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Enumeration of *Salmonella* from Poultry Carcass Rinses *via* Direct Plating Methods

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ORIGINAL ARTICLE

Enumeration of *Salmonella* from poultry carcass rinses *via* direct plating methods*

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Abstract

Aim: To evaluate direct plating methods for the estimation of *Salmonella* load in poultry carcass rinses.

Methods and Results: Two direct plating tools, the spiral plate count method (SPCM) and the hydrophobic grid membrane filtration (HGMF) method, were adapted to support quantification of *Salmonella* during poultry processing. Test samples consisted of 180 broiler carcasses from a commercial abattoir, 60 from each of three points in the processing line [pre-inside–outside bird wash (pre-IOBW), prechill and postchill]. The SPCM was used to estimate *Salmonella* load in pre-IOBW rinses, while HGMF was used to estimate *Salmonella* levels in prechill and postchill rinses. Carcass rinses were also evaluated for *Salmonella* prevalence by enrichment methods. Mean prevalences of *Salmonella* were 95%, 100% and 41·7%, and the geometric mean loads were $3·7 \times 10^1$, $5·6 \times 10^0$ and $5·0 \times 10^{-2}$ CFU ml^{-1} for pre-IOBW, prechill and postchill rinses, respectively.

Conclusions: The methods described are useful for estimating the concentration of viable and typical *Salmonella* in poultry carcass rinses.

Significance and Impact of the Study: Direct plating enumeration methods can facilitate the monitoring of *Salmonella* load on poultry carcasses throughout the production process, and the evaluation of new processing intervention strategies.

Introduction

Salmonella has been established as a pathogen of significance, and is a major cause of gastroenteritis in humans (Bryan and Doyle 1995; Mead *et al.* 1999). Poultry and poultry products are known reservoirs for these food-borne pathogens, and numerous reports describe the prevalence of *Salmonella* associated with live poultry, production environments and processing plants (Davies *et al.* 1998; Jiménez *et al.* 2002; Simmons *et al.* 2003). Poultry carcasses are sampled for microbiological analysis in a number of ways, including tissue collection, carcass skin swab, direct agar contact and whole carcass rinse methods (D'Aoust *et al.* 1982; McNab *et al.* 1991; Simmons *et al.* 2003). The latter is an established method that is recommended by the Food Safety and Inspection Service (FSIS) for evaluating poultry carcass hygiene (Anon 1996).

While the association of *Salmonella* with poultry carcasses has been well described, the concentration (CFU ml^{-1}) of *Salmonella* on poultry carcasses, especially at various steps in the production line, has not been extensively studied. Estimates of pathogen load are needed in order to both perceive where additional processing intervention hurdles should be implemented, and to be able to test the efficacy of new intervention strategies. Prevalence estimates are not enough to indicate the efficacy of intervention methods. A major hurdle for more thorough testing of poultry hygiene during processing, and of the intervention methods employed, has been the inability to cost-effectively enumerate pathogens from poultry carcass samples. The most probable number (MPN) method for pathogen enumeration can be very useful for estimating pathogen load; however, it is a time consuming, media-intensive procedure, and can be

potentially prone to error when molecular or enzymatic methods of pathogen detection are employed (Cochran 1950; deMan 1983; Blodgett and Garthright 1998). In contrast, enumeration methods based on direct plating, such as the spiral plate count method (SPCM) (Gilchrist *et al.* 1973) or the hydrophobic grid membrane filtration (HGMF) method (Sharpe and Michaud 1974; Entis *et al.* 1982), 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 (Brichta-Harhay *et al.* 2007). Here, we describe two high-throughput methods for the direct enumeration of viable (noninjured) and typical (nonlactose fermenting, H_2S producing) *Salmonella* from poultry carcass rinse samples, and compare enumeration by these methods with the three-tube MPN method.

