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Salmonella spp. in poultry carcass: evaluation of sample preparation methods and effect of storage under refrigeration on pathogen recovery

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Abstract

The aim of the present study was to evaluate the use of different analytical units and the influence of storage under refrigeration on the detection of Salmonella sp. in naturally contaminated poultry carcasses. One hundred and thirty samples were collected during the production process soon after chilling (post-chiller phase). Fifty-five samples were analyzed in up to 2 h after collection and 65 samples were analyzed after 72 h of storage. Pathogen screening was based on three different analytical units and a comparison was made between them. Carcasses were initially rinsed with 400 mL of diluent, and three different analytical units were incubated: total rinsing volume (TRV), a single 30 mL aliquot of the rinsing volume, and 25 g of skin from different areas of the carcass. Of all samples analyzed, 60% were positive for Salmonella sp. From the samples collected at the post-chiller phase, 57% were positive for the pathogen and 52.31% of these were detected by TRV; a better statistical performance (P<0.05) when compared to the other analytical units. Of the refrigerated samples, 63% were contaminated, but there were no significant differences between analytical units (P>0.05). There were no significant differences between the number of positive samples from the post-chiller phase and after 72 h of refrigeration. It was also seen that the use of different analytical units (one for the post-chiller phase and another for the refrigerated samples) in samples coming from the same production lot may give different results.

Introduction

Bacteria from the Salmonella genus, which were first isolated and identified at the end of the 19th century, continue to play an important role as etiological agents of disease in animals as well as in humans.[1] This genus is made up of more than 2500 serotypes is responsible for significant economic losses in livestock rearing. In humans, it is considered one of the most important etiological agents of foodborne disease.[1-3] Of all the foods that may transmit the pathogen to man, poultry products, eggs and poultry carcasses are those that play the most important roles.[4] In the last 40 years, there have been thousands of references in the scientific literature connecting Salmonella to birds,[5] to poultry products, eggs and poultry carcasses as being responsible for several outbreaks.[1] Many countries have, therefore, developed programs to reduce Salmonella levels in poultry products. As far as poultry carcasses are concerned, North American and Brazilian programs established maximum acceptable numbers of positive carcasses per sampling cycle.[6,7] However, these goals were not reached when carcasses collected from retailers were analyzed: results showed much higher levels than those determined by governmental organizations responsible for Salmonella control.[5] According to Fletcher,[5] such a discrepancy may have two origins: the analytical unit used for pathogen screening and the site from which samples were collected.

As far as the analytical units are concerned, there are various types of sampling methods: rinsing the carcasses with different volumes of diluent, followed by the incubation of either total rinsing volume (TRV) or only an aliquot of the same; collection of 25 g of skin fragments from different parts of the carcass; swabs from different areas; and, more recently, rinsing of the carcass and incubation of TRV together with the carcass. Each of these analytical units has its advantages and disadvantages, and literature reports conflicting results from studies aimed at evaluating the performance of these different analytical units.[8-10] As for sample collection, control programs stipulate that samples have to be obtained on the factory premises soon after pre-chilling.[6-11] Nevertheless, Fletcher[5] showed that this is the least recommended moment to collect samples, indicating that it might be the *least sensitive* place to detect the pathogen since carcasses have just been refrigerated immersed in chlorine solution (CS) under constant stirring. Other treatments performed during slaughter, such as successive rinsing of the whole carcass with CS, use of antimicrobial substances, or presence of residual CS in the samples, may all hamper Salmonella detection at this stage. Conversely,

Salmonella cells may recover during refrigeration at the retail point of sale and therefore be detected.[5] Besides, it is expected that pathogen cells appear in small numbers after all these treatments and are, thus, not detected.[12] This study of different analytical units and the effect of refrigeration on Salmonella detection may provide important data to explain such discrepant results in pathogen detection in samples collected in the post-chiller production phase and at the retail point of sale.

Materials and Methods

Sample collection

Samples were collected during the whole of 2008 in a poultry slaughterhouse certified by the Brazilian Federal Inspection, near Botucatu, São Paulo. A total of 130 carcasses were analyzed, and all of them were collected after the post-chiller phase in the production process. From these samples, 65 were immediately transferred to sterile plastic bags, placed inside isothermal plastic boxes containing ice,

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and taken to the research laboratory. These 65 samples were analyzed within 2 h of collection. The other 65 samples were packed on the production line, taken to the laboratory following the same procedure, and kept under refrigeration at 7ºC for 72 h, before being analyzed for the presence of *Salmonella* sp. On each day of sample collection, all samples were taken from the same production lot.

