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Reliable Detection of Viable Salmonella Enterica from Inoculated Chicken Samples by Fluorescent in Situ Hybridization (FISH) with Vivification Step

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Abstract

Inspection of food for the presence of Salmonella is a major concern in food industry. Many rapid, reliable and sensitive novel methods have been developed to detect Salmonella from food samples. However a method to detect live bacterial cells separately from dead cells yet to be developed. Although fluorescent in situ hybridization (FISH) has been identified as promising method in this regards, the viable count could be overestimated by FISH technique due to dead cells with some amount of non degraded rRNA. Some times it could be underestimated because of inactive but viable cells which not produce detectable amount of rRNA. The possibility of using antibiotic treatment step in FISH technique to overcome this problem was studied. Chicken samples inoculated by differently prepared mixtures of live and heat killed Salmonella enterica cultures were used to isolate bacterial cells with and without antibiotic treatment (Nalidixic acid 10μg/ml and Ciprofloxacin 1μg/ml for 2h at 37ºC). FISH was performed with Salmonella specific 23S rRNA probe Sal3, 5'-AATCACTTCACCTACGTG-3' labeled with fluorescein isothiocyanate (FITC) at 5' end. Cells observed with high intensity under fluorescent microscope were identified as live cells. Results of statistical analysis for antibiotic treated and untreated samples indicated that the introduction of antibiotic treatment step in FISH technique permitted a successful application to over come the problem associated with viable Salmonella enterica detection and quantification.

Key words: Salmonella, FISH, Antibiotics, Viable count

Introduction

Salmonella enterica is a major food and water borne pathogenic bacterium which causes for intestinal infection accompanied by fever, abdominal cramps and diarrhea. As consequence of many pre-harvest, harvest and post harvest factors food can be contaminated by Salmonella. Contaminated egg, meat and poultry products are the main sources of infection (Nowak et al., 2007; Wang

et al., 1996). Rapid and reliable detection of viable Salmonella in food samples is important for the prevention from disease as well as the cost of storage and transportation of infected products (Vieira-Pinato et al., 2007). Plate count method traditionally used for the determination of viable count of Salmonella has disadvantages such as, requirement of long incubation period, clumping and inhibition of cells by neighboring cells which lead to underestimation of cell number (Lahtinen et al., 2006). There are number of rapid methods for the detection of Salmonella in foods have been developed, including automated detection methods (Peng & Shelef, 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, those methods are inapplicable in determination of viable bacterial count.

Compared to other detection methods molecular detection techniques especially PCR based methods have become more popular in recent years (Malorny et al., 2003). Those PCR based methods are also inapplicable in viable cell detection due to the persistence of DNA and RNA even in dead cells. Fluorescent in situ hybridization (FISH) is the most commonly applied method among the non-PCR based molecular detection techniques (Ercolini et al., 2003). In this method, cells contain threshold amount of rRNA are detected microscopically using rRNA probes (Regnault et al., 2000). Since most bacteria cells contain 10^3 to 10^5 ribosomes and high number of rRNA copies (Amann et al., 1995) this technique has very high assay sensitivity. Dead bacterial cells loose their membrane integrity and their ribosomes degrade quickly (Hannig et al., 2007). This degradation of rRNA target can be used as a base for the detection of viable microorganisms separately from dead microorganisms by FISH (Viaria-Pinato et al., 2007; Hannig et al., 2007). So, FISH technique could identify as a promising tool in determination of viable count of microorganisms. There are some other reports, which state that some dead bacteria also could have metabolic activity and could have some amount of rRNA (Regnault et al., 2000). This may lead to over estimation of the viable bacterial count by FISH. On the other hand, there are some reports which state that some live bacteria could be in inactive state and not produce enough rRNA for the detection by FISH technique which may lead to negative results or under estimation of viable cell number in FISH technique (Vieira – Pinto et al., 2007).

Vivification of microorganisms by antibiotic treatment prior to detection by FISH technique has been used in some researches in order to increase the detectable signal by increasing the amount of rRNA in the cells (Regnault et al., 2000). This antibiotic treatment could activate the inactive cells and reduce the under estimation of viable count due to inactive but viable cells. More ever this could increase the fluorescent signal of live cells compared to dead cells,

which could lead for more reliable detection of viable cells separately from dead cells. The aim of this work was to study the effect of vivification step on determination of viable count of Salmonella from inoculated chicken samples by FISH technique.

