Enumeration of *Salmonella* and *Escherichia coli* O157:H7 in ground beef, cattle carcass, hide and faecal samples using direct plating methods†


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**Keywords**


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**Abstract**

**Aim:** To develop and validate high throughput methods for the direct enumeration of viable and culturable *Salmonella* and *Escherichia coli* O157:H7 in ground beef, carcass, hide and faecal (GCHF) samples from cattle.

**Methods and Results:** The hydrophobic grid membrane filtration (HGMF) method and the spiral plate count method (SPCM) were evaluated as rapid tools for the estimation of pathogen load using GCHF samples spiked with known levels of *Salmonella* serotype Typhimurium. Validation studies showed that for a single determination of each sample type the low end of the detection limits were approx. $2 \times 10^0$ CFU g$^{-1}$ for ground beef, $5 \times 10^{-1}$ CFU (100 cm$^2$)$^{-1}$ for *Salmonella* and $8 \times 10^{-1}$ CFU (100 cm$^2$)$^{-1}$ for *E. coli* O157:H7 on carcasses, $4 \times 10^1$ CFU (100 cm$^2$)$^{-1}$ for hide and $2 \times 10^2$ CFU g$^{-1}$ for faecal samples. In addition, ground beef ($n = 609$), carcass ($n = 1520$) and hide ($n = 3038$) samples were collected from beef-processing plants and faecal samples ($n = 3190$) were collected from feed-lot cattle, and these samples were tested for the presence of *Salmonella* and *E. coli* O157:H7 by enrichment and enumeration methods.

**Conclusions:** The direct enumeration methods described here are amenable to high throughput sample processing and were found to be cost-effective alternatives to other enumeration methods for the estimation of *Salmonella* and *E. coli* O157:H7, in samples collected during cattle production and beef processing.

**Significance and Impact of the Study:** Use of the methods described here would allow for more routine testing and quantification data collection, providing useful information about the effectiveness of beef processing intervention strategies.

**Introduction**

In order to quantify the risks associated with the harvesting of animals that may harbour or shed *Salmonella* or *Escherichia coli* O157:H7, estimates of the distribution and concentration of these pathogens in ground beef, carcass, hide and faecal (GCHF) samples is needed. Beef processors need to know the levels of pathogens entering their plants and have access to the necessary tools to quantify these levels throughout processing, in order to have greater control of their process. At present, the majority of beef pathogen enumeration experiments are conducted using the most probable number (MPN) dilution technique (Barkocy-Gallagher et al. 2003; Arthur et al. 2004; Fegan et al. 2004). This method provides an indirect estimate of the number of organisms present in a sample (Cochran 1950; deMan 1983; Blodgett 2001; Wohlsen et al. 2006). One of the drawbacks encountered when this method is employed for the enumeration of a particular pathogen is that it relies on the enrichment of...
low numbers of target organisms, and the ensuing competition between the target organism to be enumerated and the background flora of the sample likely hinders pathogen detection (Arroyo and Arroyo 1995). Previous enumeration studies of beef carcass samples in our laboratory using traditional MPN methods were problematic and yielded inconsistent results (Barkocy-Gallagher et al. 2003). Another disadvantage to the MPN procedure is that it is a time-consuming and media-intensive process, when performed appropriately, which does not make it amenable to high throughput processes (Fig. 1). The high cost of the traditional MPN method, both in terms of labour and supplies, has resulted in limited data collection on the enumeration of pathogen load during the beef processing.

Enumeration methods based on direct plating have the advantage of being expedient, as they provide a measure of viable bacterial counts without an enrichment step, and they can be performed at a fraction of the cost of traditional MPN methods. Direct plating methods do, however, suffer from a different caveat. One of the hurdles of direct plating methods is the inherent variability in the concentration of background flora. This can result in some plates having a very high number of colonies, making pathogen detection difficult, whereas other plates have a very low colony number and the analysis would have benefited from evaluating more of the sample. While an obvious solution to this problem is to plate multiple dilutions of a sample, this is not a feasible alternative when large numbers of samples are being analysed. The direct plating methods described here address this problem of bacterial load variability.

The spiral plate count method (SPCM) (Gilchrist et al. 1973), which is used here for the evaluation of samples with a generally high concentration of background flora, such as hide and faecal samples, dispenses the sample being evaluated in a logarithmic spiral pattern. It delivers a larger amount of the inoculum in the centre of the plate and a decreasing amount towards the edge of the plate, and the resulting dilution effect is advantageous for the detection of target pathogens in samples of this type.

On the opposite end of the spectrum, the hydrophobic grid membrane filter (HGMF) method (Sharpe and Michaud 1974; Entis et al. 1982; Szabo et al. 1990; Blackburn and McCarthy 2000; Sharpe et al. 2000), allows the evaluation of samples with generally low background flora, such as carcass or ground beef samples, and effectively concentrates the sample being evaluated on a hydrophobic grid membrane. In this study, samples were applied to HGMF using the FiltaFlex filtration apparatus (FiltaFlex Ltd, ON, Canada), which greatly decreases processing time in comparison with other filtration devices that require sterilization between samples. The wax-grid pattern on the ISO-GRID membrane (Neogen Corp., Lansing, MI, USA) inhibits the lateral growth of colonies, making it easier to detect the presence of pathogens and preventing the formation of a bacterial lawn on the plate. The HGMF contains 1600 grid cells and each grid cell may contain zero, one or more bacterial cells. Thus each cell containing growth of the pathogen of interest, in this case either typical Salmonella or E. coli O157:H7, is considered a positive grid cell, referred to here as a colony forming unit (CFU). The number of CFUs is used to estimate the number of pathogens in a sample and is referred to as the HGMF–MPN (McDaniels et al. 1987).

