clinical cases and that in fresh beef mince raises the possibility of an hypothesis that the risk of transmission of multidrug-resistant STEC from cattle to humans is low via the foodborne route, with a level of selection pressure occurring later in the chain between consumption and sampling for clinical diagnosis. Alternatively, it may be influenced by the aforementioned bias, due to the way that diagnostic methods are applied to samples from human clinical cases.

4.4 Potential risk factors

The low numbers of the pathogenic isolates *Campylobacter* and *Salmonella* limit the extent of the statistical analysis that can be performed. Therefore, analysis of any associations that may indicate potential risk factors has been limited to the STEC positive samples.

The higher numbers of presumptive STEC positive samples facilitated more in-depth statistical analysis. When adjusted for Retail Category, Season and the random effect of Geographic Area, there was strong evidence ($p<0.001$) that samples of minced beef with a generic *E. coli* count above the limit of detection had higher odds of being presumptive STEC positive compared to minced beef samples with a generic *E. coli* count below the limit of detection. As for *Salmonella* & *Campylobacter,* this is biologically plausible; the lower standards of hygiene indicated may lead to increased contamination with faecal pathogens if they are present. In addition, it is possible that the more *E. coli* organisms that are present, the more likely that there will be *E. coli* with *stx* genes present in the population. There was no clear dose-response relationship between generic *E. coli* count and presumptive STEC as, although the point estimates for the first two categories above the baseline increased consecutively, the 95% confidence intervals overlapped. However, both the lower and the upper limits of the confidence intervals increased, therefore, it is possible that with a larger sample size a trend may be seen. Additionally, the small size of the largest category may

have contributed to a lack of an increased OR for this category. An alternative is that there is no true population dose-response relationship between generic *E. coli* count and presumptive STEC. In this case, once generic *E. coli* are present in sufficient numbers to be detected, further increases in generic *E. coli* count would not change the odds of a sample being presumptive STEC positive.

There was strong evidence that Retail Category was associated with the odds of a sample of minced beef being presumptive STEC positive when adjusted for Season, generic *E. coli* count and the random effect of Geographic Area. This was driven by the odds for a sample purchased from an outlet belonging to Retail Category 3 being three times (Adjusted OR 3.13 (95% CI 1.92 to 5.12) that of one purchased from the baseline category, Retail Category 1. Retail Category 3 premises only sold pre-packed minced beef. Pre-packed mince had a higher proportion of presumptive STEC positive samples than loose mince samples. However, the evidence for an association between packaging and presumptive STEC was weak (p=0.169), so packaging was not considered for inclusion in the multivariable model development. If the study power had been higher, the type of packaging may, or may not, have shown a stronger association with presumptive STEC samples and, if the former, been included for consideration; it may not have been retained. Retail Category 3 premises also had fewer samples with generic *E. coli* below the limit of detection, so it is possible that the model does not fully adjust for this effect. Given the interplay seen at the univariable level between presumptive STEC status, ACC, generic *E. coli* count, packaging atmosphere, packaging type and Retail Category, it would be inappropriate to consider this finding as a cause for concern about fresh mince sourced specifically from Retail Category 3. There would need to be further investigation of the role of these factors. It is also possible that additional factors within the distribution and supply chain - that it was not possible to capture in this survey - have led to clustering.

The evidence for an association between a minced beef sample being presumptive STEC positive and Season was driven by a reduction in the odds for those samples collected in Winter (January, February and December) compared to the baseline of Spring (March to May, inclusive). It might be hypothesised that this is due to environmental temperature influencing the cold chain and bacterial growth. However, all samples were transported in a cool box and the median temperature of the cool box and the interquartile range was the same in the anticipated extremes of environmental temperature – summer and winter (median 4°C, IQR 2°C to 5°C). Again, there are many other hypotheses and factors for which season may be a proxy, some of which occur earlier in the supply chain, pre-purchase.

The survey was planned around the primary aim of establishing a baseline for the microbiological quality of retail fresh beef mince on sale in Scotland. Given the low estimated prevalence of the pathogens the statistical power of the analysis to investigate variation and potential risk factors was low. Type II errors are likely i.e. some potential risk factors and associations may not have been identified, although they exist. Furthermore, as with all cross-sectional studies, the study design only allows hypotheses to be raised about the

associations that are detected. A causal relationship cannot be inferred, and there is no temporal component (cause versus effect). There is potential for biologically plausible factors that have been identified to be risk factors. However, it would not be appropriate to develop risk mitigation measures based solely on these outcomes; further studies would be required.

4.5 Public health and policy implications

Minced meat is a raw product which is expected to be cooked before consumption. It is often an economical product containing trim as well as other surface parts of, potentially, multiple carcases. Thorough cooking should ensure food safety, even if surface pathogens are distributed through a batch by the mechanical process of the mincing of intact cuts to produce the minced meat. At the time of this 2019 microbiological survey of fresh beef mince on retail sale in Scotland, EU Rules regarding Food Hygiene covered all stages of the production, processing, distribution and placing on the market of food intended for human consumption. The legal basis behind the general obligation to provide safe food was a suite of Regulations (Regulation (EC) No. 852/2004[35], 853/2004[36] and 854/2004[37]). The Microbiological Criteria Regulation (Regulation (EC) No. 2073/2005[38]) established microbiological criteria for certain microorganisms and provided rules to be complied with by food business operators when implementing the general and specific hygiene measures referred to Regulation (EC) 852/2004[25]. These European Union Regulations were implemented directly in each Member State by national legislation that outlined the requirements for hygiene of foodstuffs and the implementation of procedures by food businesses to prevent unsafe foods.

As discussed earlier, the absence of *Salmonella* in specified samples is a food safety criterion that applies to minced meat intended to be eaten cooked, with the definition of 'absence' based on multiple repeated sampling and a threshold number of positives. The baseline prevalence established in the 2019 survey and the results of the WGS should provide reassurance that existing controls along the chain from farm to retail point of sale are contributing to managing the potential risk to the consumer. However, achieving a state where the risk is negligible is not feasible. Therefore, in addition to hygienic production controls, these products will require labelling with advice on cooking and safe handling, and consumers will need to be educated about their responsibilities, in order to minimise the risk to human health.

There are no European regulations that require testing for the presence of STEC in the final minced meat product. Process hygiene criteria that apply, as part of the Hazard Analysis and Critical Control Point (HACCP) approach, to carcases of cattle (ACC and Enterobacteriaceae) after dressing but before chilling and for the minced meat product at the

[35] http://data.europa.eu/eli/reg/2004/852/2009-04-20
[36] http://data.europa.eu/eli/reg/2004/853/2014-11-17
[37] http://data.europa.eu/eli/reg/2004/854/2015-01-01
[38] http://data.europa.eu/eli/reg/2005/2073/oj

end of the manufacturing process (ACC and *E. coli*) are deemed adequate. Regular audits and inspections by various bodies monitor enforcement of EU Food Hygiene legislation and industry standards at slaughterhouses and food retail and catering businesses.

