Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Jan - Mar 2014 / v.16 / n.1 / 45-48

Miniaturized Most Probable Number for the Enumeration of Salmonella sp in Artificially Contaminated Chicken Meat

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Keywords

Chicken meat, most probable number, *Salmonella.*

Submitted: March/2013
Approved: September/2013

ABSTRACT

Salmonella is traditionally identified by conventional microbiological tests, but the enumeration of this bacterium is not used on a routine basis. Methods such as the most probable number (MPN), which utilize an array of multiple tubes, are time-consuming and expensive, whereas miniaturized most probable number (mMPN) methods, which use microplates, can be adapted for the enumeration of bacteria, saving up time and materials. The aim of the present paper is to assess two mMPN methods for the enumeration of *Salmonella* sp in artificially-contaminated chicken meat samples. Microplates containing 24 wells (method A) and 96 wells (method B), both with peptone water as pre-enrichment medium and modified semi-solid Rappaport-Vassiliadis (MSRV) as selective enrichment medium, were used. The meat matrix consisted of 25g of autoclaved ground chicken breast contaminated with dilutions of up to 10^6 of *Salmonella* Typhimurium (ST) and *Escherichia* coli (EC). In method A, the dilution 10^{-5} of *Salmonella* Typhimurium corresponded to >57 MPN/mL and the dilution 10^{-6} was equal to 30 MPN/mL. There was a correlation between the counts used for the artificial contamination of the samples and those recovered by mMPN, indicating that the method A was sensitive for the enumeration of different levels of contamination of the meat matrix. In method B, there was no correlation between the inoculated dilutions and the mMPN results.

INTRODUCTION

Bacteria of the genus *Salmonella* play a major role in poultry farming. Avian-adapted serovars cause gastroenteritis, septicemia and eventually impair productivity, while human-adapted typhoidal serovars cause gastroenteritis and are major barriers for the exports of poultry products. The identification of *Salmonella* in products of animal origin by qualitative methods (conventional microbiological tests) is widely acknowledged, but the enumeration of this microorganism is not used on a routine basis (Borowsky *et al.*, 2005). Enumeration at the poultry farm level up to processing is crucial to estimate the extent of contamination of meat cuts and carcasses and to assess the efficacy of Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Points (HACCP) used for the control of pathogens (Borsoi *et al.*, 2010).

Enumeration methods such as the most probable number (MPN) allow estimating bacterial population based on statistical probabilities and can be combined with *Salmonella* identification methods (Cavada *et al.*, 2010). However, as the MPN method uses multiple tubes, it is not very practical for multiples analysis, especially when several samples are simultaneously analyzed in *Salmonella* control programs.

Miniaturized most probable number (mMPN) methods, which use microplates, can be adapted for the enumeration of microorganisms, saving time and materials (Oscar, 2004; Skovgaard, 2005). Fravalo *et al.* (2003) proposed an mMPN method using the modified semi-solid Rapapport Vassiliadis (MSRV) medium for the selection of motile *Salmonella* strains and later growth in chromogenic media. Pavic *et al.* (2009) validated an mMPN method based on ISO 6579:2002 using MSRV and found no significant differences between traditional MPN (tNMP) and mNMP. The methods developed by Fravalo *et al.* (2003) and Pavic *et al.* (2009) employed mNMP for enumeration and MSRV as enrichment medium in pig slurry and poultry meat matrices, respectively. Nevertheless, there are no studies comparing these methods in chicken meat. Therefore, the aim of this paper is to assess two mNMP methods for the enumeration of *Salmonella* sp in artificially-contaminated chicken meat samples.

MATERIAL AND METHODS

The method adapted from Fravalo *et al.* (2003), referred to as method A (24-well microplates), and the method adapted from Pavic *et al.* (2009), referred to as method B (96-well microplates), were used. The meat matrix consisted of25g of autoclaved ground chicken breast inoculated with positive (*Salmonella* Typhimurium ATCC 14028, ST) and negative (*Escherichia* coli ATCC 25922, EC) controls. In the stationary phase, the controls were inoculated in 9 mL of peptone water at 1% (PW 1%)for 24h at 37°C,with dilution up to 10^{-6}. The conventional qualitative microbiological analysis (ISO 6579: 2002) was carried out concomitantly with methods A and B.

Method A

(adapted from Fravalo *et al.*, 2003)

An aliquot of 225 mL of PW 1% was added to the meat matrix and1 mL of $10^{-5}$and 10^{-6}dilutions of ST and EC controls was used for contamination. A volume of 17.5mL was transferred to a sterile tube, of which 10 mL were used for the conventional microbiological analysisand2.5mL were transferred to the first three wells in the first row of the 24-well microplates. In each well,0.5 mL were transferred to 2 mL of buffered PW 1%, previously poured into the subsequent rows, with three successive dilutions(Figure 1). The samples were pre-enriched for16-20 h at 37°C and kept in an orbital shaker for 3-5 minutes. A volume of 20µLwas transferred from each well to the corresponding well in a microplate containing 2mL of MSRV (Worcman-Barninka *et al.*, 2001), and incubated for24-48 h at 42°C. Aliquots from wells in which there was color change in the MSRV were streaked onto chromogenic agar (Laborclin®), XLD and brilliant green agar, and colonies suggesting the presence of *Salmonella* were submitted to biochemical tests in TSI (*Triple Sugar Iron*), LIA(*Lysine Iron Agar*), SIM (*Sulfide- Indole-Motility*), and urea broth, and to a serological test with *Salmonella* polyvalent O antiserum.

