

Research Paper

Assessing the growth and recovery of *Salmonella* Enteritidis SE86 after sodium dichloroisocyanurate exposure

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Abstract

The objective of the present study was to assess the growth and the recovery of *Salmonella* (*S.*) Enteritidis SE86 in different diluents, culture media and using different plating methods after the exposure to 200 mg/kg sodium dichloroisocyanurate (NaDCC). Before and after NaDCC exposure, SE86 was cultured at 30 °C and 7 °C in the following diluents: Peptone water (P), Saline solution (SaS), Peptone water+Saline solution (P+SaS), Peptone water+Tween 80+Lecithin+Sodium thiosulfate (P+N) and Saline solution+Tween 80+Lecithin+Sodium thiosulfate (SaS+N). The SaS diluent was chosen because it was able to maintain cells viable without growth and was further used for plating SE86 on non selective medium (Tryptic Soy Agar-TSA) and on selective media (Mannitol Lysine Crystal Violet Brilliant Green Agar-MLCB; Brilliant Green Agar-BGA; *Salmonella Shigella* Agar-SS and Xylose Lysine Dextrose-XLD). The Thin Agar Layer method (TAL) *i.e.*, selective media overlaid with non selective TSA was also evaluated. Results indicated that SE86 not exposed to NaDCC was able to grow in P, P+N, SaS+N and P+SaS, but not in SaS, that was able to maintain cells viable. SE86 exposed to NaDCC demonstrated similar counts after dilution in SaS and the plating on non selective TSA, selective media MLCB, BGA, SS and XLD and on TAL media. SE86, *S.* Typhimurium and *S.* Bredeney, exposed or not exposed to NaDCC, showed no significant differences in counts on TSA, XLD and XLD overlaid with TSA, suggesting that all those media may be used to quantify NaDCC-exposed *Salmonella* by plating method.

Key words: *Salmonella* Enteritidis SE86, diluents, plating methods, stressed cells, sodium dichloroisocyanurate.

Introduction

Salmonellosis is one of the most important public health problems worldwide (Greig and Ravel *et al.*, 2009; Tondo and Ritter, 2012). In Brazil, *Salmonella* spp. was identified as the main etiological agent causing foodborne diseases in the period of 1999 to 2011, being responsible for 1660 foodborne outbreaks investigated by the Brazilian Inspection Services (Brasil, 2011). Using pheno and genotyping methods (Oliveria *et al.*, 2012; Geimba *et al.*, 2004), a specific strain of *S.* Enteritidis (named SE86) was identified in more than 95% of the salmonellosis notified in State of Rio Grande do Sul (RS), southernmost State of Brazil, in the last years (Oliveira *et al.*, 2012), arising an expressive

interest in the investigation of this pathogen. Further studies demonstrated that SE86 presents high capability for thermal and acid adaptation after its exposure to sub-lethal pH and this capability was not verified in other *Salmonella* serovars (Malheiros *et al.*, 2009). SE86 was also more resistant than other *Salmonella* serovars to 200, 400 and 800 mg/kg sodium hypochlorite and was able of forming biofilms on stainless steel and polyethylene (Tondo *et al.*, 2010).

In the State of RS, between 2000 and 2002, insufficient cleanliness of utensils and equipments and cross contamination, together, were responsible for 14.15% of the foodborne outbreaks occurred in food services, indicating a

possible deficiency in the hygiene of surfaces that come into contact with foods (Cunha *et al.*, 2008). Microbiological analysis of utensil and equipment surfaces is an important tool for verifying the effectiveness of cleaning and disinfection procedures (Moore and Griffith, 2007), and the swab technique is the most suitable method for that (APHA, 2001). After disinfection of surfaces, many microorganisms are inactivated, but a part of the bacterial population may survive and remain in the VNC (Viable but Not Cultivable) condition. These bacteria may have been affected by the action of disinfectants, losing the ability to form colonies on selective media, however, they still be able to reproduce in non-selective media, being called “stressed” or “injured” (Wu and Fung, 2006; Wu, 2008). Stressed bacterial cells have great relevance to the food industry and food services, since they can not be detected in the microbiological analysis, but may be able to cause outbreaks or food quality problems, after their recovery. In this sense, the methods for analysis should allow recovery of bacterial cells, in order to obtain the number of microorganisms that are actually present in sample or on a surface (Jasson *et al.*, 2007).