In 2019, EFSA BIOHAZ panel reviewed[39] the public health risk posed by contamination of food with STEC. They concluded that "All Stx toxin subtypes were associated with some cases of severe illness suggesting all STEC strains are potentially associated with BD, HUS and/or hospitalisation." This is in line with the 2018 JEMRA report, in which it was stated that no STEC is without risk as all STEC strains are likely to pose some health risk, especially to susceptible individuals. Rather than categorising STEC as pathogenic or non-pathogenic, based on serotype, the JEMRA report suggested the use of "low", "high" and "highest" risk designations, based on virulence gene profile. In the 2019 survey reported here, eight isolates, representing almost a quarter of the STEC isolated from raw mince, were ranked as having the high or highest potential for causing severe disease. This is not unprecedented given that similar strains i.e. with matching serotype and virulence profile, have been identified in Scottish clinical cases of infection[40,41]. It should, however, be noted that while WGS provides the ability to identify virulence genes, or gene combinations that are more likely to be associated with severe illness, it does not mean that their presence is sufficient to cause a clinical outcome. Any prediction of clinical outcome will be uncertain as other factors, e.g. gene expression in the bacteria, and the immune status of the host, will also be important contributing factors. However, the demonstration of their presence does indicate that a potential risk exists.

From this 2019 Scottish retail fresh beef mince survey, the risk to the consumer of encountering a sample that is non-O157 STEC positive is approximately 10 times the risk of encountering a sample that is *Salmonella* positive. Based on the numbers of positive samples and subsequent prevalence estimates, the risk of a consumer encountering a sample associated with O157 STEC is very similar to that of *Salmonella* and that of the JEMRA high-or-highest classification isolates is approximately twice as much. It does, however, highlight the need for continued education. This is required to ensure that risk mitigation measures are understood and taken at the point of the supply chain directly before consumption i.e. either at home or in the retail or catering setting, whether that be in regard to appropriate handling to avoid possible cross contamination of other foodstuffs (particularly ready-to-eat foodstuffs), or to ensure that cooking methods are adequate.

While source attribution analysis[27] of data from EU outbreaks for which there was 'strong evidence' indicates that 'bovine meat and products thereof' is one of four main sources of STEC human infections in the EU/EEA, the specific contribution of fresh beef mince has not been elucidated. Additional data would need to be obtained to do this, for example, while

[39] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2020.5967
[40] http://www.foodstandards.gov.scot/news/foodborne-illness-strategy-scotland-consultation;
[41] https://www.foodstandards.gov.scot/downloads/WGS_Typing_and_Analysis_of_Non-O157_STEC_-_Jan_2020_v3.pdf

there are relatively recent estimates of the frequency of STEC O157 in Scottish cattle herds (Henry *et al.*, 2019), there is little known about current frequencies of non-O157 STEC isolates in cattle, which are more common in these mince samples than STEC O157. This is in contrast to the picture with human cases reported in Scotland in which, although *E. coli* O157 still predominates, non-O157 STEC account for 40% of all STEC cases[42]. In 2017, 2.5 times as many STEC O157 cases were reported when compared to non-O157 STEC[43] cases. Provisional data[44] from 2019 and 2018 indicate that the number of non-STEC O157 cases has increased, resulting in a ratio that is closer to 1.4. This may in part be due to changes in the referral pattern for diagnostic testing but is still very different to the ratio seen in the 2019 Scottish fresh beef mince survey (0.13).

As previously mentioned, this predominance of *E. coli* O157 in the human clinical picture is likely to be partly due to the diagnostic screening algorithm for O157 and STEC adopted by diagnostic laboratories in Scotland. All diarrhoeal samples are screened for O157 *E. coli*, usually by culture followed by a latex agglutination reaction. In contrast, non-O157 STEC are only screened for in patients with more severe disease (bloody diarrhoea and HUS) whose faeces are referred to SERL for PCR testing. This disparity may mean that non-O157 STEC causing less severe clinical disease will remain undetected unless routine diagnostic laboratories update their testing protocols to include PCR detection for STEC in all diarrhoeal samples. Commercially available PCR detection platforms for gastrointestinal pathogens including O157 *E. coli* and STEC have been developed and these increasingly form part of the enteric testing repertoire in many labs in England and the Republic of Ireland.

A further conclusion made by the EFSA BIOHAZ panel in the Scientific Opinion on the public health risk posed by contamination of food with STEC[23] was that "the methodology for STEC characterisation in food isolates is not currently standardised" at EU level. It will be necessary post-EU Exit to ensure that methods applied in Scotland, in surveys such as this, are standardised and agreed, appropriately for the context, so as to be acceptable to others. This will result in improved confidence in outputs, especially where there may be implications for trade. This issue of confidence in the methods used may also be of relevance for sustainable food production, reduction of waste in the food chain and consequently be linked to climate change and emissions policies.

In 2013, the Scottish Government published the VTEC/*E. coli* O157 Action Plan for Scotland, 2013-17[45]. The Action Plan identified twelve steps for transmission of VTEC (STEC) from source to receptor; and recommended controls applicable to each step. Three of the

[42] https://hpspubsrepo.blob.core.windows.net/hps-website/nss/3109/documents/2_stec-in-scotland-2019-full-report.pdf
[43] https://www.foodstandards.gov.scot/downloads/Factsheet_-_E._coli_-_Website_-_for_businesses_and_professionals_-_August_2017_.pdf
[44] https://www.hps.scot.nhs.uk/publications/hps-weekly-report/volume-54/issue-33/stec-in-scotland-2019-enhanced-surveillance-and-reference-laboratory-data/
[45] https://www.gov.scot/publications/vtec-e-coli-o157-action-plan-scotland-2013-2017

subgroups formed to focus on the implementation of recommendations were Communication, Food and Research & Surveillance. The final report on delivery of the Action Plan[46] was published in 2018. For the Communication sub-group it states that guidance for farmers and animal keepers on steps that should be taken to reduce transmission of all infectious agents are now provided via a website (www.scotlandshealthyanimals.com) while the communication recommendations related to food were stated to be part of 'business-as-usual' for FSS. For the Food subgroup, many of the implementation activities related to the other sectors of the food industry, rather than the red meat sector. All of the recommendations in the Food section had been considered and actioned, as appropriate. Overall, it was concluded that "Although the majority of reported cases are sporadic or household clusters, outbreaks of O157/STEC *E. coli* still continue to occur, including from environmental exposure and contaminated foods. Most likely sources identified in recent foodborne outbreaks include venison, unpasteurised cheese and salad leaves." It was highlighted that "there was an on-going need for effective communication strategies aimed at improving understanding, by consumers, of the routes for STEC contamination in the food chain, and the products which are likely to present the highest risk of food poisoning." The Research & Surveillance subgroup noted that, amongst other activities, there had been a programme of work on: risks associated with fresh produce; the research programme[47] on O157 *E. coli* in cattle and humans, for which the British O157 *E. coli* in cattle study (Henry *et al.,* 2017) provided the current baseline, and that WGS had been introduced into clinical surveillance in Scotland in 2017.