Here, we describe the use of these two rapid methods for the direct enumeration of viable Salmonella and E. coli O157:H7. The SPCM has been used successfully for the enumeration of E. coli O157:H7 from cattle faeces (Robinson et al. 2004a) and is here expanded further for the enumeration of Salmonella from faeces and for the enumeration of E. coli O157:H7 and Salmonella from cattle hide samples. Evaluation of hide or faecal samples using the SPCM described here can be performed for approx. a 23rd of the cost of an MPN for Salmonella and approx. one-fourteenth the cost of an MPN for E. coli O157:H7, given plating on selective media as the method of detection. The HGMF method has been used previously for the enumeration of Salmonella from various food types, but these analyses entailed a pre-enrichment step (Entis
In this study, the HGMF method was used for the direct enumeration of Salmonella and E. coli O157:H7 from carcass and ground beef samples. Evaluation of these sample types using the HGMF method described here can be performed for approx. one-fifth the cost of an MPN for Salmonella and approx. one-sixth the cost of an MPN for E. coli O157:H7, again given plating on selective media as the method of pathogen detection. We applied these enumeration methods to evaluate the pathogen load present in 8357 GCHF samples collected from several processing plants throughout the United States, and show that these methods can be readily used to assess point estimates of viable pathogen concentration at various steps during the beef processing.

Materials and methods

Bacterial inoculum

All spiking experiments were performed using a fresh, overnight culture of Salmonella serotype Typhimurium, previously isolated from cattle (Barkocy-Gallagher et al. 2002). Salmonella Typhimurium was routinely cultivated in 10 ml of tryptic soy broth (TSB, Difco, Beckton Dickinson, Sparks, MD, USA) for 18 h at 37°C. Inoculum for the spiking experiments was prepared by diluting the overnight culture through a series of eight tenfold serial dilutions in sterile normal saline (0.85% NaCl), and each serial dilution was quantified by either spread plating 100 µl or spiral plating 50 µl in triplicate.

Culture media, enrichment and confirmation of bacterial isolation

Salmonella and E. coli O157:H7 were enriched from ground beef, carcass, hide or faecal samples as previously described (Barkocy-Gallagher et al. 2002; Arthur et al. 2004; Barkocy-Gallagher et al. 2005; Bosilevac et al. 2006; Nou et al. 2006). Briefly, TSB phosphate (30 g TSB, 2.31 g KH₂PO₄, 12.54 g K₂HPO₄ per litre, final pH 7.2; used for faecal samples) or TSB was added to samples in a 1:10 ratio and incubated at 25°C for 2 h, 42°C for 6 h and then held at 4°C overnight until processed the next day. A 1-ml aliquot of each enrichment sample was removed and mixed with 20 µl of IMS beads (Dynal, Lake Success, NY, USA). Enrichment/IMS bead mixtures were incubated at room temperature, with shaking, for 15 min and then the IMS beads were removed and washed with phosphate-buffered saline (PBS) Tween 20 (Sigma, St Louis, MO, USA).

For Salmonella isolation, the IMS beads were placed into 3 ml of Rappaport-Vassiliadis Soya peptone broth (RVS, Oxoid, Basingstoke, UK) and incubated at 42°C for 18–20 h. This secondary enrichment was swabbed onto Difco Hektoen Enteric medium (Beckton Dickinson) with novobiocin at a concentration of 5 mg l⁻¹ (HEn) and Difco Brilliant Green agar with Sulfidiazine at 80 mg l⁻¹ (BGS, Beckton Dickinson), then streaked for isolation and incubated at 37°C for 18–20 h. Black colonies on HEn or pink colonies on BGS were considered putative Salmonella isolates and were confirmed by a PCR reaction that detects the Salmonella specific portion of the invA gene (Rahn et al. 1992; Nucera et al. 2006). For E. coli O157:H7 isolation, IMS beads were plated directly onto ntCHROM-O157agar (DRG International, Mountainside, NJ, USA) containing 5 mg l⁻¹ novobiocin and 2.5 mg l⁻¹ potassium tellurite and ctSMAC [Difco Sorbitol Mac- key Agar (Beckton Dickinson)] with cefsulodin at 0.05 mg l⁻¹ and tellurite at 2.5 mg l⁻¹. The plates were incubated at 37°C for 18–20 h and putative E. coli O157:H7 colonies were tested for the O157 antigen using the DrySpot agglutination test kit (Oxoid) and further confirmed as being the O157:H7 serotype with a multiplex PCR reaction (Hu et al. 1999).

Salmonella enumeration was performed on XLDₜ₅₀ medium [Xylose Lysine Desoxycholate medium (Oxoid, Remel) with 46 ml l⁻¹ tergitol (a.k.a. niaproof, Sigma), 15 mg l⁻¹ novobiocin and 5 mg l⁻¹ cefulin]. Plates were incubated at 37°C for 18–24 h and naturally contaminated samples were incubated for an additional 18–20 h at room temperature (23–25°C). Black colonies on the XLDₜ₅₀ plates were considered presumptive Salmonella. The presumptive Salmonella colonies (up to 10 per plate) were tested by PCR for the invA gene (Rahn et al. 1992; Nucera et al. 2006).

