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ORIGINAL ARTICLE

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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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. $2{\cdot}0 \times 10^{0}$ CFU g^{-1} for ground beef, $5{\cdot}0 \times 10^{-1}$ CFU $(100\ cm^2)^{-1}$ for *Salmonella* and $8{\cdot}0 \times 10^{-1}$ CFU $(100\ cm^2)^{-1}$ for *E. coli* O157:H7 on carcasses, $4{\cdot}0 \times 10^{1}$ CFU $(100\ cm^2)^{-1}$ for hide and $2{\cdot}0 \times 10^{2}$ 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

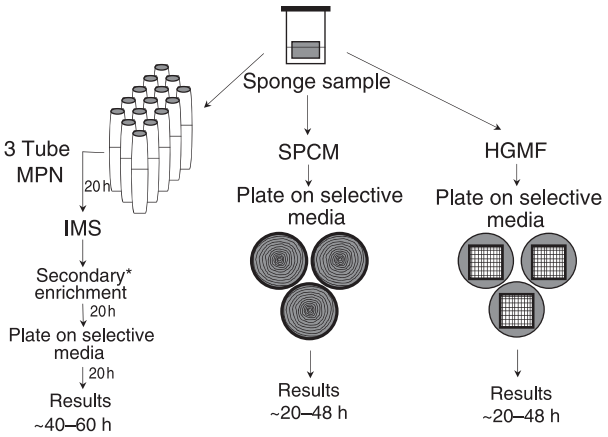

Figure 1 Comparison of the standard MPN method with the SPCM and the HGMF methods of enumeration. Depicted are the steps involved in the processing of a single sample using the three-tube MPN as opposed to the SPCM or HGMF methods of enumeration. The (*) indicates that this step is needed for *Salmonella* enumeration but not for *Escherichia coli* O157:H7. Enumeration of *E. coli* O157:H7 can be accomplished in approx. 40 h for MPN or 20 h for the SPCM or HGMF methods as described in Methods. Enumeration of *Salmonella* can be accomplished in approx. 60 h using the MPN method or in 20–48 h for the SPCM or HGMF methods as described in section 'Materials and methods'.

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* 0157: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

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et al. 1982). 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 Bickinson, Sparks, MD, USA) for 18 h at 37°C. Innoculum 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 KH_2PO4, 12·54 g K_2HPO_4 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 MacConkey Agar (Beckton Dickinson)] with cefixime 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 XLD_{tnc} medium [Xylose Lysine Desoxycholate medium (Oxoid, Remel) with 4·6 ml l^{-1} tergitol (a.k.a. niaproof, Sigma), 15 mg l^{-1} novobiocin and 5 mg l^{-1} cefsulodin]. 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 XLD_{tnc} 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

from four different abattoirs (190 per abattoir) in the USA from June 2005 to April 2006. Faecal samples were collected from a single feed-lot from September 2004 to May 2005.

Ground beef
Ground beef samples were collected from various processing plants in the United States. A 65-g aliquot of each ground beef sample was diluted 1 : 10 in 585 ml of TSB in a filter bag (Nasco, Ft. Atkinson, WI, USA) for a final volume of 650 ml. The HGMF analysis of a single 5-ml aliquot of the 650-ml sample corresponds to evaluating approx. 0·5 g (0·77%) of the original ground beef sample.

