Tropical Life Sciences Research, 21(1), 47–53, 2010

The Stability of rRNA in Heat-killed *Salmonella enterica* Cells and Its Detection by Fluorescent In Situ Hybridisation (FISH)

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Abstrak: Pembezaan sel-sel berdaya hidup daripada yang bukan berdaya hidup merupakan satu perkara yang amat membimbangkan dalam proses pengesanan patogen mikrob dalam makanan. *Fluorescent in situ hybridisation* (FISH) telah digunakan sebagai satu cara pengesanan yang berpotensi. Kebolehan FISH untuk membezakan sel berdaya hidup daripada yang bukan berdaya hidup bergantung pada degradasi secara rapid rRNA dalam sel-sel bukan berdaya hidup. Dalam kajian ini, *Salmonella enterica* yang dimatikan pada suhu 80°C, 100°C dan 121°C telah diperiksa untuk kehadiran rRNA menggunakan FISH pada pelbagai jarak waktu iaitu dari 5 minit hingga 48 jam, selepas dikenakan rawatan haba. rRNA telah dikesan oleh FISH dalam bakteria yang telah dimatikan, selama 12 jam, 3 jam dan 1 jam selepas dikenakan rawatan haba pada 80°C, 100°C dan 121°C masing-masing. Keputusan ini menunjukkan bahawa terdapat korelasi antara suhu dan kestabilan rRNA dalam bakteria yang dimatikan menggunakan rawatan haba. Secara kesimpulan, FISH ialah satu teknik yang sesuai untuk membezakan sel-sel berdaya hidup daripada yang bukan berdaya hidup, terutamanya untuk sampel-sampel yang dikenakan suhu yang tinggi.

Kata kunci: Pengiraan Daya Hidup, *Salmonella enterica*, FISH

Abstract: Differentiation of viable cells from non-viable cells is a major concern in the detection of foodborne microbial pathogens. Fluorescent in situ hybridisation (FISH) has been utilised as a promising method in this regard. The ability of FISH to differentiate viable cells from non-viable cells depends on the rapid degradation of rRNA in non-viable cells. In our work, *Salmonella enterica* that were heat-killed at 80°C, 100°C and 121°C were examined for the presence of rRNA using FISH at various times ranging from 5 minutes to 48 hours after heat treatment. rRNA was detected by FISH in heat-killed bacteria for 12 hours, 3 hours and 1 hour after treatment at 80°C, 100°C and 121°C, respectively. These results indicate that there is a correlation between temperature and stability of rRNA in heat-killed bacteria. In conclusion, FISH was determined to be a suitable method for differentiation of viable cells from non-viable cells, especially for samples subjected to extreme heat.

Keywords: Viable Counting, *Salmonella enterica*, FISH

INTRODUCTION

Salmonella enterica is a major food and waterborne pathogenic bacterium that causes intestinal infection accompanied by fever, abdominal cramps and diarrhoea. A number of rapid methods for the detection of *Salmonella* in food have been developed, including automated detection methods (Peng & Shelef

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2001), immunological methods (Jouy *et al*. 2005; Wang *et al*. 1996) and nucleic acid-based analyses (Whyte *et al*. 2002; Nam *et al*. 2005; Malorny *et al*. 2007). However, most of these methods are inapplicable in determining viable bacterial count, which is the most important factor for food pathogens. Moreover, the plate count method, which is traditionally used for counting viable *Salmonella,* has disadvantages such as the requirement for a long incubation period and the inaccuracies in the count due to clumping and inhibition of cells by neighbouring cells (Lahtinen *et al*. 2006).

Molecular detection techniques have received significant attention in recent years due to the high specificity, reliability and speed compared to traditional methods. Fluorescent in situ hybridisation (FISH) is a molecular technique that has been utilised as a promising method in differentiating non-viable and viable bacteria. FISH allows detection of rRNA microscopically using rRNA probes (Regnault *et al*. 2000). Since most bacteria contain 10^3 to 10^5 ribosomes and a high number of rRNA copies (Amann *et al*. 1995), this technique is highly sensitive. Dead bacteria lose their membrane integrity, and their ribosomes degrade quickly (Hannig *et al*. 2007). Thus, the degradation of rRNA can be used as a readout to distinguish viable microorganisms from dead microorganisms by FISH (Vieira-Pinto *et al*. 2007; Hannig *et al*. 2007).

In food processing, different temperature conditions are used to improve sensory characteristics and eliminate foodborne microbial pathogens. Since applicability of FISH to detect viable cells is based on the rapid degradation of rRNA in dead cells, assessment of the use of FISH for the detection of bacteria subjected to different food processing temperature is needed. In this research, the stability of *Salmonella* rRNA at three different commonly used processing temperatures in the food industry were used to determine the applicability of FISH.