The introduction of WGS at SMiRL has significantly improved surveillance capabilities. It provides the ability to distinguish between isolates in more detail and to place them in a wider context. WGS permits improved cluster resolution and also facilitates comparison with strains isolated in other parts of the UK, resulting in the identification of UK-wide incidents and outbreaks. During 2019, four Scottish outbreaks of infection were reported (2 of O157:H7; 1 of O26:H11; and 1 of O125:H6) and all were considered to be foodborne, although this was not confirmed microbiologically. The difficulties in linking cases of STEC infection with a contaminated foodstuff are complex. By the time a potential outbreak has been identified, a case of infection must have presented at the GP, submitted a sample from which an organism has been isolated, identified, sequenced then matched to another submitted isolate. By this point, any potential foodborne source of infection may no longer exist as it has already been consumed, thrown away or no longer be for sale. Even if a foodstuff is identified, it may contain very low levels of organism which makes it difficult not only to detect but also to isolate an organism for comparison. This makes it difficult to microbiologically link cases with a source of infection and further highlights the importance of epidemiological investigations. The SMiRL WGS databases include clinical, veterinary, food and environment strains and as

[46] https://hpspubsrepo.blob.core.windows.net/hps-website/nss/2809/documents/1_delivering-the-vtec-e-coli-o157-action-plan-for-scotland.pdf
[47] For an up-to-date (2020) summary of STEC research in Scotland see https://acmsf.food.gov.uk/sites/default/files/2020-10/ACM-1341%20STEC%20Research%20in%20Scotland.pdf

these databases expand to include more diverse types and sources of strains, comparison of these strains will improve the understanding of relationships between strains and can help direct outbreak investigations by inferring sources of infection and suggesting transmission routes (Wilson *et al.,* 2018).

As demonstrated in this 2019 Scottish retail mince survey, WGS enables survey isolates to be identified that were identical to those available in a contemporaneous human clinical outbreak: isolates that with other techniques probably would not have been identified as 'linked'. To optimise and harness the potential of WGS, ongoing provision of resources and encouragement is required to fully realise the contribution it can make to food safety and risk mitigation. These will be needed to increase the number and different sources of isolates typed by WGS, to harmonise methodologies, and to ensure that the sequences are collated and uploaded - with appropriate metadata - to centralised, validated repositories using user-friendly and efficient sharing and reporting systems[27]. For some organisations it can be a challenge to secure information governance approval to share sequence data, and even the smallest amount of associated metadata, but this is increasingly important to assist in the detection of national and international foodborne incidents.

Antimicrobial resistance has been identified as one of the current global challenges, affecting everyone, not just public health policymakers. The finding that there was little evidence for phenotypic resistance; with most non-susceptible profiles being to single, commonly used, first-line active substances that have a long history of use in ruminant populations (e.g. tetracycline and ampicillin) and no evidence for phenotypic resistance to the high priority, critically important antimicrobials tested is a positive one. It provides some reassurance that fresh beef mince on retail sale in Scotland is unlikely to currently be a major foodborne route for transmission of AMR to humans from cattle.

It should be noted that this 2019 survey was designed to reflect consumer patterns using market data from 2018. The 2020 SARS-CoV-2 pandemic has influenced and changed patterns of retail sales of meat and meat products from all species during 2020[48]. It is likely that EU exit will also have an ongoing effect, and in the longer term, other factors such as climate change emissions targets and consumer preferences with regard to alternative protein sources may all contribute to altering these patterns. This is of relevance to the translation of the estimates of prevalence for the pathogens, which were established in this survey, into risk estimates for both the population and for individuals. That translation and decisions as to whether that risk is acceptable or not are questions for risk managers and assessors. The communication of the level of risk has also, ultimately, to be their decision. Not only can qualitative phrases (e.g. low, high, likely, unlikely, rare) be interpreted differently depending on perspective, context and individual risk perception but different qualitative

[48] AHDB webinars: A changing market landscape in the red meat industry: What behavioural changes has Coronavirus driven? and How have consumer attitudes towards red meat shifted during the coronavirus pandemic?"

phrases are applied to the same quantitative value by different communities. For example, adverse event reporting (AER) classification would place the quantitative value of the prevalence estimates of these pathogens in the uncommon-common categories[49]; the Professional Head of Intelligence Assessment Yardstick, which is being used by Government advisory groups to express likelihood during the Sars-CoV-2/COVID pandemic[50] would place them in the 'remote chance' category (anything <5%), while the Advisory Committee on Food Safety advocates adoption of a double qualitative approach to risk level classification for microbiological risk assessment[51]. This enables phrases such as low, medium, high to be put in the context of others such as rare, regularly and very often, respectively but does not facilitate description of quantitative values. As AER classification facilitates such translation, it has been used to draw the conclusions and recommendations made in this report.

There are now quantitative baseline reference values for the microbiological content of Scottish fresh minced beef on retail sale in Scotland. These can be used for risk assessment, risk management and risk communication about the safety of this commodity. They can also be used for comparison purposes in the future, so that the effectiveness and impact of any additional risk mitigation activities may be evaluated.

[49] https://ec.europa.eu/health/sites/health/files/files/eudralex/vol-2/c/smpc_guideline_rev2_en.pdf; https://ec.europa.eu/health//sites/health/files/files/eudralex/vol-6/c/spcpharmaceuticals_10-07-2006_en.pdf
[50] https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/955239/NERVTAG_paper_on_variant_of_concern__VOC__B.1.1.7.pdf
[51] https://acmsf.food.gov.uk/sites/default/files/mnt/drupal_data/sources/files/multimedia/pdfs/committee/acm_1065.pdf

Conclusion

In conclusion, this survey of the microbiological content of Scottish fresh minced beef on retail sale in Scotland during 2019 has:

- provided, for the first time, a baseline measure of the microbiological status of fresh minced beef on retail sale in Scotland.

- demonstrated that, while there is always room for improvement, current measures to ensure food safety along the supply chain - from farm to retail sale - result in a product in which pathogens occur at a frequency that is comparable to that achieved in other European countries, according to the published literature. The values for *Campylobacter*, *Salmonella* were at the lower end of the range seen in European studies.

- demonstrated that because contamination with one of the three significant foodborne pathogens is found to occur at a frequency that is uncommon (*Salmonella, Campylobacter*: more than 1 but less than 10 in 1,000) to common (Shigatoxigenic *E. coli*: more than 1 but less than 10 in 100) it is important to ensure that consumers are aware of the requirements to handle these products as expected prior to consumption (i.e. hygienically in the kitchen and then thoroughly cooked).