In food services and food industries, the chlorine compounds are among the most commonly disinfectants used for disinfection of surfaces (Peng *et al.*, 2002; Rossoni and Gaylarde, 2000). In Brazil, probably the most used chlorine compounds are 200 mg/kg sodium hypochlorite or sodium dichloroisocyanurate, which can injure microorganisms present on food contact surfaces, avoiding their detection. Therefore, evaluating the effect of these sanitizers on important food pathogens such as *S. Enteritidis* SE86 is of great importance.

The aim of this study was to assess the growth and the recovery of *S. Enteritidis* SE86 in different diluents, media and by plating methods, after sodium dichloroisocyanurate exposure, aiming to identify adequate procedures to be used in swab sampling methods.

Materials and Methods

Bacterial strains

Strains of *Salmonella enterica* of three different serovars were used in this study. *S. Enteritidis* SE86 was isolated from a cabbage involved in a salmonellosis outbreak occurred in the State of RS, in 1999. This microorganism presents the same genotypic pattern of *S. Enteritidis* involved in more than 95% of the salmonellosis outbreaks occurred in the State of RS during the period of 1999 to 2006 (Oliveira *et al.*, 2012). *S. Typhimurium* and *S. Bredeney* strains were isolated from pig fecal samples also in the State of RS. These strains were provided by Prof. Dr. Marisa Ribeiro de Itapema Cardoso of the Preventive Veterinary Department of Universidade Federal do Rio Grande do Sul (UFRGS) and there is no reports indicating the involvement of them with foodborne diseases in the State of

RS. Before experiments, the strains were stored at -18 °C in 30% (v/v) glycerol, and working cultures were kept at 4 °C on Brain Heart Infusion Agar (BHI; Oxoid). Before use in the experiments, strains were activated in BHI broth at 37 °C for 18-24h, reaching a concentration of approximately 10^8 cfu.mL⁻¹. The initial concentration of cells was measure by plating the inoculum on BHI agar.

Evaluation of growth of *S. Enteritidis* SE86 in different diluents

Cultures of *S. Enteritidis* SE86 were activated in 9 mL of BHI at 37 °C for 18-24 h, reaching a concentration of approximately 10^9 cfu.mL⁻¹. Two experiments were conducted in parallel: one with *S. Enteritidis* SE86 not exposed to the disinfectant (Control) and other with *S. Enteritidis* SE86 exposed to 200 mg/kg NaDCC (Trade mark Kalyclean S313, Kalykim, Brazil). The exposure to NaDCC was performed by inoculating 1 mL of bacterial culture in 9 mL of 200 mg kg⁻¹ NaDCC, for five minutes. This exposure time was used because it was able to reduce but not inactivate all the inoculated *Salmonella*. The exposure time and bacterial concentrations were assessed previously to the experiments of multiplication. The exposed and not exposed bacterial cultures were diluted until a concentration of 10^8 cfu mL⁻¹ in five different diluent solutions: 0.1% Peptone water (P); 0.85% Saline solution (SaS); 0.85% Saline solution + 0.1% Peptone water (P + SaS); 0.1% Peptone water + 0.5% Tween 80 + 0.07% Lecithin + 1% Sodium Tiosulfate (P + N) and 0.85% Saline Solution + 0.5% Tween 80 + 0.07% Lecithin + 1% Sodium Tiosulfate (SaS + N). The diluent solutions containing exposed and not exposed *S. Enteritidis* SE86 were incubated at 30 °C and 7 °C for 1, 2, 3, 4, 5 and 6 hours. These incubation times were chosen in order to evaluate regular transport times that generally are lesser than 6 hours. These temperatures were chosen assuming that they represent temperatures easily performed under controlled (7 °C) and uncontrolled (30 °C) conditions, during the transport of swab samples. The number of colony forming units (cfu) of exposed *S. Enteritidis* SE86 was quantified by the drop technique (Silva *et al.*, 1997). To quantify not exposed *Salmonella*, 0.1 mL of each dilution was cultured on the surface of BHI plates. Before incubation for 18-24 hours at 37 °C, the plates were left to dry at room temperature, inside a laminar flow hood (LabConco, Kansas city, Missouri), previously sterilized by 15 min of UV exposure. The plates were quantified and the counts were expressed in UFC.mL⁻¹. Each measurement was performed in duplicate and each experiment was repeated at least twice.