Materials and methods

Sample collection

Poultry carcasses were obtained from three sites in the processing line of a commercial poultry abattoir in November 2006. Samples were obtained over 3 weeks, 20 of each carcass type per week, resulting in evaluation of 60 carcasses from each site. These sites included: prior to the inside–outside bird wash (pre-IOBW), prior to the chill step (prechill) and after the chill step (postchill). Carcasses were individually bagged and held on ice in coolers until processed, which was within 24 h of collection. Whole carcass rinses were obtained by adding 400 ml of sterile Difco buffered peptone water (BPW; Beckton Dickinson, Sparks, MD, USA), which was poured into and onto the carcass, which was rinsed by shaking the bag for 1 min. The rinse samples were processed as described in the following.

Salmonella direct plating enumeration

Pre-IOBW carcass rinse samples were analysed using the SPCM method, while prechill and postchill rinses were analysed using the HGMF method. These two enumeration methods were used in order to take advantage of the benefits of each. The SPCM method affords enumeration of *Salmonella* in sample types with higher concentrations of background flora, while the HGMF method concentrates more dilute samples. Accordingly, preliminary experiments showed that the use of the SPCM for estimates of *Salmonella* load in pre-IOBW rinses outperformed HGMF analysis, as the membranes clogged and were overgrown with background flora. However, the HGMF method was found to be more effective for the

enumeration of *Salmonella* from prechill and postchill rinses, where levels of background flora and debris were lower, and the concentration of *Salmonella* often fell below the detection limit of analysis with the SPCM method (data not shown).

Pre-IOBW carcass rinses were analysed by spiral plating 50 μl of rinse in quadruplicate onto xylose-lysine-deoxycholate medium (XLD_{tnc}) medium (Oxoid), containing 4·6 ml l^{-1} tergitol (a.k.a. Niaproof; Sigma, St Louis, MO, USA), 15 mg l^{-1} of novobiocin and 10 mg l^{-1} cefsulodin. The addition of tergitol, novobiocin and cefsulodin increased the selectivity of the medium, so that it outperformed other selective and differential media tested (data not shown). This medium was found to be very effective for the identification of *Salmonella*, as typical H_2S-producing *Salmonella* appear as black colonies with a clear, pink outer ring that were readily distinguished from the yellow or pink background flora. Plates were incubated at 37°C for 18–20 h, scored for the presence of typical *Salmonella* colonies, and then incubated an additional 18–20 h at room temperature and scored again (Brichta-Harhay *et al.* 2007). Up to 10 putative *Salmonella* colonies per sample were picked and confirmed using PCR for the *Salmonella*-specific portion of the *invA* gene (Rahn *et al.* 1992; Nucera *et al.* 2006), and Sensititre GNID (Gram Negative Identification) plates (Trek Diagnostic Systems, Cleveland, OH, USA) for biochemical characterization and/or serologically using the Wellcolex serogrouping kit (Oxoid, Remel). The detection limit for SPCM of pre-IOBW carcass rinse samples is approximately $5·0 \times 10^0$ CFU ml^{-1}.

Prechill carcass rinse samples were enumerated using the HGMF method with ISO-GRID membranes (Neogen, Lansing, MI, USA), and the FiltaFlex Spread Filter Apparatus (FiltaFlex Ltd. Almonte, Ontario, Canada). A 1-ml sample aliquot of carcass rinse was placed in a 50-ml conical tube with 23 ml of BPW and 1% (v/v) Tween 80 (Sigma). The resulting 24-ml solution was dispensed onto four HGMF membranes, 6 ml per membrane, such that 250 μl of original carcass sample was applied to each membrane. The samples were filtered and the membranes transferred to XLD_{tnc} agar plates. XLD_{tnc} plates were incubated, and putative *Salmonella* were isolated and confirmed as earlier. The detection limit for HGMF of prechill rinses is approximately $1·0 \times 10^0$ CFU ml^{-1}.