Analytical units

In each lot of carcasses analyzed in the laboratory, 50% of the samples were rinsed with 400 mL of 1% buffered peptone water (Difco, Becton Dickinson) for 3 min. This volume was divided into two sterile plastic bags as follows: a 30 mL aliquot (AL, American Method)[7] and 370 mL (total rinsing volume, TRV). From the same rinsed carcass, 25 g of skin (SK) were collected from the inner and outer wing region, cloaca, neck and chest, placed in sterile plastic bags, with the addition of 225 mL of 1% buffered peptone water (Figure 1), and homogenized for 3 min in a Stomacker. The other 50% of the samples underwent the opposite process: SK was collected before rinsing the carcasses. After rinsing as described above, rinsing volume was divided into AL and TRV.

Salmonella isolation

All analytical units (AL, TRV, and SK) were incubated at 35ºC for 24 h. Then, 0.1 mL and 1 mL from all pre-enriched samples were transferred to tubes containing 10 mL of Rappaport-Vassiliadis broth (Difco, Becton Dickinson) and tetrathionate broth (Difco, Becton Dickinson), respectively. Both were then kept at 42ºC for 24 h. Each broth was then cooled on plates containing xylose lysine desoxycholate (Difco, Becton Dickinson), bismuth sulfite agar (Difco, Becton Dickinson) and mannitol lysine crystal violet brilliant green agar (OXOID), incubated at 35ºC for 24 h. Suspicious colonies were replicated in tubes containing triple sugar iron (TSI, Difco, Becton Dickinson) and lysine iron agar (LIA, Difco, Becton Dickinson) and incubated for 24 h at 35ºC. Tubes that presented typical *Salmonella* sp reaction underwent biochemical testing (indole, Voges-Proskauer, methyl red, citrate, urea, phenylalanine and motility) and serological screening with O and H polyvalent antisera (Probac, Brazil).[13]

Statistical analysis

The number of positive samples for *Salmonella* sp. obtained with each analytical unit according to the type of sample (post-chiller or after 72 h of refrigeration), as well as between the types of samples, were compared by the Goodman[14,15] test for contrast between and within binomial populations, using 5% as level of significance.

Results

From the total number of carcasses analyzed (n=130), after obtaining positive results in at least one analytical unit in each sample, and without considering the moment of analysis (post-chiller or after 72 h of refrigeration), 78 (60%) carcasses were contaminated with *Salmonella* sp. Positive *Salmonella* sp samples obtained with different analytical units in the 130 carcasses are shown in Table 1.

The analytical unit pre-established by the Department of Agriculture of the United States (pathogen screening in a 30 mL aliquot of rinsing fluid) gave the worst results, detecting only 30% of positive samples, whereas TRV and SK detected 50 and 38% positive samples, respectively. From the 78 positive carcasses, 39 (62.8%) were detected with the AL, 65 (83.3%) with TRV, and 49 (65.3%) with SK. These results were obtained no matter which sequence was used to produce the analytical units. As for the statistical analysis, a significant difference (P<0.05) was observed in AL performance compared to TRV, with no difference between TRV

and SK (P>0.05). When only the samples that were collected during the post-chiller phase were considered, of the 65 analyzed carcasses, 34 (52.3%) were positive for *Salmonella* sp. when TRV was used, 20 (30.7%) were detected with SK, and 17 (26.1%) were detected with AL (Table 2). From the 37 positive samples (sum of all results), 34 (91.9%) were detected using TRV, 20 (54%) with SK, and 17 (45.9%) with AL. There was a statistical difference in performance between these methods (P<0.05) with TRV performing better than the others. This

Table 1. Number and percentages of carcasses positive for *Salmonella* sp. according to analytical unit.

Analytical unit	Results* Positive	Total
TRV	65 (50%)[b]	130
AL	39 (30%)[a]	130
SK	49 (38%)[ab]	130