Methodology

Salmonella enterica was cultured for 4 h at 36 ^{0}C to obtain mid exponential phase culture in 150 ml of Tryptic Soy Broth Yeast Extract medium (TSBYE). Then the culture was serially diluted (10^{-1} – 10^{-10}) in sterile distilled water and enumerated using Rambach agar plates at 37 ^{0}C overnight. Bacterial concentration was estimated by calculating the average number of red colonies on plates containing 30 to 300 colonies. After enumeration the same bacterial culture kept at refrigerator was used to prepare 250 ml of 10^7 cfu/ml bacterial dilution. 125 ml of dilution was separated, heated in boiling water bath for 15 minutes to kill the microorganisms, plated in Rambach agar medium and confirmed absence of viable microorganisms. Then ten different mixtures contain 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10% live bacterial cells were prepared using the heat killed and live microorganism samples. Then, 1ml of each prepared dead and live microorganism mixtures were used to inoculate 5 g of chicken meat samples.

Inoculated meat samples were mixed with 15 ml of 1xPBS solution and homogenized by vortexing for 5 min. Then, 1 ml of each sample was treated with antibiotic (Nalidixic acid 10 µg/ml and Ciprofloxacin 1 µg/ml for 2 h at 37 ^{0}C). Then, 1 ml of antibiotic treated and non treated sample suspensions were centrifuged at 12500 rpm for 3 min and bacterial pellets were obtained. The bacterial pellets were then fixed with three volumes of 4% paraformaldehyde (w/v) solution in PBS for 1 h at 4 ^{0}C. Fixed cells were then washed twice with 1 ml of 1x PBS by centrifugation at 12500 rpm for 3 min. Pellets were re-suspended in one volume of PBS and equal volume of ice cold 98% ethanol was added and stored at -20 ^{0}C.

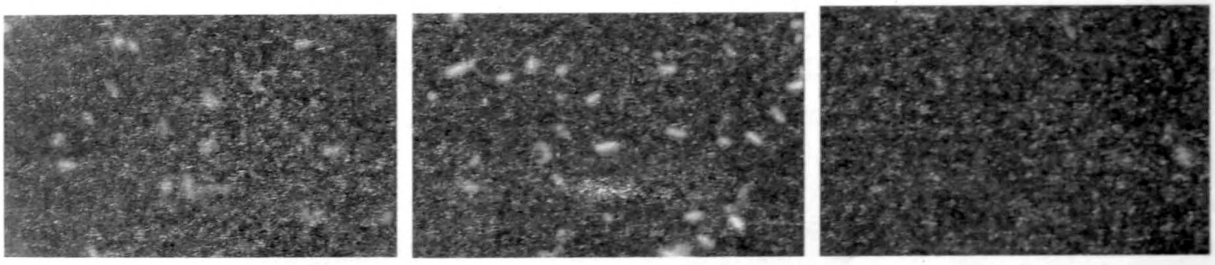
The fixed bacterial samples were used for hybridization with fluorescent probe. Eight well teflon slides were used as hybridization support, 3 µl of each fixed cell sample were spread in wells and oven dried in 37 ^{0}C for 10 min. Then cells were dehydrated by successive passages through 50%, 80%, and 98% (v/v) ethanol for 3 min in each solution and slides were air dried in room temperature in vertical position. After air drying 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, P^{II} 7.2, 0.1% SDS) containing 5 ng/ µl of the Sal3 Probe, 5'-AATCACTTCACCTACGTG-3' labeled with FITC at 5' end were added to each well. Then slides were incubated for 2 h at 46 ^{0}C in humid chamber. After incubation slides were washed by buffer solution (0.9 M NaCl, 20 mM Tris-HCl, P^{II} 7.2, 0.1% SDS) at 46 ^{0}C for 15 min, rinsed two

times with autoclaved ddH_2O and air dried at room temperature in the vertical position in a dark place. Then 10 µl of 2 µg/ml DAPI working solution was added per well, kept at room temperature in dark place for 3 min, and removed extra DAPI solution by rinsing the slides with ddH_2O. After that slides were air dried at room temperature in vertical position in dark and 3 µl of antifade reagent were added per well and sealed with a cover slip. Finally, slides were examined by oil immersion fluorescent microscopy. Five random microscopic fields were observed per one well and photographs were taken.

Results and Discussion

Owing to its speed and sensitivity, FISH technique is considered as a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology (Amann et al., 1990; Amann et al., 2001; Moter and Gobel, 2000). The versatility of FISH technique is testified by the wide spectrum of applications currently found in literature; so far, it has been applied to the study of microbial symbiosis, and in wastewater treatment (Amann et al., 2001). Moreover, FISH is routinely applied in medicine as a diagnostic tool for the identification of bacteria in complex communities (Moter and Gobel, 2000). Despite these huge applications, the use of FISH in food is very limited (Ercolini et al., 2003). Even in those limited applications there is no literature available for application of FISH technique for viable cell detection. In our experiment we assessed the ability of using vivification step in FISH technique to differentiate viable cells from dead cells.