Carcass
Carcass samples (20 ml each) were obtained by swabbing approx. 8000 cm^2 of carcass with two sterile sponges (Nasco, Fort Atkinson, WI, USA), each prewetted with 10-ml sterile Difco-buffered peptone water (BPW, Beckton Dickinson) as previously described (Arthur *et al.* 2004). Evaluation of a 500-μl aliquot of the 20-ml sponge sample corresponds to approx. 200 cm^2 of sample area or 2·5% of the original sample, and a 300-μl sample to 120 cm^2 or 1·5%, given an 8000-cm^2 original sample size. For the enumeration of target pathogens from naturally contaminated carcass samples using HGMF, 500 μl (for *Salm.* Typhimurium) or 300 μl (for *E. coli* O157:H7) of carcass sponge sample was added to 7 ml of PBS with 1% (v/v) Tween 80 (Sigma). Two factors contribute to the difference in volumes evaluated for *Salmonella* and *E. coli* O157:H7 enumeration. The first is that detection of typical H_2S-producing *Salmonella* as a black colony is aided by the use of XLD_{tnc} medium because the background flora is generally yellow and the black colonies are thus easily observed. The second is that by and large the background flora is very well selected against with XLDtnc medium. This is not the case with the detection of *E. coli* O157:H7 on ntCHROMagar where the background flora tend to be dark blue, making the lighter mauve *E. coli* O157:H7 difficult to detect. In addition, the ntCHROMagar appears to be less able to select against the background flora. As a result, a greater sample volume can be evaluated consistently for *Salmonella* enumeration than for *E. coli* O157:H7.

Hide
Hide samples were obtained at beef-processing plants by swabbing approx. 1000 cm^2 with a sterile sponge (Nasco), prewetted with the 20-ml BPW. Hide swabs were collected from the brisket plate, and were collected from stunned animals that were on the line, prior to hide removal. Evaluation of a 50-μl aliquot of the 20-ml sponge sample using the SPCM, corresponds to approx. 2·5 cm^2 of sample area or 0·25% of the original sample.

Faeces
Faecal grab samples were obtained from feed-lot cattle. Pathogen enumeration of faecal samples was conducted by hand mixing 10 g of faecal sample with 90 ml of phosphate-buffered TSB in a filter bag (Nasco). A 1-ml aliquot of each faecal slurry was removed, vortexed and the debris allowed to settle for 3 min before further processing. Evaluation of a 50-μl aliquot of the original 10-g faecal sample using the SPCM, corresponds to approx. 0·005 g of sample or 0·05%.

Validation of direct enumeration methods using samples spiked with *Salmonella*

Hydrophobic grid membrane filtration (HGMF) method for the enumeration of Salmonella *from ground beef and carcass samples*
Ground beef was evaluated by spiking 65 g samples that had been placed in filter bags, with dilutions of an overnight culture of *Salm.* Typhimurium such that the final concentrations ranged from 10^0, 10^1, 10^2 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 XLD_{tnc} 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 *Salm*. Typhimurium so that the final concentrations were in the range of 10^{-1},

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10^0, 10^1 and 10^2 CFU $(100\ cm^2)^{-1}$. 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_{tnc} 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 10^1, 10^2, 10^3, 10^4 and 10^5 CFU $(100\ cm^2)^{-1}$. Each aliquot was vortexed, allowed to settle for 3 min and then spiral plated in quintuplicate on to XLD_{tnc} 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 at final concentrations of 10^1, 10^2, 10^3, 10^4 and 10^5 CFU g^{-1}. A sixth 10-g aliquot served as an uninoculated control. The spiked faecal samples were diluted in the ratio of 1 : 10 by the addition of 90 ml of phosphate-buffered TSB and samples were then homogenized by hand. One millilitre of the homogenate was removed from each bag, vortexed, allowed to settle for at least 3 min and then spiral plated onto XLD_{tnc} medium, (50 μl of sample which is equivalent to 0·005 g or 0·05% of the original sample). Eight 50-μl replicates were plated from each bag and the experiment was repeated four times.

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 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_{tnc} medium. XLD_{tnc} 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_{tnc} 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.

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^{TM} 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^{TM} 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

and 10^2 CFU $(100\ cm^2)^{-1}$ range. 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 HGMF–MPN = $1600 \cdot \log_e[1600/(1600\text{-}x)]$, 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 *Salm.* Typhimurium and then determining the CFU g^{-1} or $(100\ cm^2)^{-1}$. The mean observed values for CFU of *Salm.* 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 $\geq 10^3$ 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.