- demonstrated that, at the point of retail sale, the frequency with which an indicator of faecal contamination (generic *E. coli* counts) can be detected in samples is much lower than that in which an indicator of the general background microbiological status of the meat, including spoilage organisms (ACC) can be detected.

- provided some reassurance that fresh beef mince on retail sale in Scotland is unlikely to currently be a major foodborne route for transmission from cattle to humans of AMR to critically important antimicrobials, given the evidence for antimicrobial resistance observed in the bacteria that were tested.

- illustrated the value of WGS in facilitating both the placing of survey findings in the wider context of livestock and public health and in the provision of finer detail about the isolates that were detected.

It should be noted that the study design – a cross-sectional survey - does not enable conclusions to be drawn about causal relationships. It only allows hypotheses about the potential for factors to be considered as 'risk factors' to be raised. These will need to be investigated further before any risk mitigation measures based on biologically plausible associations are proposed.

Recommendations for further work

A number of recommendations for further work have emerged:

- risk managers will need to consider whether the risk currently presented by these products is acceptable, or whether additional risk mitigation measures can feasibly be implemented in the food chain.

- a systematic investigation into the food chain - from farm to fork – might contribute to identifying where interventions and risk mitigation measures are currently proving effective, where gaps exist, whether there are any points at which risk mitigation measures could feasibly be implemented and what the cost-benefit, in terms of risk reduction, might be.

- however, the risk posed by these products may not be of the highest priority - further studies could investigate the comparative or relative risk of products i.e. which products present the highest risk of food poisoning in Scotland - either by meat-producing species, product types, or both.

- the outcomes of the survey support the need for ongoing effective communication strategies for consumers, as a risk mitigation measure, to ensure that they handle the product appropriately. This may require studies that involve social scientists and science communications specialists.

- the *E. coli* O157 sequences from this survey could be compared to those found in the British *E. coli* O157 in cattle study (Henry *et al.,* 2019). This would add further context.

- the frequencies of occurrence of the non-O157 STEC isolates could be compared with those that have been produced by the Wellcome Trust grant study[52] (D. Hoyle, University of Edinburgh), in which value is being added to the samples obtained from the British *E. coli* O157 in cattle study (Henry *et al.,* 2019). Study title: "'Prevalence and diversity of Shiga-toxin and non-O157 *Escherichia coli* carriage in cattle".

- comparison of non-O157 STEC isolate sequences from this survey with any from the Wellcome Trust study referred to above. The sequencing of these isolates is ongoing at SMiRL (Edinburgh), at the time of writing of this report.

Both of the above Wellcome Trust study-associated suggestions could be considered as ways to investigate whether the observed O157 STEC/non-O157 STEC ratio found in the retail mince is really so different from that seen in human clinical cases. As explained earlier, this may be wholly, or partly a bias due to the decisions made with regard to which cases are

[52] https://aem.asm.org/content/87/10/e03142-20.long

clinically screened, and for what. The most effective way to determine whether this is the case would be to:

- run a study in which diagnostic clinical labs screened all diarrhoeal faeces by PCR and/or if multi-gastro PCR platforms were to be introduced. Aspects of the differences seen between antimicrobial resistance patterns in non-O157 STECs, which have been highlighted, could also be investigated by comparing frequencies of AMR genes in the relevant sequences.

- further analysis to refine the prevalence estimates to account for test sensitivity and specificity, if they are known, or via the use of latent class analysis if there are other data sources in different populations in which they have been used in the same way.

- there is a whole programme of work that could be done - given suitable resources, collaborative will and an appropriate leadership - to bring the various strands of AMR research in STEC, non-STEC and generic *E. coli* in Scotland that have been completed, or are ongoing together into a truly holistic picture.

Outputs/Impact:

- there is now an archive of the pathogenic isolates and generic *E. coli* that can be used for further study. It will be maintained for five years (to end 2026) and can be used for further study, if additional questions arise.

- an investigation by Dr K. Scott, University of Aberdeen, to screen for AMR genes across the whole microbial community in the samples.

- provision of materials to enable further sequencing of the two mince survey isolates that matched the 2019 human clinical outbreak strains, by Public Health England, using Oxford nanopore sequencing methods.

- the establishment of effective working relationships within the project team, and of work flow processes between the project partner laboratories, to provide a Scottish network that is confident in their abilities to work together to deliver similar surveys, both within Scotland and UK-wide.

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Appendices

Appendix 1: Sample Size Calculation

Appendix 1.1 Sample size calculations and considerations

Basic sample size calculations are based on assumptions of independence (i.e. that the samples are not clustered), that simple random sampling is achievable and that the prevalence distribution approximates to a normal distribution. The indicative sample size depends on the anticipated true prevalence, the confidence and the precision, or tolerance, required of the prevalence estimate obtained. Larger sample sizes facilitate improved precision of the estimate, for a population with the same true prevalence.

Sample size calculations (SSC) provide indications of what is possible, or feasible, with the resources available and/or vice versa, what resources are required to do a study that is statistically robust. SSC can also provide an indication of whether comparisons can, or cannot, be made. Sample sizes will need to be larger to achieve the same effect when a sampling design does not meet the basic assumptions. However, increasing sample size cannot account for bias in design.

1. When the true prevalence (TP) is low, then in order to obtain a precise estimate, the sample size will need to be high: when the TP is high then a smaller sample size is required.

e.g. TP = 0.12%

to achieve a 95% confidence interval (C.I.)

within a tolerance of +/- 0.06% (i.e. 50% of the TP)

Sample size required= 12,790[53]

TP = 20.00%

to achieve a 95% confidence interval (C.I.)