*Different letters in the same column show statistical difference (P<0.05); TRV, total rinsing volume. AL, 30-mL aliquot; SK, 25g of skin.
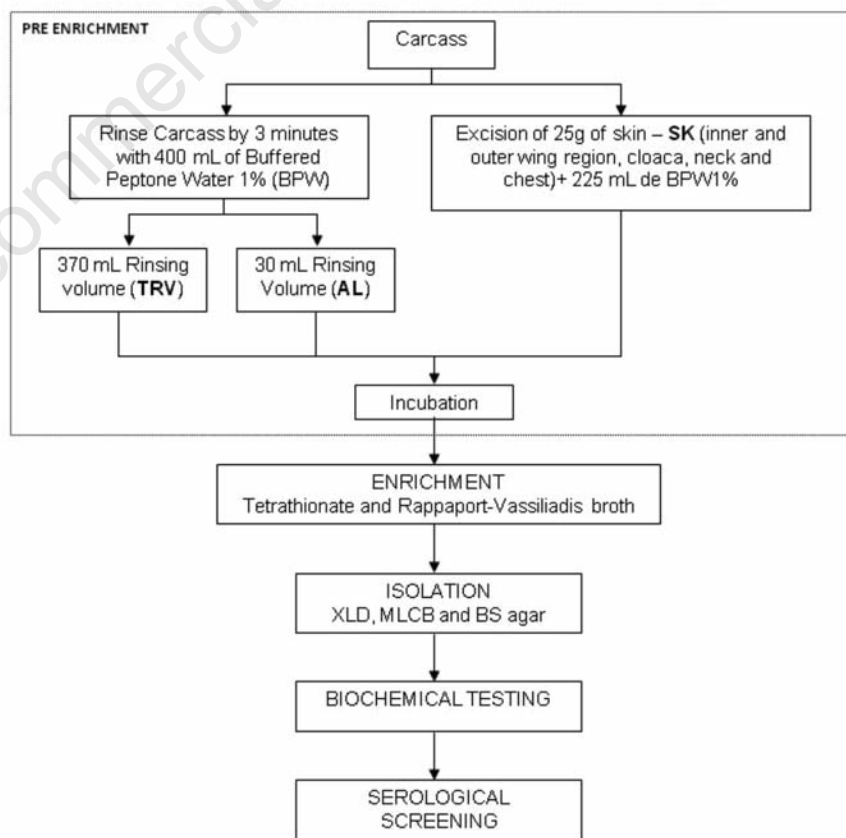
Figure 1. Protocol for *Salmonella* spp. analysis on chicken carcasses collected in the production process (post-chiller) and kept under refrigeration at 7ºC for 72 h. TRV, total rinsing volume; AL, 30 mL aliquot; SK, 25g of skin.

superior performance was only observed in carcasses collected soon after cooling, in the post-chiller phase. No statistical difference between analytical units was observed between carcasses refrigerated for 72 h (Table 2). From 65 refrigerated carcasses, 41 (52.5%) were positive for *Salmonella* sp. when TRV was used, 29 (44.6%) when SK was used, and 22 (33.8%) with AL. From the 41 positive carcasses, considering all results from all analytical units, 31 (75.6%) were detected using TRV, 29 (70.7%) with the use of SK, and 22 (53.7%) with AL. There was no statistically significant difference (P>0.05) between the number of *Salmonella* sp positive carcasses in samples collected during the production process (post-chiller phase) and in refrigerated samples (Table 2). Even if we take each result in isolation from both sample types (use of SK and AL), there was still no significant difference in the higher number of positive carcasses after refrigeration (P>0.05). On the other hand, if different analytical units are used to analyze carcasses in the post-chiller phase, then later, after refrigeration, the number of positive samples may differ (Table 3). These data show the discrepancy between results obtained when one analytical unit is used in the production process and another analytical unit is used after refrigerated storage, which is exactly what happens at the retail point of sale. As for the results of this study, the use of AL to analyze samples in the post-chiller phase, followed by the use of TRV for samples from the same lot after 72 h of refrigeration, led to an increase in the number of positive samples (82.3%). Conversely, when TRV was used to analyze samples in the post-chiller phase, and then AL was used to assess them after 72 h of refrigerated storage, the number of positive samples decreased by 31.2%. Similarly, there was a 70.6% increase in the number of positive samples when AL was used to analyze samples in the post-chiller phase and SK was used to analyze samples after 72 h of refrigeration. On the other hand, when TRV was used in the post-chiller phase and AL was used after refrigeration, a 31.2% decrease in the detection of positive samples was observed.

Discussion

These data showed a high number of positive samples for *Salmonella* spp. In general, there is a large variation in the number of *Salmonella* sp. positive carcasses in Brazil. Some studies show up to 50% positive samples, lower than that found in the present study.[16-19] This may be due to different factors, such as pathogen prevalence in birds still on the poultry farm, weather conditions, animal transport, environmental conditions before slaughter, the slaughterhouse facilities, and the industrial processing of carcasses, besides the analytical methodology used.[16,20,21]

Slaughtering logistics may also contribute to the difference in the number of positive carcasses. When carcasses that resulted positive are processed before negative ones or before those with low pathogen counts, cross-contamination may lead to a higher number of contaminated carcasses.[9,22] The analytical units used can have an important influence on results. AL gave a worse performance than the other two techniques; this poor result using AL was also observed by Simmons *et al*.[10] The same authors have reported that AL had detected only 13% positive samples whereas TRV detected 38% positive samples. Both techniques were applied to the same samples in order to compare and contrast these methods.