Enumeration of E. coli O157:H7 was performed on ntCHROM-O157agar. Incubation temperatures of 37°C and 42°C were evaluated and results showed that 42°C incubation reduced the presence of background flora, enhancing the ability to detect E. coli O157:H7, which were observed as flat, mauve colonies without distinct centres. These putative E. coli O157:H7 colonies were tested using the DrySpot agglutination test kit as described earlier. Colonies that gave a positive agglutination reaction were then subcultured to ctSMAC and further confirmed as the O157:H7 serotype with a multiplex PCR reaction (Hu et al. 1999).

Sample collection

All samples were shipped in coolers, on ice and were received and processed within 24 h of collection. Ground beef samples were obtained from four different commercial ground beef producers in the USA from January to May of 2006. Carcass samples were collected from four different abattoirs (190 per abattoir) in the USA from January to April of 2006. Hide samples were collected...
enumeration. The first is that detection of typical H2S-producing flora. As a result, a greater sample volume can be evaluated consistently for the enumeration of target pathogens from naturally contaminated samples and these were inoculated with E. coli O157:H7. Typhimurium such that the final concentrations ranged from 10^6, 10^5, 10^4 and 10^3 CFU g^{-1}. The spiked samples were allowed to sit at 4°C for approx. 15 min to allow for the possible attachment of the bacterial cells to the meat surfaces. Next, 585 ml of TSB was added per sample (1:10 dilution) and the bags were mixed for 30 s using a laboratory blender (BagMixer 400VW, Interscience Laboratories Inc., Weymouth, MA, USA) at medium speed (seven strokes per second). A 9-ml aliquot of the resulting suspension was removed to a 15-ml conical tube, containing 3 ml of PBS Tween 80, for a final volume of 12 ml. The tubes were mixed by inversion and then allowed to sit at room temperature for 5–10 min so that the meat and fat debris could settle. HGMF analysis was performed by applying 7 ml of the 12-ml sample (equivalent to evaluating 0.5 g or 0.77% of the original 65-g ground beef sample), to an ISO-GRID membrane (Neogen, Lansing, MI, USA) and then filtering the sample using a FiltaFlex Spread Filter apparatus (FiltaFlex Ltd, Ontario, Canada). The membranes were transferred to XLDnc agar plates and incubated at 37°C for 18–20 h. An uninoculated control also was evaluated along with the test samples to test for the presence of naturally contaminated ground beef. Each sample was tested in replicates of ten and the experiment was repeated three times.

Pooled carcass samples were used to validate the HGMF method for this sample type. Three-millilitre aliquots were made from each of the pooled carcass samples and these were inoculated with Salmonella Typhimurium so that the final concentrations were in the range of 10^{-1},
$10^0$, $10^1$ and $10^2$ CFU $(100 \text{ cm}^2)^{-1}$. One millilitre of each aliquot was added to 11 ml of PBS Tween 80, for a final volume of 12 ml. This mixture was applied to ISO-GRID membranes (Neogen), 6 ml per membrane (equivalent to 0.5 ml or 2.5% of the original sample). Thus, six ISO-GRID membranes were prepared from each original 3-ml aliquot. Samples were filtered using a Spread Filter apparatus as described earlier, and the membranes were transferred to XLD$_{inc}$ medium. An uninoculated control also was evaluated along with the test samples to test for natural Salmonella contamination and the spiking experiment was repeated four times.

Validation of the SPCM for the enumeration of Salmonella from hide and faecal samples

Hide samples were pooled and five aliquots of the pooled sample were spiked with an overnight culture of Salm. Typhimurium at final concentrations of $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ and $10^5$ CFU $(100 \text{ cm}^2)^{-1}$. Each aliquot was vortexed, allowed to settle for 3 min and then spiral plated in quintuplicate on to XLD$_{inc}$ medium with the spiral plate set in logarithmic mode, plating a 50-μl aliquot for each replicate (equivalent to evaluating 2.5 cm$^2$ of hide or 0.25% of sample area). A 1-ml control was examined along with the test samples and the experiment was repeated three times.

Faecal samples were analysed by collecting and pooling 200 g of faecal grab samples. For each of three pooled faecal samples, five aliquots at 10 g each were placed into filter bags (Nasco) and spiked with an overnight culture of Salm. Typhimurium. Samples were adjusted for the per cent of verified isolates per positive sample, and then reported as CFU g$^{-1}$ or 100 cm$^2$.

Evaluation of carcass background flora growth at 4°C over time

The aerobic plate count (APC) was evaluated from several naturally contaminated carcass samples that were held at 4°C for 96 h from the time of collection. Samples were taken at 24-, 48-, 72- and 96-h time points and dilutions were made from each sample $(10^{-1}, 10^{-2}, 10^{-3}$ and $10^{-4})$. APC of the samples was measured by plating a 1-ml aliquot of each dilution onto APC Petrifilm (3M™ Healthcare, St Paul, MN, USA). Petrifilm were incubated at 37°C for 18–20 h and the APC counts evaluated using a Petrifilm Counter (3M™ Microbiology).