Evaluation of culture media and plating methods

Exposed and not exposed *S. Enteritidis* SE86 cultures were diluted in SaS until 10^8 cfu.mL⁻¹ and plated (0.1 mL) in duplicate by surface plating method on TSA (nonselective medium), on four selective media [Mannitol Lysine

Crystal Violet Brilliant Green Agar (MLCB; Oxoid); Brilliant Green Agar (BGA; Oxoid), *Salmonella Shigella* Agar (SS; Oxoid) and Xylose Lysine Dextrose (XLD; Oxoid) and on selective media (MLCB, BGA, SS and XLD) overlaid with TSA (TAL method). The cell concentration was determined by a growth curve. The TAL plates were prepared overlaying 14 mL of nonselective medium (TSA) onto 25 mL of each selective medium. The plates were incubated at 37 °C for 18-24 hours and bacterial colonies were quantified and expressed in cfu mL⁻¹.

Evaluation of growth and recovery of *S. Enteritidis* SE86, *S. Typhimurium* and *S. Bredeney* on XLD by different plating methods and culture media

After the choose of the best diluent and the best plating method for the growth of exposed and not exposed *S. Enteritidis* SE86, other two serovars of *Salmonella* (*S. Typhimurium* and *S. Bredeney*) were tested by the same techniques, aiming to compare their growth. All the bacteria were individually exposed to NaDCC and diluted as described previously. After, 0.1 mL of the dilutions was plated in duplicate on the surface of TSA, XLD and XLD overlaid with TSA (TAL). The plates were incubated for 24 hours at 37 °C. After incubation, the UFC.mL⁻¹ were quantified and the results of each serovar in each medium was evaluated.

Statistical analysis

All experiments performed to evaluate the growth of microorganisms were carried out at least twice, and all results from counts were made in duplicates. The mean values were calculated and the analysis of variance (ANOVA) and a Tukey Test were carried out to compare the differences between the mean values. The differences were considered significant with p values were less than 0.05.

Results

Multiplication and recovery of *S. Enteritidis* SE86 in different diluents

The differences between counts of not exposed and exposed *S. Enteritidis* SE86 were approximately 2 log cfu.mL⁻¹, demonstrating the effect of NaDCC on the bacterial populations.

S. Enteritidis SE86 not exposed to NaDCC (Control) showed no significant multiplication ($p < 0.05$) in any of the diluents tested in the first three hours of experiment. However, the diluents P + SaS, P, SaS + N and P + N sustained significant multiplications (more than 1.5 log) after four, five, five and six hours at 30 °C, respectively. In opposite, *S. Enteritidis* SE86 did not multiply in the diluent SaS, at 30 °C, during the six hours of incubation, but the cells remained viable. P + SaS was the diluent that sustained higher counts of *S. Enteritidis* SE86, increasing 2.0 log UFC.mL⁻¹ after six hours of incubation at 30 °C (Figure 1).