Santos *et al*.[23] found the same results in Brazil detecting 34.8% positive samples with this technique, whereas both SK and TRV detected 56.5% positive samples. According to Simmons *et al*.,[12] the number of positive carcasses may be underestimated by the United States Department of Agriculture when AL is used for *Salmonella* sp. screening. This may especially be the case when there are few pathogen cells in the carcasses. As for the results found with SK and TRV, there are conflicting data reported in the literature. D'Aoust[8] observed a much better performance of TRV when compared to SK. Data from this study show a statistically significant (P<0.0001) difference in results; of 70 carcasses positive for *Salmonella*, 65 were detected by TRV and just 52 were detected when using SK.

Similar results were obtained in Brazil by Pinto *et al*.[24] and Souza Júnior *et al*.[25] These authors observed a better performance with TRV compared with SK. Conversely, Santos *et al*.[23] did not observe any difference once both analytical units were able to detect the same ratio of *Salmonella* sp. positive carcasses (56.5%).

When only the samples that were collected during the production process (post-chiller) were considered, we did not find any differences between SK and TRV but only between these two methods and AL. Literature shows that during this phase, *Salmonella* cells are generally found in small numbers on the carcasses, and they are often injured due to the various processes they have undergone during processing.[5] According to Fletcher, in a study performed by the North-American Federal

Table 2. Number and percentages of carcasses positive for Salmonella sp. according to analytical unit and type of sample (post-chiller or refrigerated for 72 h).

Sample	Method	Response Positive	Total
	SK	20 (30.77%)[aA]	65
Post-Chiller	AL	17 (26.15%)[aA]	65
	TRV	34 (52.31%)[bA]	65
	SK	29 (44.61%)[aA]	65
Refrigerated	AL	22 (33.85%)[aA]	65
	TRL	31 (47.80%)[aA]	65

[a,b]comparison of methods, types of sample, and response category. [A,B]comparison between types of fixed sample, methods and the response category. TRV, total rinsing volume; AL, 30 mL aliquot; SK, 25g of skin.

Table 3. Improvement/decrease in the detection of positive samples for Salmonella sp. in poultry carcasses using different analytical units at different phases (post-chiller and after refrigeration for 72 h).

Analytical unit Post-chiller x refrigeration*	Post-chiller (positive samples)	Refrigeration (positive samples)	Improvement/decrease in detection of positive samples (%)
SK x AL	20	22	+10
SK x TRV	20	31	+55
AL x SK	17	29	+70.6
AL x TRV	17	31	+82.3
TRV x SK	34	29	-14.6
TRV x AL	34	22	-31.2

TRV, total rinsing volume (370 mL); AL, 30 mL aliquot; SK, 25 g of skin. *Refrigeration for 72 h.

Inspection Service, 42% of the carcasses presented less than 12 *Salmonella* cells, and in 43% of the samples, counts ranged from 12 to 120 cells/carcass.[5] These low levels of contamination, as well as the presence of injured cells, require a more sensitive method of analysis and the use of analytical units that may promote pathogen detection. Collection and incubation of the total volume of fluid used to rinse the whole sample was the analytical unit that gave the best results and this, therefore, seems to solve the problem of detecting small numbers of *Salmonella* cells.

This hypothesis is based on the fact that when the carcass is rinsed, the whole product is evaluated and not just a single region, as occurs when using SK. With TRV, it is possible to recover a higher number of pathogen cells from contaminated skin. Similarly, the incubation of TRV, compared with AL (7.5% of the total volume) may also more easily detect *Salmonella*.[5] It could be hypothesized that at least part of the *Salmonella* cells (those that were injured) could be recovered and detected[5] after refrigeration for 72 h. Therefore, after this storage period, performance of the analytical units SK and AL was similar to TRV.

For biological purposes, TRV continues to be the analytical unit most capable of detecting the highest number of positive carcasses, consequently yielding the lowest numbers of false-negative results. Therefore, this study proved the hypothesis that more positive carcasses for *Salmonella* would be detected after refrigeration if the same analytical unit were used to analyze post-chiller samples and samples stored for 72 h under refrigeration.[5] Based on these results, we can hypothesize that the use of different analytical units may be responsible, among other factors, for the discrepancy in data found in the literature. The use of TRV as a preferred method for samples in the production process and for those coming from retailers seems to be the best to evaluate the real status of *Salmonella* sp. contamination in carcasses, providing a more reliable source of information for pathogen control in official government programs.

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