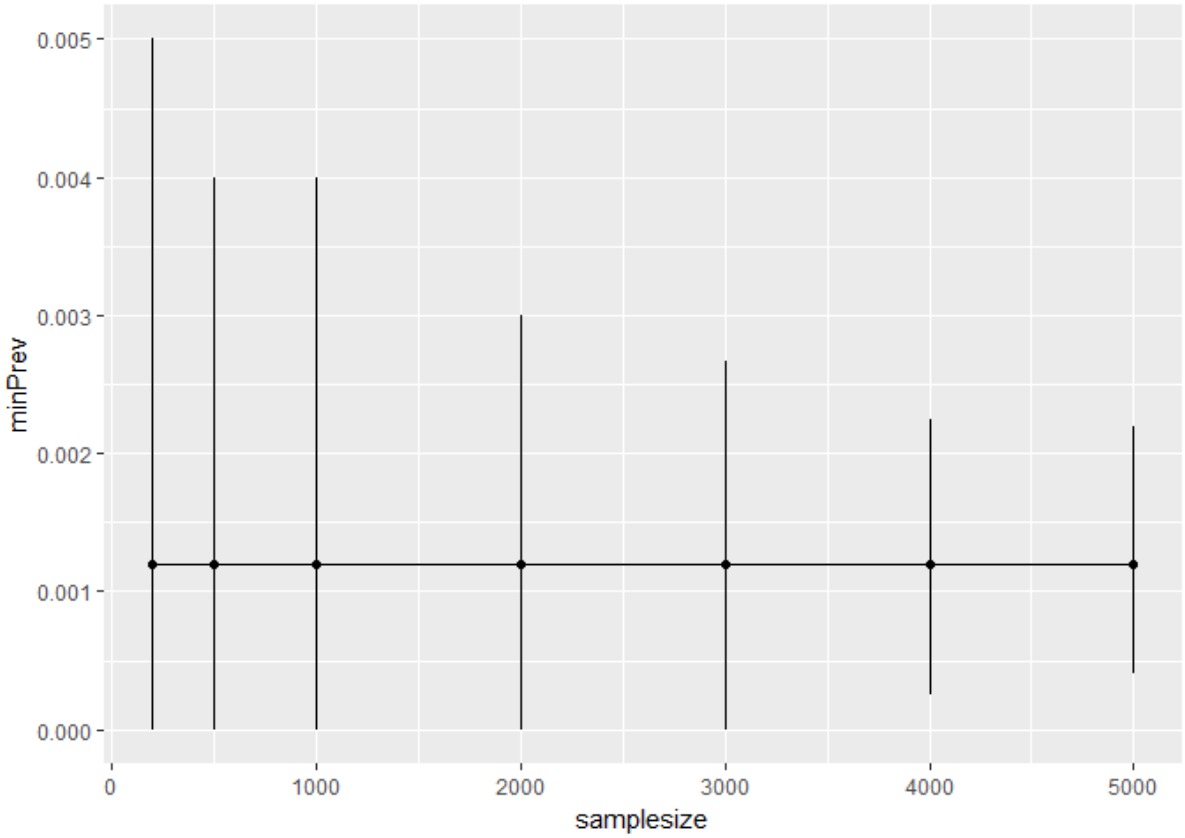
within a tolerance of +/- 5% (i.e. 25% of the TP)

Sample size required = 246[52]

[53] https://epitools.ausvet.com.au/

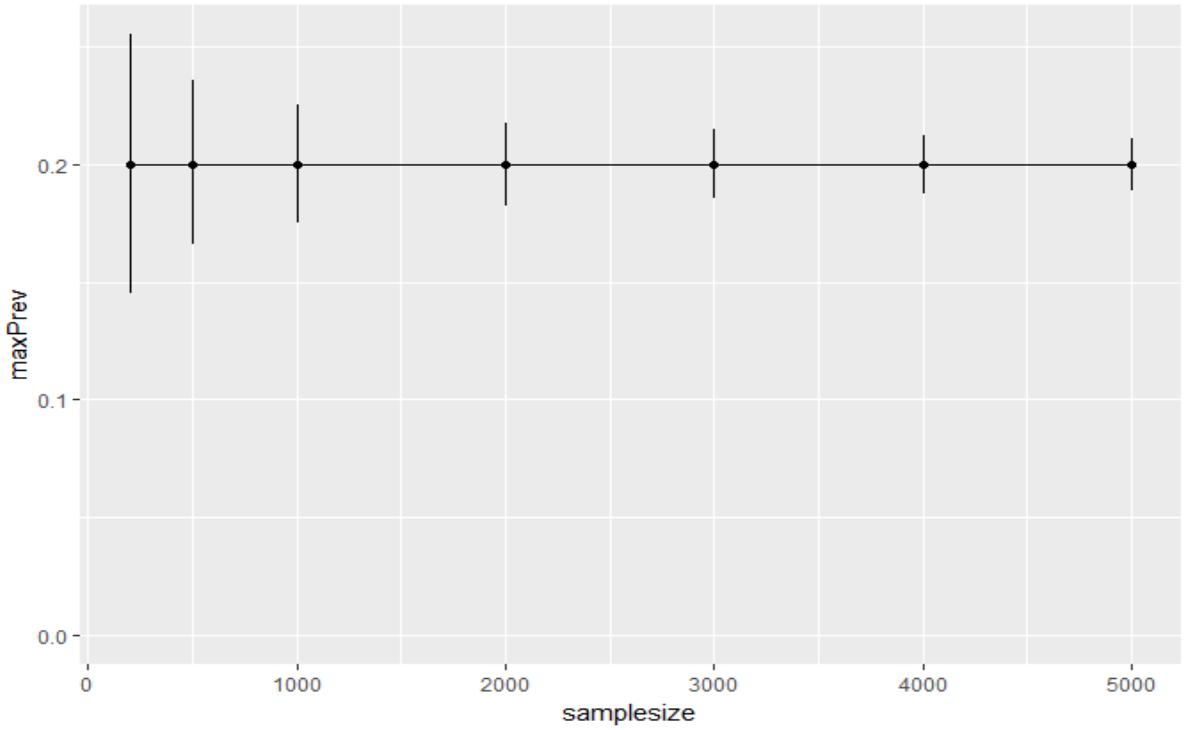
2. Using simulation to capture the asymmetry in C.I at low prevalences, and with no account of clustering, a graphical representation can be used to demonstrate the decrease in 95% percentile range for the estimated prevalences as the sample size is increased (App. Figure 1.1.1).

App. Figure 1.1.1 The 95% percentile range of estimated prevalence around a low true prevalence of 0.12% decreases as the sample size is increased (minPrev = proportion)