Postchill carcass rinse samples were enumerated using the HGMF method. Here, 40 ml of postcarcass rinse sample was evaluated per carcass (10 ml per ISO-GRID membrane, four membranes per carcass). Membranes were transferred to XLD_{tnc} agar, incubated, and putative *Salmonella* were isolated and confirmed as earlier. The detection limit for HGMF of postchill rinses is approximately $2·5 \times 10^{-2}$ CFU ml^{-1}.

Salmonella three-tube MPN enumeration

To compare the three-tube MPN method with SPCM and HGMF, eight randomly selected carcass samples from each of the three sample sites were chosen for MPN analysis in sample week 3. For pre-IOBW and prechill carcass rinse samples, 1-ml aliquots were placed into each of the three tubes containing 9 ml of tryptic soy broth (TSB; Beckton Dickinson). For postchill samples, the first three tubes contained 10 ml each of carcass rinse sample. Ten-fold serial dilutions were performed out to eight dilutions. The TSB dilution tubes were incubated at 25°C for 2 h, 42°C for 6 h and held at 4°C for 8–10 h. Next, 1 ml from each enrichment tube was subjected to immuno-magnetic separation (IMS), secondary enrichment in Rappaport–Vassiliadis soya peptone broth (RVS; Oxoid, Basingstoke, UK) and plated as described in the following. Two putative *Salmonella* isolates were picked per sample, and the isolates were confirmed using *invA* PCR and biochemical or serological methods.

Salmonella enrichment

The carcass rinse samples were evaluated for the presence of *Salmonella* by combining 30 ml of rinse with 70 ml of TSB in a WhirlPak bag (Nasco, Fort Atkinson, WI, USA) and incubating at 25°C for 2 h, then 42°C for 6 h and then held at 4°C for 8–10 h (Barkocy-Gallagher *et al.* 2002; Brichta-Harhay *et al.* 2007). Next, 1 ml from each enrichment was subjected to IMS using 20 μl of anti-*Salmonella* IMS beads (Invitrogen, Grand Island, NY, USA) and a KingFisher-96 Magnetic Particle Processor (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A detailed description of the KingFisher IMS program used here and a flow chart of the processing scheme is available from the corresponding author. The IMS beads were transferred to 3 ml of RVS, incubated at 42°C $\sim$16 h and tested for the presence of *Salmonella* by swabbing Difco Hektoen Enteric agar (Beckton Dickinson) containing 5 mg l^{-1} of novobiocin (HEn) and Difco brilliant green agar with 80 mg l^{-1} of sulfadiazine (BGS; Beckton Dickinson), and streaking for isolation. The plates were incubated at 37°C for 18–20 h, and two suspect *Salmonella* colonies per sample were picked and confirmed as described earlier.

Calculations and statistical analysis

Putative *Salmonella* colonies appearing on XLD$_{tnc}$ from SPCM or HGMF enumeration analysis were counted over 2 days of incubation. Up to 10 colonies from each plate were picked for confirmation, as it has been found previously that enumeration analysis using the methods

described can result in the appearance of black, H$_2$S-producing *Salmonella*-like colonies, which on confirmation were found most often to be *Citrobacter* (Brichta-Harhay *et al.* 2007). For SPCM of pre-IOBW rinses, estimates of the CFU ml^{-1} of *Salmonella* were calculated by converting CFU per 50 μl from each of the four plates, to CFU ml^{-1}, and then calculating the mean CFU ml^{-1} and 95% confidence interval (CI) of the mean. The 95% CI values were calculated by multiplying the standard error of the mean (SEM) from each data set, by the *t*-distribution constant ($t = 2.353$ for $n - 1 = 3$), and then adding or subtracting this value from the respective mean. If any colonies were not found to be *Salmonella*, then the calculated CFU ml^{-1} was corrected by multiplying the total number of putative *Salmonella* per plate, by the ratio of the number of confirmed *Salmonella* to the total number picked for confirmation.