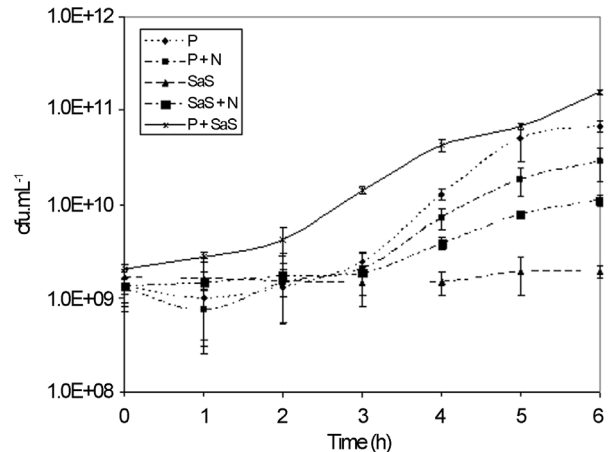


Figure 1 - Multiplication of *Salmonella* Enteritidis SE86 not exposed to NaDCC at 30 °C in Peptone water (♦), Saline solution (▲), Peptone water+Saline solution (*), Peptone water+Tween 80+Lecithin+Sodium thiosulfate (•) and Saline solution+Tween 80+Lecithin+Sodium thiosulfate (■).

S. Enteritidis SE86 exposed to NaDCC showed to be stressed because no significant multiplication ($p < 0.05$) in any of the diluents were observed during the six hours at 30 °C, but cells still viable. *S. Enteritidis* SE86 exposed and not exposed to NaDCC showed no significant growth in the diluents ($p < 0.05$), during the six hours of incubation at 7 °C, however cells remained viable.

Evaluation of culture media and plating methods

S. Enteritidis SE86 not exposed and exposed to NaDCC showed no significant differences ($p < 0.05$) among counts on non selective media (TSA), on selective media (BGA, SS XLD and MLCB) and on selective media XLD, SS, BGA overlaid with TSA (TAL media). TSA counts were higher than counts observed on selective media and TAL media, but this difference was not significant. MLCB overlaid with TSA demonstrated lower counts when compared with other TAL media.

Recovery of *S. Enteritidis* SE86, *S. Typhimurium* and *S. Bredeney* grown in XLD by different plating methods

The three serovars *S. Enteritidis* SE86, *S. Typhimurium* and *S. Bredeney*, not exposed (Figure 2) and exposed (Figure 3) to NaDCC showed no significant difference in counts on non selective TSA, on selective medium XLD and XLD overlaid with TSA (TAL).

Discussion

In the present study, *S. Enteritidis* SE86 was able to grow at 30 °C in all diluents tested, except SaS. In order to quantify microorganisms present in a diluted sample, an appropriate diluent should not promote bacterial multiplication or death, should be ease to prepare, should present low