MATERIALS AND METHODS

Bacteria Culture

S. enterica obtained from BIOTECH, NASTDA, Thailand were cultured in tryptic soy broth yeast extract medium (TSBYE) at 37°C for 24 hours and serially diluted (10^{-1} to 10^{-10}) in sterile distilled water. Bacteria were enumerated using Rambach agar plates at 37°C overnight. Bacterial dilutions containing 10^7 cfu/ml were prepared using the same bacterial culture and kept refrigerated for subsequent experiments. The samples were subjected to heat treatments at 80°C, 100°C (heated in water bath) and 121°C (autoclaved) for 15 minutes in all of our studies. After heat treatment, bacteria were cultured in Rambach agar plates to confirm the absence of any live bacteria. rRNA in heat-treated cultures was detected using FISH at different intervals after heat treatments: 5 minutes, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours and 48 hours. A live bacterial dilution of the same concentration as above was used as a control. The control sample was also tested using FISH at the same time intervals.

Fluorescent In Situ Hybridisation (FISH)

Samples (1 ml each) from heat-treated cultures were centrifuged at 12,500 rpm for 3 minutes and bacterial pellets were obtained. Each bacterial pellet was then fixed with approximately 3 times its volume of 4% paraformaldehyde (w/v) solution in phosphate buffered saline (PBS) for 3 hours at 4°C. Fixed cells were then washed twice with 1 ml of 1X PBS by centrifugation at 12,500 rpm for 3 minutes. Pellets were re-suspended in 1 volume of PBS and an equal volume of ice-cold 98% ethanol and stored at –20°C.

Fixed bacterial samples were used for hybridisation with the fluorescent probe. First, 3 µl of fixed sample were put into each of the 8 wells on Teflon slides and oven-dried at 37°C for 10 minutes. These cells were dehydrated by successive passages through 50%, 80% and 98% (v/v) ethanol for 3 minutes per solution. Slides were then air-dried at room temperature in a vertical position. After air-drying, 10 µl of hybridisation buffer [0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.1% sodium dodecyl sulphate (SDS)] containing 5 ng/µl of the Sal3 Probe, 5'-AATCACTTCACCTACGTG-3' labelled with flourescein isothiocyanate (FITC) at the 5' end (Vieira-Pinto *et al.* 2007), was added to each well. The slides were then incubated for 2 hours at 46°C in a humid chamber. After the incubation, slides were again washed with the same buffer solution at 46°C for 15 minutes, rinsed twice with autoclaved distilled water and air dried at room temperature in a vertical position in the dark. Next, 10 µl of 2 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) solution was then added to each well and kept at room temperature in the dark for 3 minutes. Excess DAPI solution was removed by rinsing the slide with distilled water. The slides were again air-dried at room temperature in a vertical position in the dark, 3 µl of anti-fade reagent was added to each well, and the slides were sealed with a cover slip. Finally, slides were examined by fluorescent microscopy.

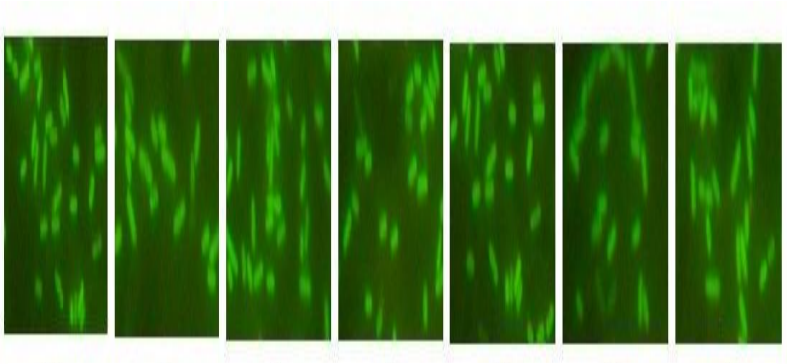
RESULTS AND DISCUSSION

Molecular techniques are adaptable for the detection of microbial pathogens in various sources. Application of molecular techniques in the detection of foodborne pathogens has received significant attention in the last few years. However, for determination of viable cells in food testing, reliability of the results obtained by molecular techniques is questionable compared to the traditional culturing-based detection. This is because residual DNA and/or RNA in dead cells contribute to false positive results using molecular techniques when determining viability. As pathogenic bacteria are killed by different processing methods, especially heat treatment in food production, this inaccuracy becomes a major issue. The study of DNA and rRNA stability in heat-killed cells and its effect on the molecular detection of viable cells are thus very important.