For HGMF analysis of prechill and postchill carcass rinse samples, estimates of the CFU ml^{-1} *Salmonella* were calculated by counting putative *Salmonella* appearing on membranes over 2 days, and then picking up to 10 colonies per plate for confirmation. The HGMF membranes contain 1600 grid cells and each grid cell may contain 0, 1 or more bacterial cells. Each cell containing growth of typical H$_2$S-producing *Salmonella* is considered a positive grid cell and referred to as a CFU. For prechill samples, the CFU per 250 μl (per membrane) were converted to CFU ml^{-1} and the mean CFU ml^{-1} and 95% CI were calculated. For postchill samples, the CFU per 10 ml were converted to CFU ml^{-1}, and the mean CFU and 95% CI were calculated and adjusted as earlier.

The per cent prevalence of *Salmonella* contamination was calculated for each carcass type in each sample week, and is reported as the mean and standard deviation (SD) over the 3-week sampling period. The geometric mean and 95% CI were calculated for samples from which *Salmonella* was enumerated, either by the direct plating methods described or the MPN method. The MPN values were calculated using Thomas' formula to approximate MPN counts (Thomas 1942) given by:

$$\text{MPN/ml} = P/\sqrt{NT}$$

where P is the number of positive tubes, N is the volume of sample (ml) in the dilution tubes that were negative and T is the total volume of sample (ml) in all the dilution tubes. Estimates of the 95% CI were obtained by the formula of Cochran (1950), and were calculated by:

$$\log(\text{MPN/ml}) \pm (1.96)(0.55)(\sqrt{(\log a)/n})$$

where a is the dilution factor (in this case, 10) and n is the number of tubes per dilution (in this case, 3). Enumeration counts were reported either as MPN ml^{-1}

or CFU ml^{-1}, or as the log transformed values, and were analysed using the unpaired *t*-test in order to determine if the mean counts were significantly different ($P < 0.05$).

Results

We evaluated the prevalence and load of *Salmonella* on poultry carcasses collected from three sites in a poultry abattoir, including pre-IOBW, prechill and postchill carcasses, over a 3-week sampling period. As seen in Table 1, the overall incidence of *Salmonella* on carcasses sampled was $95 \pm 5\%$ for pre-IOBW, $100 \pm 0.0\%$ for prechill and $41.7 \pm 17.6\%$ for postchill rinse samples. Direct plating methods yielded enumeration results for $46.7 \pm 12.6\%$, $48.3 \pm 22.5\%$ and $28.3 \pm 15.3\%$ of pre-IOBW, prechill and postchill rinse samples, respectively. As prevalence estimates were determined by enrichment, and enumeration values by direct plating methods, these differences might be expected. The overall geometric mean load of *Salmonella* in rinse samples yielding enumeration data was found to be 3.67×10^1 CFU ml^{-1} ($n = 28$) for pre-IOBW, 5.60×10^0 CFU ml^{-1} for prechill ($n = 29$) and 5.03×10^{-2} CFU ml^{-1} for postchill ($n = 17$).

Putative *Salmonella* colonies appearing on XLD$_{tnc}$ from SPCM or HGMF analysis were counted over 2 days of incubation. Up to 10 colonies from each plate were picked for confirmation. Over the course of this study, 432 suspect *Salmonella* colonies were tested using *invA* PCR, 181 from pre-IOBW, 219 from prechill and 32 from postchill. Overall, 88.9% (384/432) of colonies tested were *invA* positive (93.4% of pre-IOBW isolates, 85.4% of prechill and 87.5% of postchill). All *invA*-positive isolates were confirmed as *Salmonella*, as described earlier. The 11.1% of *invA*-negative isolates were not further characterized; however, we have found *Citrobacter* to be the organism most commonly leading to false positives on XLD$_{tnc}$ medium (Brichta-Harhay *et al.* 2007).