Statistics

The repeatability of the HGMF and the SPCM enumeration methods was evaluated using inoculated samples, with each spiked sample tested in replicates of six to ten. The mean value for the CFU observed and the standard deviation (SD) of that mean is reported in all cases. The coefficient of variation (CV) was also calculated as a measure of the reliability of these enumeration data. The distribution frequency of observed CFU values from ground beef and hide samples inoculated with Salm. Typhimurium was also determined.

Inoculated ground beef samples were evaluated using the HGMF method 50 times each at the high and low end of the $10^0$ CFU g$^{-1}$ range and inoculated hide samples were evaluated using the SPCM 60 times each at the $10^1$ method for each sample type. Extensive invA PCR analysis of putative Salmonella isolated from naturally contaminated samples showed the need for confirmation testing of black colonies on XLD$_{inc}$ medium. XLD$_{inc}$ plates were examined for the presence of black colonies over 2 days, incubating the first day at 37°C and the second day at 25°C. Confirmation of presumptive Salmonella enumeration isolates with invA PCR or biochemical tests showed the majority of black colonies on XLD$_{inc}$ medium to be Salmonella, however, some degree of heterogeneity was observed within samples. To examine this phenomenon further, carcass samples ($n = 168$) were tested for sample heterogeneity by picking up to ten black, suspect Salmonella colonies per sample. The presumptive Salmonella colonies were tested by PCR for the invA gene (Rahn et al. 1992; Nucera et al. 2006). The genera of black colonies isolated from naturally contaminated samples that were found to be negative for the invA PCR reaction, were determined using Sensititre GNID test plates (TREK Diagnostics, Cleveland, OH, USA) as per manufacture’s instructions. The CFU counts for confirmed Salmonella were adjusted for the per cent of verified isolates per positive sample, and then reported as CFU g$^{-1}$ or 100 cm$^2$.

Detection and enumeration of Salmonella and E. coli O157:H7 in naturally contaminated samples

GCHF samples ($n = 8357$) were collected from several processing plants throughout the United States. The prevalence of Salmonella and E. coli O157:H7 in these samples was determined using the enrichment methods described earlier. A single aliquot from each sample was analysed for the pathogen load estimates of Salmonella and/or E. coli O157:H7, using the specific enumeration
and $10^2$ CFU (100 cm$^2$)$^{-1}$. The data sets were evaluated in groups of ten observations and the mean frequencies and the SDs were plotted as box and whisker plots describing these data. These data were further analysed with Prism 4 software (GraphPad Prism Version 4.0, GraphPad Software Inc., San Diego, CA, USA) and distribution lines were calculated and fitted to the data sets.

The number of HGMF positive growth cells or CFU may be converted to HGMF–MPN by the formula $\text{HGMF–MPN} = 1600 \log_e [1600/(1600-x)]$, where $x$ is the number of CFUs (McDaniels et al. 1987). This conversion had little effect on the pathogen load estimates determined in this study, as they were generally very low, and thus the values were reported as CFU. The CFU counts were adjusted for the per cent of isolates verified per positive sample and then reported as CFU g$^{-1}$ or 100 cm$^2$.

**Results**

The reliability and repeatability of the SPCM and the HGMF enumeration methods was tested by inoculating GCHF samples with *Salmonella* Typhimurium and then determining the CFU g$^{-1}$ or (100 cm$^2$)$^{-1}$. The mean observed values for CFU of *Salmonella* Typhimurium (per membrane for HGMF of ground beef and carcass samples and per 50-μl test for SPCM of faecal and hide samples) enumerated from each sample type were compared with the predicted values for each spiking experiment (Fig. 2a–d). Generally, the observed values reflected the predicted values. The greatest variation between the observed and predicted values was seen for the spiked hide and carcass samples when target pathogen contamination levels were near the limit of detection for each method (Fig. 2b,d).

In order to further examine the reliability of the enumeration data, the CV was calculated for each sample type. The CV is a unitless measure that describes relative variability, and is defined as the SD divided by the mean, expressed as a per cent, with low values indicating greater reliability or less variability. As seen in Fig. 3, the values observed for HGMF analysis of spiked carcass and ground beef samples were most reliable when the inoculum levels were $\geq 10^1$ CFU g$^{-1}$ or (100 cm$^2$)$^{-1}$, whereas the observed values for spiral plate analysis of spiked hide and faecal samples were most reliable when the inoculum levels were $\geq 10^2$ CFU g$^{-1}$ or (100 cm$^2$)$^{-1}$. When contamination levels were lower than those stated, the calculated CV values for those samples were greater than 25%, which is considered the cut-off point for reliable measurements (Clough et al. 2005). Thus the data in Fig. 3 illustrate the increase in precision that is concomitant with the increase in bacterial counts.

![Figure 2](image_url) Comparison of the levels of *Salmonella* used to inoculate samples [CFU g$^{-1}$ or (100 cm$^2$)$^{-1}$], and the mean observed CFU recovered per sample filtered for the HGMF (a, b) or 50-μl spiral plated for the SPCM method (c, d). The (●) symbol represents the mean of the CFU values observed at each spiking level, with error bars indicating the SD from the mean. (a) HGMF of spiked ground beef samples; (b) HGMF of spiked carcass samples; (c) SPCM of spiked hide samples; (d) SPCM of spiked faecal samples. ——, Predicted; ●, observed.
The frequency distribution of observed enumeration values (CFU per sample evaluated) for SPCM analysis of ground beef and HGMF of cattle hide samples that were inoculated with low levels of *Salmonella* was determined, and box and whisker plots of the mean and the SD of these observations are shown in Fig. 4. For both sample types evaluated, a shift in the distribution of observed values for CFU was seen. At very low levels of contamination, the distribution of observed values demonstrated a Poisson-like character, where the most frequently observed value for CFU was 0 (Fig. 4a,b – top). At higher levels of contamination, the observed values for CFU exhibit Gaussian-like character where the values for CFU observed follow more of a normal distribution (Fig. 4a,b – bottom).