Non-decimal serial dilutions were calculated by the simplified MPN formula (Thomas, 1942), that is, MPN / g or mL = P / √NT, where P is the number of positive tubes, N is the sum of the amount of sample inoculated into all negative tubes and T is the sum of the amount of sample inoculated into all tubes. The application of this formula was compared with the results obtained by the MPN Calculator (Curiale, 2012), using the combination of positive wells in series of 3, with inoculated amounts of 1,0.5,0.1,and 0.02 mL in four dilutions.

Method B

(adapted from Pavic *et al.*, 2009)

For inoculation in the miniaturized system of 96-well microplates, 900 uL of buffered PW 0.1% were added to each well and 100 uL were transferred from each contaminated matrix to the first three wells in the microplate. Serial dilutions were obtained and the sample was homogenized with aspirations repeated 10 times up to dilution 10^{-8}. The microplates were covered in plastic film and incubated for24h at 37°C. The total volume of each well (± 1 mL) was transferred to a new microplate containing 500 uL of MSRV and incubated for 24-48h at 42°C (Figure 2). Aliquots of wells in which there was color change in the MSRV were streaked onto chromogenic agar (Laborclin®), XLD and brilliant green agar. The plates were incubated for 24h at 37°C and the colonies suggesting the presence of *Salmonella* were identified as in method A.

RESULTS AND DISCUSSION

In method A (adapted from Fravallo *et al.*, 2003), the dilution 10^{-5} of *Salmonella* Typhimurium corresponded to >57 MPN/mL and the dilution 10^{-6} was equal to 30 MPN/mL. There was a correlation between the counts used for the artificial contamination of the samples and those recovered by mMPN, indicating that the method was sensitive for the identification of different levels

of contamination of the meat matrix. Fravallo *et al.* (2003) tested fecal samples and environmental swabs obtained from a pig slaughter house and from wattles of turkeys and found similar MPN results to those of the inoculum. Only one sample with an inoculum of0.8 / g of *Salmonella* Typhimurium was not detected by mMPNin the MSRV medium, which, however, was detected by the conventional microbiological analysis. The replacement of the Rappaport-Vassiliadis broth with the MSRV medium allows for efficient, quick and sensitive detection of motile *Salmonella* spp. (Vassiliadis *et al.* 1978), as demonstrated by De Smedt *et al.*(1986), who showed that MSRV allowed detecting100% of *Salmonella* spp. at concentrations of 60 CFU/mL.

In method B (adapted from Pavic *et al.*, 2009), it was not possible to establish a correlation between the inoculated dilutions and mMPN results. These results are at odds with those obtained by Pavic *et al.* (2009), who were able to retrieve *Salmonella* regardless of the type of matrix used. Those authors reported a 92% correlation and less than±1 log of difference when using *S.* Typhimurium, Infantis, Montevideo, Muenster and *Salmonella* sub sp II 1,4,12,27:b: [e,n,x] (Sofia) in naturally- and artificially-contaminated samples (carcasses, scalding tank water, feces, ceca and feed) of poultry products. When developing the technique, the authors found out that, independently of the type of matrix or of the level of coexisting flora, mMPN was able to detect and enumerate *Salmonella* both in naturally- and artificially-contaminated samples.

Skjerve & Olsvik (1991) state that the type of matrix can affect the sensitivity and specificity of the isolation protocol, where the selected matrices represent critical poultry facilities (USDA-FSIS, 2008), ranging from poorly humid and highly humid samples (carcasses and scalding tank water, respectively) to highly competitive environments (ceca and feces).

On the other hand, the method adapted from Fravalo *et al.* (2003) proved to be applicable and practical for the enumeration of *Salmonella*, in line with Robinault *et al.* (2005) and Fablet *et al.* (2006). The authors assert that the method is suitable for the enumeration of *Salmonella* in pig slurry, allowing for the count of bacteria even at low concentrations, proving to be a safe, easy and low-cost method, as miniaturization expedites the analyses and reduces the volume of reagents, enabling the simultaneous analysis of several samples.

The adequacy of the mMPN method adapted from Fravalo *et al.* (2003) can be inferred from the study of Cavada *et al.* (2010), who compared the conventional MPN methods developed by Escartín *et al.* (2000), Koivunen *et al.* (2003) and Sanguinetti *et al.* (2005) for enumeration of *Salmonella* in pig slurry and found that only the technique proposed by Escartín *et al.* (2000) was able to enumerate *Salmonella* at similar counts as those obtained by artificial contamination, whereas in the other methods, the inoculum was way beyond detectable levels. However, the method proposed by Escartín *et al.* (2000) is costly compared with miniaturized techniques and does not allow for the simultaneous analysis of several samples due to the large number of tubes and culture media required. Pavic *et al.* (2009), after assessing the costs and feasibility of mMPN, showed that this method required 64% fewer culture media and that it was 56% less laborious than the traditional MPN (tMPN).

The technique described by Fravallo *et al.* (2003) and used in the present paper allowed proper enumeration of *Salmonella* for hazard analysis. The use of quantitative methods for the control of contamination levels and microbiological risks observed in the production chain is essential for determination of critical points of contamination by *Salmonella* at the farm level up to the storage of chicken parts and carcasses under refrigeration or freezing. The enumeration of *Salmonella* throughout the slaughtering process allows the review of quality control measures at the company level and the adoption of new strategies, as it indicates the critical points of contamination and multiplication of this pathogen during the slaughtering process.

In the method adapted from Fravallo *et al.* (2003),the dilution $10^{-5}$of *Salmonella* Typhimurium corresponded to > 57 MPN/mL and the dilution 10^{-6} was equal to 30 MPN/mL. There was a correlation between the counts used for the artificial contamination of the samples and those recovered by mMPN, indicating that the method is sensitive for the enumeration of different levels of contamination of the meat matrix. In the method adapted from Pavic *et al.* (2009), no correlation could be established between the inoculated dilutions and mMPN results.

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