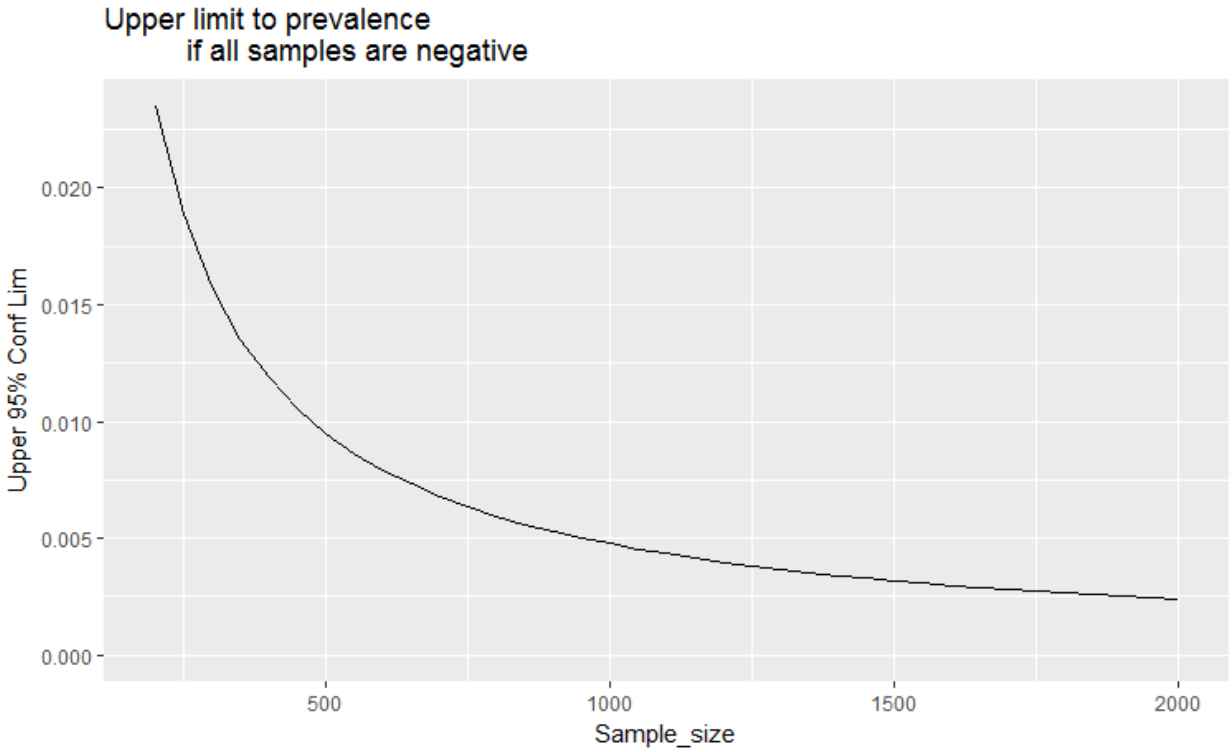
Thus a sample size of 1000 gives a 95% percentile range for the estimated prevalence that includes zero and goes as high as 0.4%. Therefore it is possible to get an estimated prevalence of zero when the true prevalence is 0-12% and 1000 samples are taken.

App Figure 1.1.2 The 95% percentile range for the estimated prevalence around a high true prevalence of 20% decreasing as the sample size is increased (maxPrev = proportion)



Given limitations on sample size and assuming that true prevalence will be low, it is useful to know what confidence interval can be achieved, if there are zero positives detected out of a specified number of samples.

App. Figure 1.1.2 The upper 95% confidence limit (Upper 95% Conf. Lim. = proportion) for the prevalence estimate if all samples test negative.



Given the basic assumptions, we can be 95% sure that the true prevalence lies beneath the line. Therefore, from Figure 4, if all of 250 samples test negative then we can be 95% sure that the prevalence is less than 2%. If all of 1000 samples test negative we can be 95% sure that the prevalence is less than 0.48% and if all of 2000 samples test negative we can be 95% sure that the prevalence is less than 0.24%.

Appendix 2: Geographic Areas

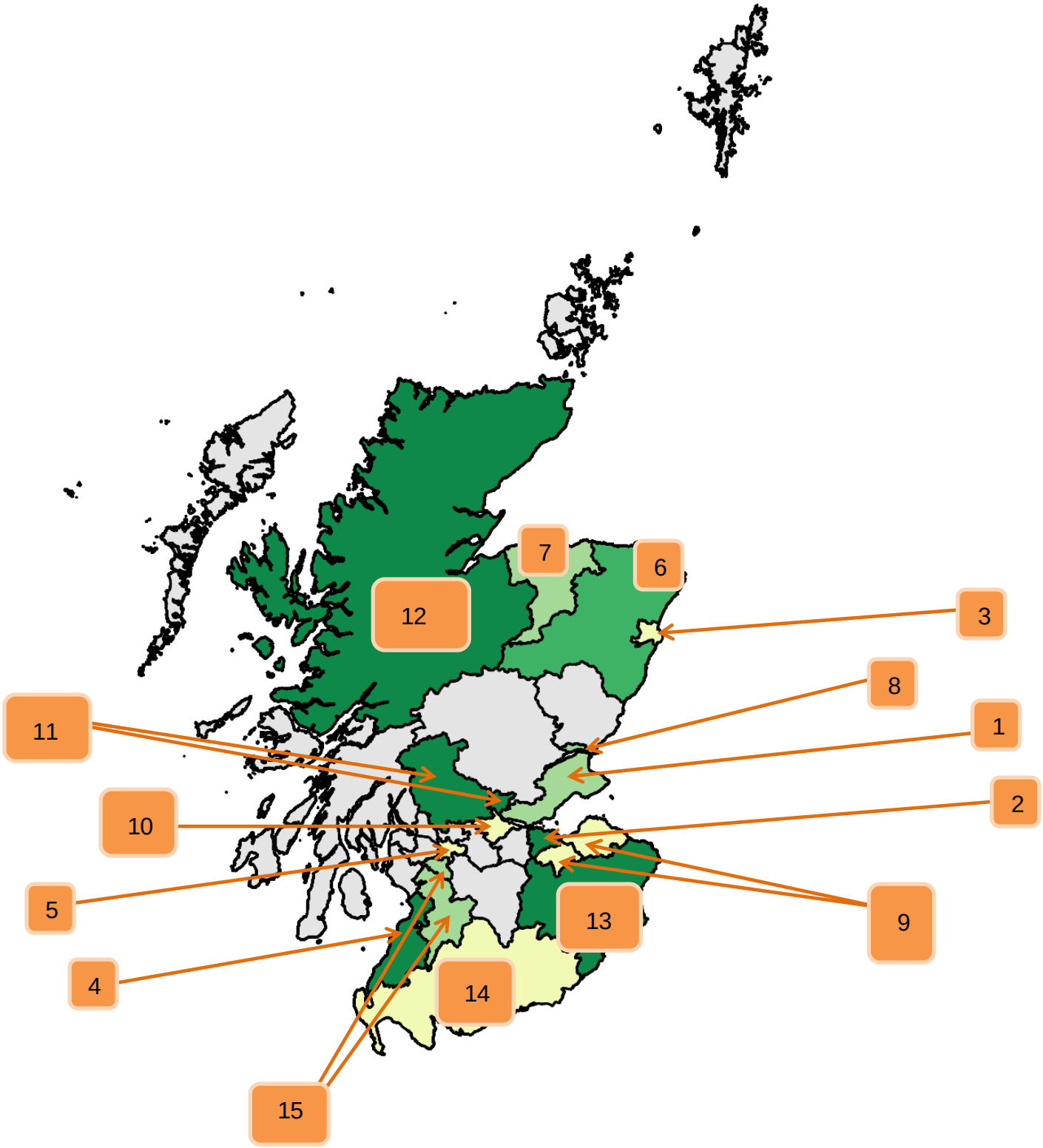
App Table 2.1 Amalgamation of Unitary Authority Council Areas into "Geographic Areas". All other Geographic Areas consist of their corresponding single UA and keep the same name.