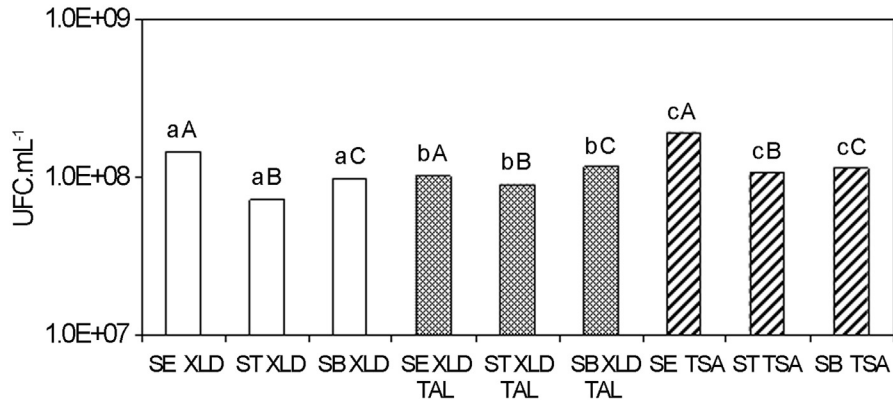


Figure 2 - Multiplication of *Salmonella* Enteritidis SE86 (SE), *Salmonella* Typhimurium (ST) and *Salmonella* Bredeney (SB) not exposed to sodium dichloroisocyanurate plated on XLD, XLD overlayed with TSA (TAL) and TSA. Values with different letters differ significantly ($p < 0,05$). a,b,c: Comparison among serovars on the same medium plated by the same method. A, B, C: Comparison of the growth of the same *Salmonella* serovar inoculated on different media.

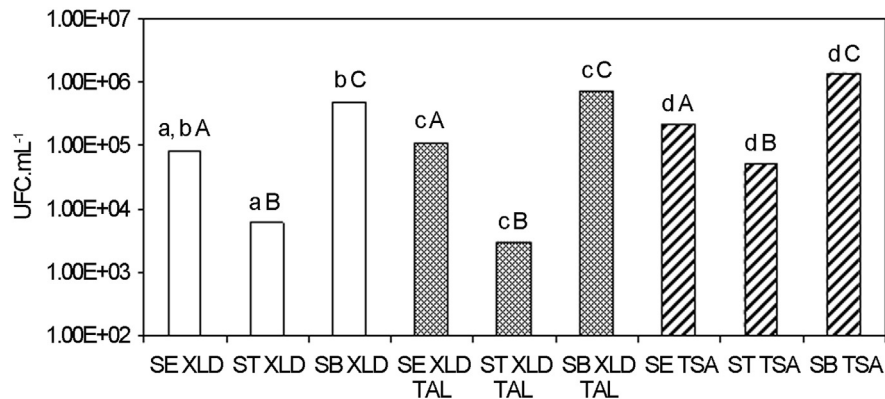


Figure 3 - Multiplication of *Salmonella* Enteritidis SE86 (SE), *Salmonella* Typhimurium (ST) and *Salmonella* Bredeney (SB) exposed to sodium dichloroisocyanurate for 5 min and plated on XLD, XLD overlayed with TSA (TAL) and TSA. Values with different letters differ significantly ($p < 0,05$). a,b,c: Comparison among serovars on the same medium plated by the same method. A, B, C: Comparison of the growth of the same *Salmonella* serovar inoculated on different media.

cost, and ideally, should be able to recover stressed cells (Wu *et al.*, 2001; Wu, 2008). Based on these appointments and according to our results, The most appropriate diluent was SaS, because it did not cause decreasing in the NaDCC exposed cell numbers and did not sustain the multiplication of not exposed cells. Additionally, this diluent is easy to be prepared and have a lower cost when compared to other diluents.

After the exposure to NaDCC, *S. Enteritidis* SE86 showed no significant multiplication in any of the diluents tested, even after six hours of incubation at 30 °C. As no significant multiplication occurred in any of the diluents, the cell recovery was not assessed, and it was not possible to distinguish dead cells from stressed or viable but not cultivable (VNC) cells. In order to recover stressed cells, bacteria present an extension in its lag phase, and this is called the recovery period. After this period, cells start to multiply at an equal rate to the not stressed cells (Tomlins and Ordal,

1971). As an example, a study have demonstrated a four to five hours lag phase of *S. Typhimurium* growing in Trypticase Soy Broth (TSB) and on TSA, after heat stress at 48 °C for 30 min (Clark *et al.*, 1968; Clark and Ordal, 1969). The experiments conducted in this study may suggest that the extent of the lag phase of *S. Enteritidis* SE86 exposed to NaDCC became longer than six hours, because not exposed present SE86 presented shorter lag phases in different diluents. It is possible that longer periods of incubation could be able to show the exact duration of the lag phase necessary to recover the stressed cells. However, it was not the purpose of this work, since the time of six hours was chosen because it is often recommended as an appropriate period of sample transport inside diluents (Silva *et al.*, 1997; Lightfoot and Maier, 2003).