Since RNA is less stable in dead cells, RNA is considered a better target for the detection of viable bacterial pathogens (Mckillip *et al*. 1998). A number of studies have used mRNA (Nadal *et al*. 2007; Liu *et al*. 2006; Zhao *et al*. 2006) and rRNA (Nieminen *et al*. 2006; Ercolini *et al*. 2003; Cocolin *et al*. 2007) in this regard. Compared to mRNA, rRNA is a more suitable indicator for viable

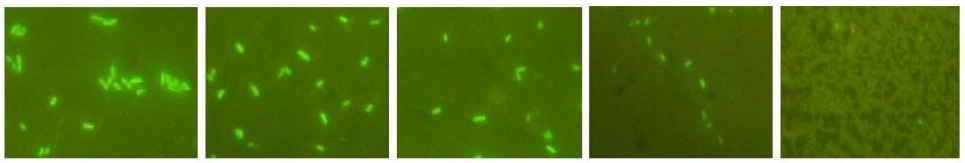
bacteria due to its high copy number, constitutive expression, low stability compared to DNA and its presence in all viable cells, while mRNA is present only when protein expression is expected (Amann *et al*. 1995; Mckillip *et al*. 1998). The stability of rRNA in heat-killed cells depends on the bacterial species and the temperature of the heat treatment. The ability to detect residual rRNA in heat-killed cells after heat treatment is different for different molecular techniques (Mckillip *et al*. 1998). All heat-treated bacterial samples were cultured on Rambach agar plates, and there were no colonies observed. This showed that there were no living bacterial cells in any of the heat-treated samples.

There was no significant variability in staining of control samples, and they gave positive results throughout the experimental period (Fig. 1). *S. enterica* culture treated at 80°C for 15 minutes gave positive results by FISH for up to 12 hours after heat treatment (Fig. 2). However, no fluorescent signals were observed after 24 hours. The culture subjected to 100°C for 15 minutes was positive for FISH for up to 3 hours after the treatment (Fig. 3), but no detectable signal was observed at and after 6 hours. For the autoclaved culture (121°C, 15 minutes), detectable signals were observed for only an hour after heat treatment (Fig. 4).



(a) (b) (c) (d) (e) (f) (g)

Figure 1: The detection of *S. enterica* in the control sample (live cells) at different times using FISH: (a) 5 minutes, (b) 1 hour, (c) 3 hours, (d) 6 hours, (e) 12 hours, (f) 24 hours and (g) 48 hours.

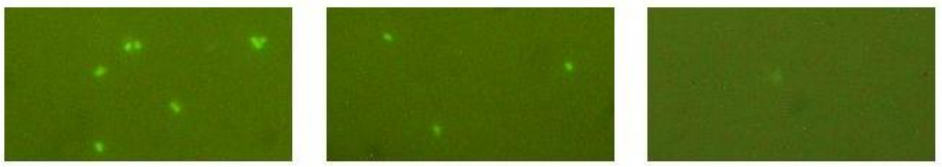


(a) (b) (c) (d) (e)

Figure 2: The detection of *S. enterica* in heat-killed cells (80°C for 15 minutes) by FISH at indicated times after heat treatment: (a) 5 minutes, (b) 1 hour, (c) 3 hours, (d) 6 hours and (e) 12 hours.

(a) (b) (c)

Figure 3: The detection of *S. enterica* in heat-killed cells (100°C for 15 minutes) by FISH at indicated times after heat treatment: (a) 5 minutes, (b) 1 hours and (c) 3 hours.



(a) (b) (c)

Figure 4: The detection of *S. enterica* in heat-killed cells (121°C for 15 min) by FISH at indicated times after heat treatment: (a) 5 minutes, (b) 1 hour and (c) 3 hours.

In all samples tested, bacterial cells were stained to different levels and different intensities. The number of well-stained cells was lower compared to the total number of cells, and the signal decreased with time. The fluorescent intensity of the cells decreased with increased temperature. Compared to the culture treated at 80°C, the staining was very low in the culture treated at 100°C. In autoclaved culture, the staining was very low and hardly detectable. Residual rRNA was found to be detectable by FISH for 12 hours after *S. enterica* were treated at 80°C, compared to 3 hours and 1 hour at 100°C and 121°C respectively. When rRNA was not detectable, ribosomal units may have remained intact and protected the rRNA from internal and external RNase, as explained by Mckillip *et al.* (1998). This could occur when heat treatment is at less extreme temperatures, since rRNA lose their integrity at extreme temperatures. However, when considering autoclaved samples, there may be an effect of high pressure of the autoclave on cellular matter, which could increase degradation of rRNA. Hence, the results obtained for autoclaved samples could be due to a combined effect of normal degradation of rRNA in dead cells and disruption of cellular matter by high pressure.

CONCLUSION

FISH was found to be successful in differentiating viable *S. enterica* cells from heat-killed cells. Although rRNA could not be confirmed as a good indicator for viability of heat-killed cells at lower temperatures, it could be considered a good viability indicator for bacteria that are heat-killed under extreme heat conditions. FISH can be recommended for detection of *S. enterica* in food samples subjected to extreme heat conditions after 3 hours of treatment.

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