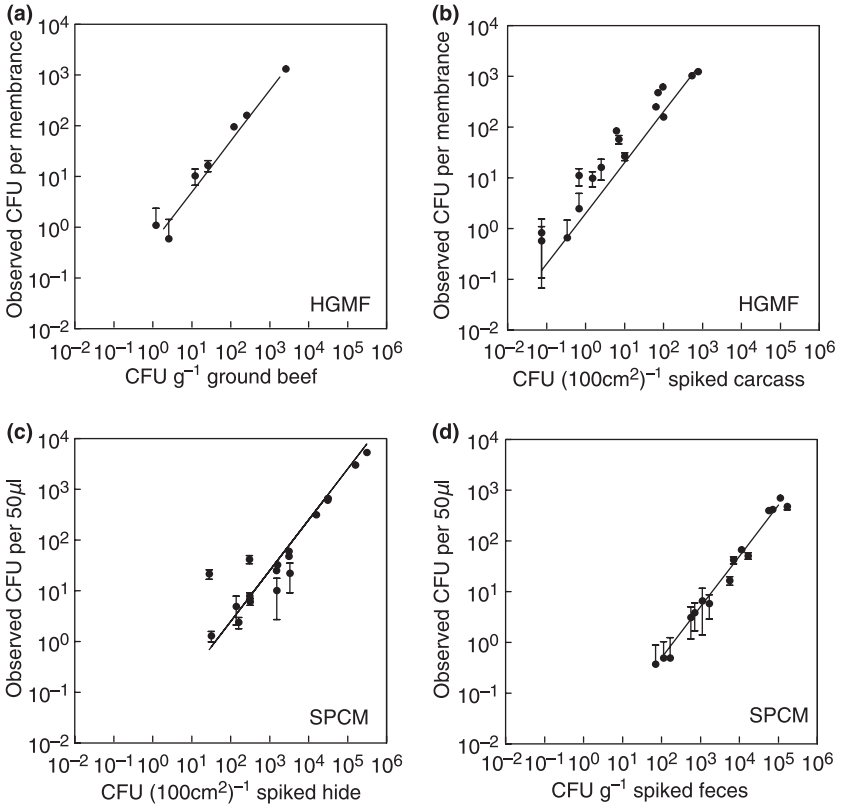

Figure 2 Comparison of the levels of *Salmonella* used to inoculate samples [CFU g^{-1} or $(100\ cm^2)^{-1}$], and the mean observed CFU recovered per sample filtered for the HGMF (a, b) or 50-μl spiral plated for the SPCM method (c, d). The (●) symbol represents the mean of the CFU values observed 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.

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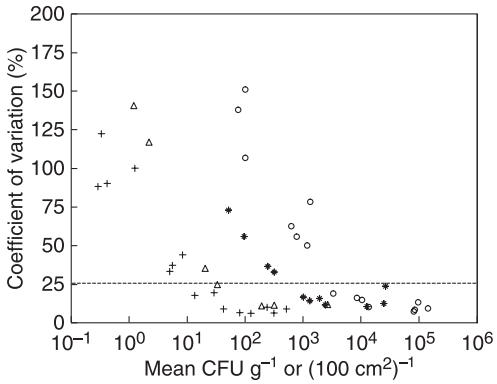

Figure 3 Coefficient of variation analysis of enumeration results from inoculated GCHF samples using HGMF and SPCM methods. Coefficient of variation for counts of *Salmonella* recovered from inoculated ground beef (△), carcass (+), hide (*) or faecal (○) samples, plotted against the mean level of contamination (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 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 $1{\cdot}6 \times 10^0$ CFU $(100\ cm^2)^{-1}$, $8{\cdot}0 \times 10^1$ CFU $(100\ cm^2)^{-1}$ and $1{\cdot}6 \times 10^3$ CFU g^{-1}, respectively. The median counts for the pathogen load estimates of *Salmonella* on carcasses