At 7 °C, not exposed *S. Enteritidis* SE86 (Control) showed no significant growth in the diluents, during the six hours of incubation. Similar results demonstrated that pure

Buffered Peptone Water and also added with Tween 80 + Lecithin and Tween 80 + Lecithin did not sustain multiplication of *Bacillus cereus* and *Escherichia coli*, at 4 °C, during 6 hours (Greig and Ravel, 2009). In other study, no significant increase in the numbers of cells of *Salmonella* spp., *Escherichia coli* O157:H7 and *Staphylococcus aureus* was observed during nine hours of storage at 4 °C, after disinfection of shredded cabbage, using 0.1% calcined calcium, for 20 min, combined with 100 mg kg⁻¹ of Sodium hypochlorite, for 20 min (Fukuyama *et al.*, 2009). The results of the present study suggest that the temperature of 7 °C can be used to transport samples without allowing the multiplication of *S. Enteritidis* SE86. It is possible that this result may be valid for other microorganisms, since the American Public Health Association recommended refrigeration temperature for the transport of bacterial samples collected by swabs from surfaces, but the exact temperature is not define (APHA, 2001).

When the culture media and the plating methods were evaluated, no significant difference was shown in the counts of *S. Enteritidis* SE86 exposed to NaDCC, however there were increased numbers on TSA when compared with selective media and TAL. This quantitative difference suggests that there was few stressed cells, but the selective media did not inhibit significantly the multiplication of *S. Enteritidis* SE86, making it suitable for its cultivation, even by direct plating. Corroborating this result, the ISO 6579/2002 recommends the use of XLD plates for the enumeration of *Salmonella*.

In the present study, selective media overlaid with TSA (TAL) also showed no significant difference in counts of *S. Enteritidis* SE86 exposed to NaDCC, when compared with TSA or selective media. This result is interesting, since the TAL method has been reported as important for the recovery of stressed bacterial cells (Osaili *et al.*, 2010; Kang and Fung, 1999; Kang and Fung, 2000). According to the present work, XLD showed no significant difference in counts obtained on non-selective TSA and selective media overlaid with TSA, with or without NaDCC exposure. XLD plates are recommended by the Brazilian Ministry of Agriculture (MAPA) for *Salmonella* investigation and by ISO 6579/2002 for *Salmonella* quantification, suggesting its adequacy to be used in the enumeration of stressed *S. Enteritidis* SE86, at least for *in vitro* experiments. However, in order to evaluate its adequacy to real surface or food samples, more experiments are necessary.

When the growth and recovery of *S. Enteritidis* SE86, *S. Typhimurium* and *S. Bredeney* were evaluated, no significant differences in counts on the XLD plates, non-selective TSA and TAL were observed, demonstrating that the selective media did not inhibit the growth of these strains of *Salmonella*. This result is interesting and corroborates that such media could be suitable for the quantification of *Salmonella* exposed or not exposed to NaDCC. Other studies are

necessary to evaluate the behavior of these microorganisms, stressed by disinfectants on surfaces of food services.

In conclusion, this study indicated that 0.85% Saline (SaS) solution did not support *S. Enteritidis* SE86 growth and maintain cells viable for six hours, suggesting being an adequate diluent for the collection and transport of swab samples for the investigation of *S. Enteritidis* SE86. The temperature of 7 °C demonstrated to be able to maintain stable the numbers of *S. Enteritidis* SE86, suggesting to be adequate for the transport of swab samples. Finally, the direct plating on XLD medium demonstrated the same *Salmonella* counts that non selective TSA and TAL media, suggesting to be adequate for the quantitative analysis of different *Salmonella* serovars, exposed or not exposed to NaDCC, at least in laboratory conditions. Further studies are necessary to test these procedures for the analysis of *Salmonella* in natural conditions.

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