Geographic Area	Contributing Unitary Authorities (Population)
"Islands"	Shetlands (23,080), Orkneys (22,000) & Nah-Eileanan Siar (26,950)
Mid and East Lothian	Midlothian (90,090) & East Lothian (104,840)
Inverclyde and North Ayrshire	Inverclyde (78,760) & North Ayrshire (135,790)
Argyll & Bute and West Dunbartonshire	Argyll & Bute (86,810) & West Dunbartonshire (89,610)
Clackmannanshire and Stirling	Clackmannanshire (51,450) & Stirling (94,000)

App Table 2.2. The 15 randomly selected geographic areas (GA) for sampling in planned order

Planned week within block	Project GA	Constituent UAs	Number of samples Round 1	Number of samples Round 2	Number of samples Round 3
1	Fife	Fife	22	22	22
2	City of Edinburgh	City of Edinburgh	22	22	23
3	Aberdeen City	Aberdeen City	22	23	22
4	South Ayrshire	South Ayrshire	23	22	22
5	Glasgow City	Glasgow City	23	22	22
6	Aberdeenshire	Aberdeenshire	22	23	22
7	Moray	Moray	22	22	22
8	Dundee City	Dundee City	22	22	23
9	M_E.Lothian	Mid Lothian + East Lothian	23	22	22
10	Falkirk	Falkirk	22	23	22
11	Clack_Stirl	Clackmannanshire + Stirling	22	22	22
12	Highland	Highland	22	22	22
13	Scottish Borders	Scottish Borders	22	22	22
14	Dumfries and Galloway	Dumfries and Galloway	22	22	23
15	E.Renf_E.Ayrsh	East Renfrewshire + East Ayrshire	22	23	22

App Figure 2.1. Geographic distribution of the selected Geographic Areas to be sampled.



Appendix 3. Sample plan versus samples collected

In Round 1, 2 and 3, there were 6, 3 and 13 samples respectively collected from certain Retail Categories in specific GAs that were additional to the plan. However, there was also some under sampling in specific GAs and Retail Categories which resulted in an under representation of 9, 5 and 12 samples in Rounds 1, 2 and 3, respectively. Accounting for the over and under sampling of specific Retail Categories in specific GAs, the net difference between the sampling plan and the samples collected was -3, -2 and +1 for Rounds 1, 2 and 3, respectively.

In the following tables green and red highlighting represents where over-sampling and under-sampling occurred, respectively.

App Table 3.1 Round 1 sample plan:

Geographic Area	Retail Category				
	1	2	3	4	5
Fife	6	6	4	2	4
City of Edinburgh	6	6	4	2	4
Aberdeen City	6	6	4	2	4
South Ayrshire	6	6	4	3	4
Glasgow City	6	6	4	3	4
Aberdeenshire	6	6	4	2	4
Moray	6	6	4	2	4
Dundee City	6	6	4	2	4
Mid and East Lothian	6	6	4	3	4
Falkirk	6	6	4	2	4
Clackmannanshire & Stirling	6	6	4	2	4
Highland	6	6	4	2	4
Scottish Borders	6	6	4	2	4
Dumfries and Galloway	6	6	4	2	4
East Renfrewshire & Ayrshire	6	6	4	2	4

App Table 3.2 Round 1 samples actually collected:

Geographic Area	Retail Category 1	2	3	4	5
Fife	6	6	4	2	4
City of Edinburgh	6	6	4	2	4
Aberdeen City	6	6	4	2	4
South Ayrshire	7	6	4	3	4
Glasgow City	6	6	4	4	7
Aberdeenshire	6	6	4	2	4
Moray	6	5	4	2	4
Dundee City	6	6	4	1	4
Mid and East Lothian	5	6	4	3	4
Falkirk	4	5	4	2	4
Clackmannanshire & Stirling	6	6	4	2	4
Highland	6	6	4	2	4
Scottish Borders	6	6	4	2	4
Dumfries and Galloway	6	5	4	0	4
East Renfrewshire & Ayrshire	6	6	4	3	4

App Table 3.3 Round 2 sample plan:

Geographic Area	Retail Category 1	2	3	4	5
Fife	6	6	4	2	4
City of Edinburgh	6	6	4	2	4
Aberdeen City	6	6	4	3	4
South Ayrshire	6	6	4	2	4
Glasgow City	6	6	4	2	4
Aberdeenshire	6	6	4	3	4
Moray	6	6	4	2	4
Dundee City	6	6	4	2	4
Mid and East Lothian	6	6	4	2	4
Falkirk	6	6	4	3	4
Clackmannanshire & Stirling	6	6	4	2	4
Highland	6	6	4	2	4
Scottish Borders	6	6	4	2	4
Dumfries and Galloway	6	6	4	2	4
East Renfrewshire & Ayrshire	6	6	4	3	4

App Table 3.4 Round 2 samples actually collected:

Geographic Area	Retail category				
	1	2	3	4	5
Fife	6	6	4	2	4
City of Edinburgh	6	6	4	2	4
Aberdeen City	6	6	4	3	4
South Ayrshire	6	6	4	2	4
Glasgow City	6	6	4	2	4
Aberdeenshire	6	6	4	3	4
Moray	6	5	4	2	4
Dundee City	6	6	4	3	4
Mid and East Lothian	5	6	4	2	4
Falkirk	6	6	4	3	4
Clackmannanshire & Stirling	6	6	4	1	4
Highland	6	6	4	2	4
Scottish Borders	6	6	4	2	4
Dumfries and Galloway	6	5	4	4	4
East Renfrewshire & Ayrshire	6	6	4	2	4

App Table 3.5 Round 3 sample plan:

Geographic Area	Retail category				
	1	2	3	4	5
Fife	6	6	4	2	4
City of Edinburgh	6	6	4	3	4
Aberdeen City	6	6	4	2	4
South Ayrshire	6	6	4	2	4
Glasgow City	6	6	4	2	4
Aberdeenshire	6	6	4	2	4
Moray	6	6	4	2	4
Dundee City	6	6	4	3	4
Mid and East Lothian	6	6	4	2	4
Falkirk	6	6	4	2	4
Clackmannanshire & Stirling	6	6	4	2	4
Highland	6	6	4	2	4
Scottish Borders	6	6	4	2	4
Dumfries and Galloway	6	6	4	3	4
East Renfrewshire & Ayrshire	6	6	4	2	4

App Table 3.6 Round 3 samples actually collected:

Geographic Area	Retail category				
	1	2	3	4	5
Fife	6	6	4	1	5
City of Edinburgh	6	6	4	3	4
Aberdeen City	6	6	4	2	4
South Ayrshire	6	6	4	1	4
Glasgow City	6	6	4	1	1
Aberdeenshire	6	6	4	2	4
Moray	6	4	4	2	4
Dundee City	6	6	4	3	4
Mid and East Lothian	8	6	4	2	4
Falkirk	8	6	4	4	4
Clackmannanshire & Stirling	6	6	4	4	4
Highland	6	6	4	2	4
Scottish Borders	6	6	4	2	4
Dumfries and Galloway	6	5	4	1	6
East Renfrewshire & Ayrshire	6	6	4	1	6

Appendix 4. The five samples eligible for exclusion

These five samples were identified by selecting GAs and Retail Categories that were overrepresented. Samples were preferentially removed from those obtained in the unplanned Round of sampling. If there were no samples collected in the unplanned Round from the identified GA and Retail Category, then samples were selected from oversampled planned Rounds (Round 1, 2 or 3). Once the GA, Retail Category and Round had been identified, based on oversampling, the samples to be removed from the analysis were randomly selected. The five samples that were excluded originated from the UK and were prepacked in a modified atmosphere except sample 3 (App Table 4.1). It was loose and the country of origin was unknown. All five samples weighed 500g and were negative for *Salmonella*, *Campylobacter*, presumptive and confirmed STEC and *E. coli* O157.