A comparison of *Salmonella* enumeration estimates obtained for pre-IOBW rinses using SPCM, and HGMF for prechill and postchill rinses, with those obtained using the three-tube MPN enumeration method, was performed in sample week 3 (Fig. 1). SPCM analysis of 20 pre-IOBW rinse samples (19 of which were found to be contaminated with *Salmonella* by enrichment) yielded enumeration results for seven (36.8%), and the geometric mean CFU ml^{-1} *Salmonella* was 3.72×10^1 (95% CI = 11.8 –117.3). MPN analysis of eight randomly chosen
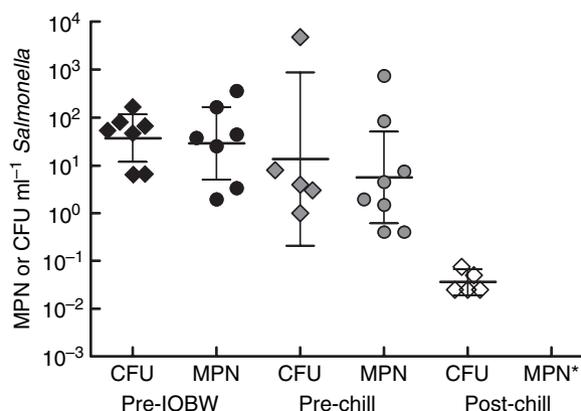
Figure 1 Comparison of *Salmonella* enumeration values [CFU ml^{-1} or (MPN) ml^{-1}] as determined by direct plating methods (SPCM or HGMF) or indirect methods (MPN) of poultry carcass rinses in sample week three. Black, grey and white symbols represent values determined for pre-IOBW, prechill and postchill carcass rinse samples, respectively. Values determined using the SPCM or HGMF methods are indicated by ($\diamond$) and those determined using the three-tube MPN are indicated by ($\bigcirc$). The thick bars indicate the geometric means of the calculated enumeration values and the error bars represent the 95% CI of the means. (*) No enumeration values were obtained for the MPN evaluation of the postchill carcass rinse samples. MPN, most probable number; SPCM, spiral plate count method; HGMF, hydrophobic grid membrane filtration; IOBW, inside–outside bird wash; CI, confidence interval.

Table 1 Summary of prevalence and enumeration results determined for carcass rinse samples examined in this study

	Pre-IOBW ($n = 60$)	Prechill ($n = 60$)	Postchill ($n = 60$)
Salmonella prevalence	95.0 ± 5.0	100.0 ± 0.0	$41.7 \pm 17.6*$
Enumeration positive	46.7 ± 12.6	48.3 ± 22.5	28.3 ± 15.3
Geometric mean CFU ml^{-1}	3.67×10^1	5.6×10^0	5.03×10^{-2}
95% CI	17.1–78.9	2.5–12.6	0.04–0.07
Log CFU ml^{-1}	1.56	0.75	−1.29

Samples were evaluated over 3 weeks (20 of each carcass type per week) and the average per cent positive (±SD) are reported. The geometric mean load of *Salmonella* (CFU ml^{-1}), 95% CI and the log conversion of the mean load for each carcass type are also presented.

IOBW, inside–outside bird wash; CI, confidence interval.

*Postchill samples that were considered prevalence positive for *Salmonella* contamination included those found to be positive by enrichment, and/or by direct plating enumeration methods ($n = 25$).

pre-IOBW samples yielded enumeration results for seven, and the geometric mean MPN ml^{-1} of *Salmonella* was 2.89×10^1 (95% CI = 5·1–166·0). An unpaired *t*-test of the two data sets showed no significant difference between the means (*P* = 0·5840). Similarly, HGMF analysis of 20 prechill rinses, all of which were found to contain *Salmonella*, yielded results for five samples (25%) and showed the geometric mean CFU ml^{-1} to be 1.36×10^1 (95% CI = 0·2–888·2). MPN analysis (*n* = 8) showed the geometric mean MPN ml^{-1} to be 5.64×10^0 (95% CI = 0·6–50·5) (*P* = 0·2745). MPN analysis of postchill rinse samples failed to result in enumeration data for *Salmonella*; hence, no comparison could be made. However, HGMF analysis of 20 rinse samples (eight of which were found to contain *Salmonella*), yielded enumeration results for five samples (62·5%) and showed the geometric mean CFU ml^{-1} to be 3.58×10^{-2} (95% CI = 0·02–0·07).