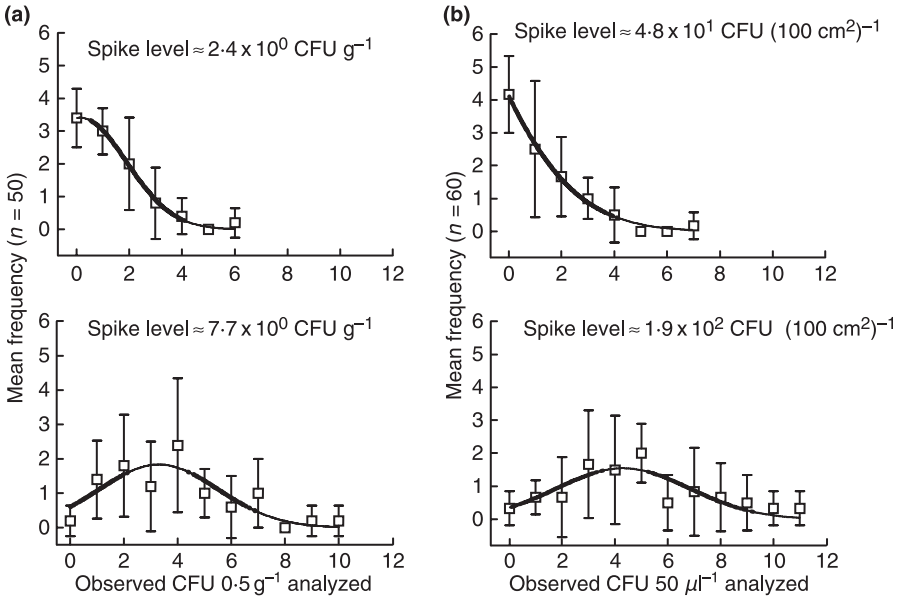

Figure 4 Frequency Distribution of observed values with HGMF and SPCM measurements. Mean observed frequency (□) and error bars representing the SD from the mean CFU values observed from samples inoculated with low levels of *Salmonella*. (a) HGMF of ground beef samples spiked at $2{\cdot}4 \times 10^0$ CFU g^{-1} (top) and $7{\cdot}7 \times 10^0$ 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 $4{\cdot}8 \times 10^1$ CFU $(100\ cm^2)^{-1}$ (top) and $1{\cdot}9 \times 10^2$ 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.

Table 1 Enrichment and enumeration results for naturally contaminated GCHF samples

Sample type	Number examined	*Salmonella*		*E. coli* O157:H7	
		Number enrichment positive (%)*	Number enumerated (%)†	Number enrichment positive (%)*	Number enumerated (%)†
Ground beef	609	17 (2·8)	1 (5·9)	ND	ND
Carcass	1520	757 (49·8)	144 (19·0)	256 (16·8)	40 (15·6)
Hide	3038	2721 (89·6)	448 (16·5)	1427 (46·9)	86 (6·0)
Faecal	3190	ND	ND	532 (16·7)	122 (22·9)

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

			Number of samples observed in each range CFU g^{-1} or 100 cm^2 (%)*								CFU g^{-1} or 100 cm^2	
Organism	Sample type (number enrichment positive)	Analysis type	10^{-1}	10^{0}	10^{1}	10^{2}	10^{3}	10^{4}	10^{5}	10^{6}	Median observed value	Max. observed value
Salmonella	Ground Beef (17)	HGMF	BD	1 (5·9)	0	0	0	†	†	†	0	$2{\cdot}0 \times 10^{0}$
	Carcass (757)	HGMF	71 (9·4)	64 (8·4)	7 (0·9)	2 (0·3)	0	†	†	†	$1{\cdot}6 \times 10^{0}$	$4{\cdot}8 \times 10^{2}$
	Hide (2721)	SPCM	BD	BD	258 (9·5)	162 (5·9)	24 (0·9)	4 (0·15)	0	0	$8{\cdot}0 \times 10^{1}$	$3{\cdot}4 \times 10^{4}$
E. coli	Carcass (256)	HGMF	13 (5·1)	24 (9·4)	3 (1·2)	0	0	†	†	†	$1{\cdot}6 \times 10^{0}$	$4{\cdot}6 \times 10^{1}$
O157:H7	Hide (1427)	SPCM	BD	BD	58 (4·1)	26 (1·8)	2 (0·14)	0	0	0	$8{\cdot}0 \times 10^{1}$	$9{\cdot}8 \times 10^{3}$
	Faeces (532)	SPCM	BD	BD	BD	43 (8·1)	44 (8·3)	15 (2·8)	19 (3·6)	1 (0·2)	$1{\cdot}6 \times 10^{3}$	$5{\cdot}7 \times 10^{6}$