App Table 4.1 Description of the five samples that could be excluded as they contributed to over-representation.

Sample Number	1	2	3	4	5
GA	Falkirk	Falkirk	Falkirk	East Ren & Ayr	Mid & East Lothian
Retail Category	1	1	4	5	1
Round	Extra	Extra	Extra	3	Extra
ACC (cfu/g)	5.0×10^5	1.6×10^5	4.7×10^7	5.9×10^4	8.4×10^5
Generic *E. coli* (cfu/g)	<10	<10	5.3×10^2	10	50

Appendix 5. Copy of leaflet sent to independent retailers

Food Standards Scotland
For safe food and healthy eating

Survey of beef mince at retail level in Scotland

Food Standards Scotland (FSS) has commissioned a survey of the microbiological quality of Scottish beef mince at retail level.

The primary aim of this project is to generate baseline data on the significant microbiological pathogens and hygiene indicator bacteria present in raw beef mince on retail sale to the consumer in Scotland. A secondary objective is to analyse any patterns of variation (e.g. seasonal or geographical) in order to identify any risk factors associated with microbial contamination.

This data is needed to provide an up to date and statistically robust evidence base on the prevalence of these organisms. This will improve our understanding of key transmission pathways and support future risk assessment.

The survey consists of a one year sampling programme from January to December 2019. The sampling plan is structured to provide geographic coverage across the whole of Scotland, with samples of raw beef mince acquired from major and smaller retail establishments, based on market share.

All samples will be examined for the presence and levels of microbiological pathogens (STEC (including *E. coli* O157 and non-O157s), Campylobacter, and Salmonella) and hygiene indicator organisms (aerobic colony count and generic *E. coli*). The presence of antimicrobial resistance (AMR) will be determined in any pathogens and a subset of 100 isolates of generic *E. coli* isolated from samples.

Your premises have been selected and relevant samples of fresh beef mince have been purchased for testing.

If there are any adverse results, they will be provided to you as soon as they become available. This will allow you to identify where corrective actions may be appropriate. Please note that, where required, FFS will also inform the relevant enforcement authorities.

At the end of the project, a final report will be published, highlighting the proportion of samples taken at each of the major retailer groups and the cohort of smaller independent outlets. Overall prevalence figures will be provided for each of the pathogens, with additional information on trends in indicator organisms and AMR. Please be assured that the report will not identify individual retailers sampled in the survey and all results will be published in anonymised form.

If you have any further questions regarding this survey please do not hesitate to contact the Food Standards Scotland project managers.

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Photographs: AdobeStock

Appendix 6. Instructions for Scientific Services and samplers.

Appendix 6.1 Background and overview

Sampling Areas: Sampling will be done in three Rounds; each Round will consist of 15 operating weeks. Each week within a Round will be devoted to a particular Geographic Area (GA),

- within each Round the GAs will be sampled in the same order (1-15)
- therefore, each GA will be sampled three times: once within each Round

Most GAs are Unitary (Local) Authority areas but a few are made up of two adjacent Unitary Authority areas due to low numbers of households.

Categories of retailers: There are five categories of retailers. The total number of samples to be collected each week will be 22 or 23, as specified in each week's sampling plan. The weekly sampling plan will state how many samples are to be obtained from each category of retailer and will list all the retailers for each category of retailer for that week's GA in a randomly selected order. In practice the number of samples for each category of retailer won't change much from week to week and is summarised as follows:

- Category 1: 6 samples;
- Category 2: 6 samples;
- Category 3: 4 samples;
- Category 4: 2 samples, or 3 samples, as specified in each week's sampling plan;
- Category 5: 4 samples;

Timings: Within each category of retailer it is important that the retailers are visited in the order within their Retail Category as listed in sampling plan for that week until the desired number of samples is achieved. Within each operational week it doesn't matter what the order of samples *between* the Retail Category is. As an example it might, for logistical reasons, suit samplers to visit the first two retailers within Category 1, and the first three in Category 3 on day one, and then on day two to visit the first two retailers in Category 2 and the next retailer in Category 1, etc. This is fine. The only restriction is that *within* a Retail Category, the retailers should be visited in the order presented in the sampling plan.

Appendix 6.2 At a retail premises

When visiting a retail premises, one sample of every product type of fresh mince beef that is available should be purchased. A separate form for each product type sampled from a retailer should be included.

If a retailer cannot be sampled this should be recorded along with the reason:

e.g. because they don't stock fresh beef mince; they are no longer trading etc.

The number of samples purchased contributes to the overall number of samples required for that category of retail premise.

It is possible that where a number of retail premises have already been visited in that category purchasing one sample of every product type of fresh mince beef that is available will exceed the total required. In this case, only purchase sufficient samples to meet the total required for that category. Selection in such a case should be in the order of discovery.

Appendix 6.3 Instructions to samplers

1. For each week of each Round, confirm receipt of the weekly sampling plan and check week number to find the Geographic Area to be tested.
2. From the sampling plan locate the retailers within each Retail Category to assist in optimising the order of visits whilst ensuring that within each Retail Category the retailers are going to be visited in the specified order.
    a. The order of taking samples ***within*** a category is important but the order of taking samples ***between*** categories can be chosen to reduce travelling (see the background info above for an example if this isn't clear).
3. For each category of retailer, visit each shop/premise in order.
4. Within each shop/premise, purchase one sample of each product type of fresh beef mince that is available until the total number of samples required for that Retail Category in that week have been collected.
    a. The minimum weight that should be purchased is 500g.
5. If there are no fresh beef mince products available in the shop/premise, please record this and move on to the next shop in the Retail Category.
6. If, in that shop/premise, the purchase of one sample of every product type of fresh mince beef that is available will exceed the total required for the Retail Category, in that week, then only purchase sufficient samples to meet the total.
    a. Selection of the samples to purchase in such a case should be in the order of discovery.
7. For each sample collected follow the accredited Scientific Services SOPs for sample collection and submission to the laboratory for testing.
8. Please take a photo of information on the packet taken. Complete a sample submission form for each sample collected. The submission form for this project is slightly different to the standard form. Follow your usual protocol for labelling and transporting samples back to base.