Discussion

Evaluation of pre-IOBW and prechill carcass rinses showed that 95% and 100% of the samples contained *Salmonella*, respectively. These prevalence values were perhaps not unexpected; however, prevalence of *Salmonella* in postchill samples (41·7%) was somewhat higher than previously reported. Simmons *et al.* found the prevalence of *Salmonella* contamination on retail poultry carcasses in Georgia to be 33·9% (Simmons *et al.* 2003), while FSIS base line studies in 1994 (Anon 1996) and 1998 (Anon 1998) on *Salmonella* in retail poultry found 20% and 10·4% prevalence, respectively. A 1990 study of retail poultry in Ohio found the prevalence of *Salmonella* to be 43% (Bokanyi *et al.* 1990) in agreement with the results reported here. The consistency between the *Salmonella* prevalence values reported here, and those in other studies, reflects the efficacy of the selective enrichment procedure (2 h 25°C; 6 h 42°C; hold 4°C) described. While many *Salmonella*-enrichment procedures employ a 37°C incubation, the selective 42°C incubation temperature does not appear to be detrimental to the growth of viable and typical *Salmonella*.

The direct plating methods described represent rapid and cost-effective means of enumerating *Salmonella* from poultry carcass rinse samples. The SPCM and HGMF methods can provide results in 24–48 h, in comparison with approximately 60 h for the three-tube MPN method, and the evaluation of carcass rinse samples using SPCM or HGMF can be performed for approximately one-twentieth and one-fiftieth the cost, respectively, in comparison with a three-tube MPN analysis. However, the direct enumeration methods do have limitations. As shown in Table 1, enumeration results were obtained for approx.

50% of the pre-IOBW and prechill samples, of which 95–100% were contaminated with *Salmonella*, while about 30% of postchill samples evaluated, or 68% of those found to contain *Salmonella*, resulted in enumeration data. Thus, while direct methods are rapid, they yield results approximately 30–60% of the time, while enumeration methods based on enrichment, such as the MPN method, provide results more consistently. These factors should be considered when determining the sample number in enumeration protocols.

A comparison of direct enumeration methods, with the three-tube MPN method demonstrated that values obtained by each method were similar (Fig. 1). MPN analysis of *Salmonella* in postchill rinses did not yield enumeration results; hence, no comparison could be made. However, HGMF enumeration data of postchill rinses indicated that the load of *Salmonella* in these carcass samples was very low. Over the 3-week collection period, the levels of *Salmonella* in postchill rinses averaged 0·05 ± 0·005 CFU ml^{-1}. With *Salmonella* levels in this range, a 30-ml aliquot of postchill rinse would have a low probability of containing *Salmonella*. In keeping with these results, of the 25 postchill samples (41·7%) found positive for contamination with *Salmonella*, nine (15·1%) were found positive by both enumeration and enrichment methods, eight (13·3%) by enrichment only and eight (13·3%) by HGMF only. These data suggest the need for more rigorous testing of postchill samples, as current methods could miss *Salmonella*-positive carcasses. It has been suggested that the whole carcass enrichments are the best way to detect *Salmonella* contamination (Cox *et al.* 1978) on postchill carcasses or retail poultry, especially as some *Salmonella* may not have been released from poultry carcass skin in the shaking process (Lillard 1986). Other reports indicate that *Salmonella* is best detected when neck skin samples and carcass rinses are analysed in parallel (Jørgensen *et al.* 2002). The low *Salmonella* CFU observed for postchill rinse samples in this study supports these conclusions.

In summary, the use of the direct enumeration methods described can provide baseline information on the levels of typical and viable *Salmonella* present in poultry carcass rinses, and could facilitate the monitoring of *Salmonella* on carcasses throughout the production process, ultimately helping to improve poultry carcass hygiene.

Acknowledgements

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