BD, indicates that this range was below the detection limit of the enumeration method using the stated initial sample size, sample aliquot volume evaluated and performing a single test for each sample.

*Per cent in each range calculated by dividing the number of samples that were enumeration positive by the total number of samples that were enrichment positive.

†Indicates that this range is beyond the maximum detection limit of the enumeration method.

and hides were $1{\cdot}6 \times 10^{0}$ CFU $(100\ cm^2)^{-1}$ and $8{\cdot}0 \times 10^{1}$ 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 $3{\cdot}4 \times 10^{4}$ CFU $(100\ cm^2)^{-1}$ and $4{\cdot}8 \times 10^{2}$ CFU $(100\ cm^2)^{-1}$ for *Salmonella* and $9{\cdot}8 \times 10^{3}$ CFU $(100\ cm^2)^{-1}$ and $4{\cdot}6 \times 10^{1}$ 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 ($2{\cdot}0 \times 10^{0}$ 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_2S-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_2S-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_2S-producing organism. Identification of 15 of the black colonies that were negative for *invA* PCR showed 11 (73%) to be *Citrobacter*,

Table 3 Analysis of sample heterogeneity for *Salmonella* and other H_2S-producing organisms observed from carcass enumeration samples. The identity of *Salmonella* isolates and other H_2S-producing organisms was determined by performing *invA* PCR analysis and with Sensititre GNID test panels. The following table summarizes the number and percent of H_2S-producing organisms isolated from carcass samples that either were completely *Salmonella* or were not *Salmonella*, or were a mixture of *Salmonella* and other H_2S-producing organisms

Observation of black colonies at:	Number of samples observed (%)	
	24 h (n = 104)	48 h (n = 64)
Organism profile		
Salmonella	71 (68·3)	21 (32·8)
Not *Salmonella*	14 (13·5)	33 (51·6)
Mixed	19 (18·3)	10 (15·6)

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 cm^2) using HGMF results in the analysis of approx. 200 cm^2 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

Table 4 Summary of the amount of sample analysed and the limit of detection (LOD) for each enumeration method used with the sample type indicated. The LOD that can be observed for each sample type evaluated as described, is given for samples examined with a single test (n = 1)

Sample type	Sample size	Enumeration method	Sample size evaluated per test (%)	Lower LOD for a single determination
Ground Beef	65 g	HGMF	0·5 g (0·77)	$2{\cdot}0 \times 10^{0}$ CFU g^{-1}
Carcass	8000 cm^2	HGMF	120–200 cm^2 (1·5–2·5)	$5{\cdot}0$–$8{\cdot}0 \times 10^{-1}$ CFU (100 cm^2)$^{-1}$
Hide	1000 cm^2	SPCM	2·5 cm^2 (0·25)	$4{\cdot}0 \times 10^{1}$ CFU (100 cm^2)$^{-1}$
Faeces	10 g	SPCM	0·005 g (0·05)	$2{\cdot}0 \times 10^{2}$ CFU g^{-1}

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 10^1 to 10^3 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 $9{\cdot}8 \times 10^3$. 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 10^3 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 $3{\cdot}4 \times 10^4$. 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 1·6 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 $\leq 10^0$ 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 1·6 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; Synge 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*

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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