Naturally contaminated ground beef (*n* = 609), carcass (*n* = 1520) and hide (*n* = 3038) samples were collected from beef-processing plants throughout the USA and faecal grab samples (*n* = 3190) were collected from feed-lot cattle. Samples were examined for the presence of *E. coli* O157:H7 and/or *Salmonella* by the enrichment and the enumeration methods (Table 1). Hide samples showed the highest per cent of contamination with 89·6% and 46·9% being enrichment positive for *Salmonella* and *E. coli* O157:H7, respectively. The enumeration results for the naturally contaminated samples are summarized in Table 2. The median counts for the pathogen load estimates of *E. coli* O157:H7 from carcass, hide and faeces are 1·6 ± 100 CFU (100 cm$^2$) and 1·6 ± 103 CFU g$^{-1}$, respectively. The median counts for the pathogen load estimates of *Salmonella* on carcasses

Figure 3 Coefficient of variation analysis of enumeration results from inoculated GCHF samples using HGMF and SPCM methods. Coefficient of variation for counts of *Salmonella* recovered from inoculated ground beef (△), carcass (+), hide (*) or faecal (○) samples, plotted against the mean level of contamination (CFU g$^{-1}$ or 100 cm$^2$) for each sample type. The dashed line indicates the 25% cut-off with values below this line demonstrating greater repeatability.

The frequency distribution of observed enumeration values (CFU per sample evaluated) for SPCM analysis of ground beef and HGMF of cattle hide samples that were inoculated with low levels of *Salmonella* was determined, and box and whisker plots of the mean and the SD of

Figure 4 Frequency Distribution of observed values with HGMF and SPCM measurements. Mean observed frequency (□) and error bars representing the SD from the mean CFU values observed from samples inoculated with low levels of *Salmonella*. (a) HGMF of ground beef samples spiked at 2·4 × 10$^9$ CFU g$^{-1}$ (top) and 7·7 × 10$^9$ CFU g$^{-1}$ (bottom). Top – the mean CFU observed per HGMF membrane (0·5 g sample evaluated per membrane) was 1·2 CFU. Bottom – the mean observed CFU per HGMF membrane (0·5 g sample evaluated per membrane) was 3·8 CFU. (b) SPCM analysis of cattle hide samples spiked at 4·8 × 10$^1$ CFU (100 cm$^2$)$^{-1}$ (top) and 1·9 × 10$^2$ CFU (100 cm$^2$)$^{-1}$ (bottom). Top – the mean CFU observed per 50-μl aliquot analysed was 1·2 CFU per plate. Bottom – the mean CFU observed per 50-μl aliquot analysed was 4·8 CFU per plate.
and hides were $1.6 \times 10^6$ CFU (100 cm$^2$)$^{-1}$ and $8.0 \times 10^4$ CFU (100 cm$^2$)$^{-1}$, respectively. *Salmonella* was generally more prevalent and at higher levels on hides and carcasses than *E. coli* O157:H7, as the maximum values of CFU observed for hides and carcasses were $3.4 \times 10^8$ CFU (100 cm$^2$)$^{-1}$ and $4.8 \times 10^6$ CFU (100 cm$^2$)$^{-1}$ for *Salmonella* and $9.8 \times 10^7$ CFU (100 cm$^2$)$^{-1}$ and $4.6 \times 10^7$ CFU (100 cm$^2$)$^{-1}$ for *E. coli* O157:H7. Prevalence of *Salmonella* in ground beef samples was found to be relatively low at 2.8% and only a single ground beef sample of the 609 samples evaluated was found to contain *Salmonella* in the countable range ($2.0 \times 10^4$ CFU g$^{-1}$).

It was also determined that the enumeration analysis of carcass sponge samples should be performed within 24 h of collection. This determination was made as a result of experiments examining the growth of aerobic bacteria in carcass sponge samples at 4°C over time. Results showed that sponge sample APC values increased approx. half a log for every 24 h that they were held at 4°C. The resulting background flora were able to grow on the selective media used and this made pathogen detection difficult (data not shown).

Carcass samples ($n = 168$) were examined for heterogeneity of H$_2$S-producing organisms, by picking up to ten black suspect *Salmonella* colonies per sample. As seen in Table 3, of the 104 samples that had black colonies after 24 h incubation, all colonies tested from 71 samples (68.3%) were identified as *Salmonella*, all colonies tested from 14 samples (13.5%) were not *Salmonella* and colonies from 19 samples tested (18.3%) were mixed, containing *Salmonella* and a non-*Salmonella* H$_2$S-producing organism. Of the 64 samples that did not have black colonies after the first 24 h incubation, but did by the end of the 48 h incubation, all colonies from 21 samples tested (33%) were identified as *Salmonella*, all colonies from 33 samples tested (51%) were not *Salmonella* and colonies from ten samples tested (16%) were mixed and contained *Salmonella* and a non-*Salmonella* H$_2$S-producing organism. Identification of 15 of the black colonies that were negative for invA PCR showed 11 (73%) to be *Citrobacter*,

### Table 1: Enrichment and enumeration results for naturally contaminated GCHF samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Number examined</th>
<th>Number enrichment positive (％)*</th>
<th>Number enumerated (％)+</th>
<th>E. coli O157:H7</th>
<th>Number enrichment positive (％)*</th>
<th>Number enumerated (％)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Beef</td>
<td>609</td>
<td>17 (2.8)</td>
<td>1 (5.9)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Carcass</td>
<td>1520</td>
<td>757 (49.8)</td>
<td>144 (9.0)</td>
<td>256 (16.8)</td>
<td>40 (15.6)</td>
<td>86 (6.0)</td>
</tr>
<tr>
<td>Hide</td>
<td>3038</td>
<td>2721 (89.6)</td>
<td>448 (16.5)</td>
<td>1427 (46.9)</td>
<td>86 (6.0)</td>
<td>86 (6.0)</td>
</tr>
<tr>
<td>Faecal</td>
<td>3190</td>
<td>ND</td>
<td>ND</td>
<td>532 (16.7)</td>
<td>122 (22.9)</td>
<td>122 (22.9)</td>
</tr>
</tbody>
</table>

ND, not determined.

*％ Enrichment positive calculated as the ratio of enrichment positive divided by the number of samples examined (n).

+％ Enumeration positive calculated as the ratio of enumeration positive divided by the number of samples that were enrichment positive.

### Table 2: Observed enumeration values for *Salmonella* and *Escherichia coli* O157:H7 from naturally contaminated GCHF samples

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sample type (number enrichment positive)</th>
<th>Analysis type</th>
<th>10$^{-1}$</th>
<th>10$^{0}$</th>
<th>10$^{1}$</th>
<th>10$^{2}$</th>
<th>10$^{3}$</th>
<th>10$^{4}$</th>
<th>10$^{5}$</th>
<th>10$^{6}$</th>
<th>Median observed value</th>
<th>Max. observed value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em></td>
<td>Ground Beef (17)</td>
<td>HGMF</td>
<td>BD</td>
<td>1 (5.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>0</td>
<td>2.0 x 10$^{6}$</td>
</tr>
<tr>
<td></td>
<td>Carcass (757)</td>
<td>HGMF</td>
<td>71 (9.4)</td>
<td>64 (8.4)</td>
<td>7 (0.9)</td>
<td>2 (0.3)</td>
<td>0</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>1.6 x 10$^{3}$</td>
<td>4.8 x 10$^{3}$</td>
</tr>
<tr>
<td></td>
<td>Hide (2721)</td>
<td>SPCM</td>
<td>BD</td>
<td>BD</td>
<td>258 (9.5)</td>
<td>162 (5.9)</td>
<td>24 (0.9)</td>
<td>4 (0.15)</td>
<td>0</td>
<td>0</td>
<td>8.0 x 10$^{3}$</td>
<td>3.4 x 10$^{4}$</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Carcass (256)</td>
<td>HGMF</td>
<td>13 (5.1)</td>
<td>24 (9.4)</td>
<td>3 (1.2)</td>
<td>0</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>1.6 x 10$^{3}$</td>
<td>4.6 x 10$^{3}$</td>
</tr>
<tr>
<td>O157:H7</td>
<td>Hide (1427)</td>
<td>SPCM</td>
<td>BD</td>
<td>BD</td>
<td>58 (4.1)</td>
<td>26 (1.8)</td>
<td>2 (0.14)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8.0 x 10$^{3}$</td>
<td>9.8 x 10$^{3}$</td>
</tr>
<tr>
<td></td>
<td>Faeces (532)</td>
<td>SPCM</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>43 (8.1)</td>
<td>44 (8.3)</td>
<td>15 (2.8)</td>
<td>19 (3.6)</td>
<td>1 (0.2)</td>
<td>1.6 x 10$^{3}$</td>
<td>5.7 x 10$^{3}$</td>
</tr>
</tbody>
</table>
two (13%) to be *E. coli* and one each (6.7%) to be *Enterobacter* and *Psychrobacter*.

### Discussion

Assuring the microbiological safety of beef has been a major focus of the beef-processing industry, and microbiological testing at several steps throughout the process is vital for the implementation and maintenance of effective HACCP procedures. Comparisons of in-plant antimicrobial interventions have traditionally been based on enrichment/prevalence data alone. Yet, interventions that do not completely eliminate pathogens can still be very effective if they significantly reduce the pathogen load on hides or carcasses. While there is an abundance of data on the prevalence of pathogens in cattle faeces and on cattle hides and carcasses, there has been little progress in quantifying pathogen levels at various steps in production or processing. A number of studies have addressed the question of *E. coli* O157:H7 and *Salmonella* load in cattle samples, however, the enumeration methods used involved limited sample numbers and pathogen enumeration of samples that had been held at 4°C, until the prevalence data for the samples were known (Fegan et al. 2003; Omisakin et al. 2003; Arthur et al. 2004; Fegan et al. 2005). The limitations encountered in these studies were due in part to the use of the three-tube MPN method for assessing pathogen load. This method is costly, time-consuming and not amenable to high throughput processes (Fig. 1) and so using it for enumeration of a large number of samples is not practical. In addition, if molecular methods such as PCR or immunological methods such as the ImmunoCard Stat! *Escherichia coli* O157 (Meridian Diagnostics, Cincinnati, OH, USA) are used as the mode of pathogen detection in MPN analyses, the results can be inconsistent and misleading because of high levels of background microflora (Blodgett and Garthright 1998). To that end, we developed two methods for the enumeration of *Salmonella* and *E. coli* O157:H7 from various sample types obtained from cattle.

Validation studies of the HGMF method for the evaluation of ground beef and carcass samples, and the SPCM for the analysis of hide and faecal samples showed that both methods were able to produce reliable and repeatable pathogen load estimates of samples that were contaminated at concentrations greater than the threshold level of detection for each given method and sample type (Fig. 2). It was also determined that enumeration of pathogens from carcass samples should be performed within 24 h of collection, as opposed to holding samples at 4°C until samples were confirmed to contain pathogens by enrichment methods. The need for enumeration testing in this time window is evidenced by the observation that certain carcass sponge background flora are able to grow at 4°C. The average APC values for carcass sponge samples held at 4°C for 96 h were observed to increase approx. half a log for every 24 h that samples were held (data not shown). These background flora were also able to grow on the selective media employed and as a result detection of pathogens in samples held at 4°C was impaired.

Validation studies of these enumeration methods also showed that the level of sample contamination resulting in consistent measurements varied for each sample type analysed (Table 2). This variation results from the fact that for each method and sample type, a different fraction or per cent of the total sample is being evaluated (Table 4). As a case in point, evaluation of 500 µl of a carcass sample (8000 cm²) using HGMF results in the analysis of approx. 200 cm² or 2.5% of the original sample, while evaluation of 50 µl of a 100-ml faecal sample (10 g) using SPCM, results in the analysis of approx. 0.005 g or 0.05% of the original sample. Accordingly, a smaller per cent of the total sample is evaluated for the faecal sample than the carcass sample and thus the overall pathogen concentration in the sample needed to obtain reliable enumeration data is greater for faecal samples than for carcass samples. This concept is further demonstrated in Fig. 4, where it is shown that when contamination levels are near the limit of detection, the distribution of observed values exhibits a Poisson-like character, where the most frequently observed value for CFU per sample is 0 (Fig. 4a,b – top). When contamination levels are greater than the detection limit, the observed values for CFU follow a normal curve and are better measures of the pathogen load of the sample (Fig. 4a,b – bottom).

The data collected in this study, using the enumeration methods described, show that the HGMF and the SPCM

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**Table 3** Analysis of sample heterogeneity for *Salmonella* and other H2S-producing organisms observed from carcass enumeration samples. The identity of *Salmonella* isolates and other H2S-producing organisms was determined by performing invA PCR analysis and with Sensititre GNID test panels. The following table summarizes the number and percent of H2S-producing organisms isolated from carcass samples that either were completely *Salmonella* or were not *Salmonella*, or were a mixture of *Salmonella* and other H2S-producing organisms.

<table>
<thead>
<tr>
<th>Observation of black colonies at:</th>
<th>Number of samples observed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h (n = 104)</td>
</tr>
<tr>
<td><strong>Organism profile</strong></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>71 (68.3)</td>
</tr>
<tr>
<td>Not <em>Salmonella</em></td>
<td>14 (13.9)</td>
</tr>
<tr>
<td>Mixed</td>
<td>19 (18.3)</td>
</tr>
</tbody>
</table>

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**Discussion**

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The data collected in this study, using the enumeration methods described, show that the HGMF and the SPCM...
can be used to generate baseline information on pathogen load in GCHF samples. As shown in Table 2, hide contamination was found to vary from $10^3$ to $10^5$ CFU (100 cm$^2$)$^{-1}$ for E. coli O157:H7 with the median level for those in enumeration range being 80 CFU (100 cm$^2$)$^{-1}$ and the highest level observed was $9.8 	imes 10^3$. These levels are similar to those described by Arthur et al., where E. coli O157:H7 levels on the majority of hide samples contaminated with E. coli O157:H7 were found to range from $10^1$ to $10^4$ MPN per 100 cm$^2$ (Arthur et al. 2004). Hide levels for Salmonella were found to range from $10^1$ to $10^4$ CFU (100 cm$^2$)$^{-1}$ with the median value for those in enumeration range being 80 CFU (100 cm$^2$)$^{-1}$ and the maximum value observed was $3.4 	imes 10^4$. These values are similar to those of Fegan et al., who described the levels of Salmonella on the hides of cattle presented for slaughter to range from 6 to 480 MPN per 100 cm$^2$ (Fegan et al. 2005). Carcass contamination levels were considerably lower than levels on hides at $10^{-1}$–$10^1$ CFU (100 cm$^2$)$^{-1}$ for E. coli O157:H7 with the median pathogen level at 16 CFU (100 cm$^2$)$^{-1}$ and the maximum observed value at 46 CFU (100 cm$^2$)$^{-1}$. These values are in agreement with those described by Arthur et al., where E. coli O157:H7 on carcasses was found to range from $10^0$ to $10^2$ MPN per 100 cm$^2$ (Arthur et al. 2004). Carcass levels for Salmonella ranged from $10^{-1}$ to $10^2$ CFU (100 cm$^2$)$^{-1}$ with the median contamination level at 16 CFU (100 cm$^2$)$^{-1}$ and the maximum observed value was 480 CFU (100 cm$^2$)$^{-1}$. With the evaluation of 1520 carcass samples, this study represents the most extensive assessment of E. coli O157:H7 and Salmonella present on cattle carcasses in the USA to date, and provides the current best estimate of carcass contamination levels.

As seen in Table 1, 89.6% and 46.9% of hide samples were contaminated with Salmonella and E. coli O157:H7, yet enumeration data were obtained for only 16.5% and 6.0% of the samples for Salmonella and E. coli O157:H7, respectively. A similar scenario was observed for carcass samples, where 49.8% and 16.8% were found to be contaminated with Salmonella and E. coli O157:H7, respectively. Of those samples that were contaminated with Salmonella and E. coli O157:H7, only 19% and 15.6% were found to be in the enumeration range, respectively. It is likely that a number of hide and carcass samples evaluated were actually contaminated at levels near the detection limit but were not able to be enumerated with the single test performed. As a result, the per cent of samples found to be in the enumeration range is potentially artifactually low.

The values obtained in this study for the enumeration of E. coli O157:H7 from faeces show that pathogen levels in contaminated samples ranged from $10^2$ to $10^6$ CFU g$^{-1}$. These data are in keeping with the results of other studies, where E. coli O157:H7 levels in faeces were found to range from $10^2$ to $10^6$ MPN per gram (Omisakin et al. 2003; Robinson et al. 2004b). There are many factors that influence the quality of enumeration data. Among these are the extents to which the sample being evaluated is representative of the lot from which it was taken, and the total number of sample replicates to be analysed. It is well documented that there is an uneven distribution of E. coli O157:H7 within cattle faeces, and that the accuracy of measurements improves when more of the sample is tested (Ogden et al. 2000; Omisakin et al. 2003; Pearce et al. 2004; Syngue and Gunn 2004; Echeverry et al. 2005; Robinson et al. 2005). These same principles apply to collection of enumeration data from samples collected throughout the beef-processing line. Taking multiple measurements from each sample can increase the sensitivity and the accuracy of the measurement, especially for those samples that carry a low pathogen load.

It should be noted that the validation experiments of these enumeration methods were conducted using spiked samples, where pathogen distribution is likely more uniform and the viable state of the target organism is known to a greater extent than with natural contaminants (Clough et al. 2005; Robinson et al. 2005). In addition, as with any use of selective media, the potential exists to select against certain Salmonella or E. coli O157:H7 or those that are in a viable but not culturable (VNC) state (Nystrom 2001). Accordingly, the HGMF and the SPCM methods described here are useful for providing a point estimate of the load of viable and typical Salmonella.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Sample size</th>
<th>Enumeration method</th>
<th>Sample size evaluated per test (%)</th>
<th>Lower LOD for a single determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Beef</td>
<td>65 g</td>
<td>HGMF</td>
<td>0.5 g (0.77)</td>
<td>$2.0 \times 10^2$ CFU g$^{-1}$</td>
</tr>
<tr>
<td>Carcass</td>
<td>8000 cm$^2$</td>
<td>HGMF</td>
<td>120–200 cm$^2$ (1.5–2.5)</td>
<td>$5.0–8.0 \times 10^{-1}$ CFU (100 cm$^2$)$^{-1}$</td>
</tr>
<tr>
<td>Hide</td>
<td>1000 cm$^2$</td>
<td>SPCM</td>
<td>2.5 cm$^2$ (0.25)</td>
<td>$4.0 \times 10^2$ CFU (100 cm$^2$)$^{-1}$</td>
</tr>
<tr>
<td>Faeces</td>
<td>10 g</td>
<td>SPCM</td>
<td>0.005 g (0.05)</td>
<td>$2.0 \times 10^2$ CFU g$^{-1}$</td>
</tr>
</tbody>
</table>
and E. coli O157:H7 that are culturable in the stated conditions.

In summary, we present here two rapid methods for the assessment of Salmonella and E. coli O157:H7 load from various sample types collected from the beef production process. While no direct comparison is given here between the methods described in this study and traditional MPN methods, the pathogen load estimates presented here are in keeping with those described in other studies, where enumeration was performed using the three-tube MPN method (Omisakin et al. 2003; Arthur et al. 2004; Robinson et al. 2004b; Fegan et al. 2005). In addition, the SPCM and the HGMF methods have the advantage of being faster, less media-intensive, less expensive and are amenable to high throughput processes. Other methods for the enumeration of Salmonella, such as real-time PCR methods (Guy et al. 2006; Wolffs et al. 2006), have the advantages of being expedient and the ability to detect VNC organisms, however, they require a prefiltration step that is necessary for the removal of potential PCR reaction inhibitors. This filtration step results in a concomitant decrease in target pathogen load and an increase in detection threshold, limiting its utility. While the direct bacterial enumeration methods described herein are themselves not novel, the application of these methods and the types of selective media used for these analyses represent a new approach to pathogen enumeration in beef production and processing and should provide useful baseline data to help monitor pathogen transfer in the beef production process as well as in other meat-processing environments.

Conclusions

We have developed and validated two methods for the enumeration of Salmonella and E. coli O157:H7 from various types of samples associated with beef production and processing. Utilization of these methods can provide valuable information about the levels of pathogen contamination, ultimately allowing producers to monitor their harvest process and ensure that it is under control.

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References