In this experiment, significant increase in fluorescent signal and cell size was observed for some bacterial cells in antibiotic treated samples. This is due to the action of Nalidixic acid and Ciprofloxacin as explained by Kogure et. al. (1979). These antibiotics inhibit DNA gyrase to prevent the cell division and increase the cell size and amount of rRNA in live cells due to cell metabolism. In this experiment, those cells which were producing high fluorescent signals were considered as live cells. (Figure: 1).



A B C

Figure 1: A, view of bacterial cells without antibiotic treatment, B, view of bacterial cells with antibiotic treatment and C, view of live bacterial cells in live and dead bacterial cell mixture.

Only those cells with high fluorescent signal were counted as live cells for all antibiotic treated samples. For non-treated samples, all detectable cells were counted as live cells without considering the intensity of fluorescent signals. Using the bacterial count of the sample with 100% live cells, expected live bacterial counts were calculated for other samples (Table: 1).

Table 1: Average live cell count with and without antibiotic treatment.

Sample number	Live cell percentage	Expected live cell number	Average live cell number (Antibiotic treated samples)	Average live cell number (Non-treated samples)
1	100	1220	1217	1218
2	90	1098	1077	1121
3	80	976	981	1035
4	70	854	833	897
5	60	732	710	767
6	50	610	589	638
7	40	488	472	518
8	30	366	361	381
9	20	244	246	273
10	10	122	119	143

In order to analyze the influence of the vivification step on viable cell count, the bacterial counts for untreated and treated samples were compared with the calculated expected counts by chi-square test. In this analysis, significant difference was observed between the expected bacterial count and bacterial counts of untreated samples at 95% confidence limit indicating the non reliability of FISH technique without vivification step in viable cell detection. However, there was no significant difference observed between expected bacterial counts and bacterial counts of antibiotic treated samples at 95% confidence limit, this indicate the association of viable count obtained by FISH technique with expected values when vivification step is introduced. In conclusion, considering the results of the study, introduction of antibiotic treatment step in FISH technique was identified as a successful application to overcome the effect of dead cells in viable *Salmonella enterica* detection and quantification from meat food samples.

References

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., Sthal, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flocytometry for analyzing mixed microbial populations. Appl.

Environ. Microbiol. 56, 1919-1925.

Amann, R.. Fuchs, B. M., Behrens, S. (2001). The identification of microorganisms by fluorescence in situ hybridization. Curr. Opin. Biotechnol. 12, 231-236.

Amann, R., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiology Review, 59(1), 143-169.

Ercolini, D., Philip, J. H., & Christine, E.R.D. (2003). Development of a fluorescence in *situ* hybridization method for cheese using a 16S rRNA probe. Journal of Microbiological Methods, 52, 267– 271.

Hannig, C., Hannig, M., Rehmar, O., Braun, G., Hellwig, E., & Al-Ahmad, A. (2007). Fluorescent microscopic visualization of initial bacterial colonization on enamel in situ. Archives of Oral Biology, doi:10.1016/j. archoralbio.2007.05.006.

Jouy, E., Proux, K., Humbert, F., Rose, V., Lalande, F., Houdayer, C., Picault, J.P., & Salvat, G. (2005). Evaluation of a French ELISA for the detection of *Salmonella Enteritidis* and *Salmonella Typhimurium* in flocks of laying and breeding hens. *Preventive Veterinary Medicine*, 71, 91–103.

Kogure, K., Simidu, U., Targa, N. (1979). A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25, 415-420.

Lahtinen, S. J., Gueimonde, M., Ouwehand, A.C., Reinikainen, J.P., & Salminen, S.J. (2006). Comparison of four methods to enumerate probiotic bifidobacteria in a fermented food product. Food Microbiology, 23, 571–577.

Malorny, B., Tassios PT, Radstrom P, Cook N, Wag-ner M and Hoorfar J (2003) Standardization of diagnostic PCR for the detection of foodborne pathogens. International Journal of Food Microbiology 83, 1, 25, 39-48.

Malorny, B., Bunge, C., & Helmuth, R. (2007). A real-time PCR for the detection of Salmonella Enteritidis in poultry meat and consumption eggs. *Journal of Microbiological Methods*, 70, 2, 245-251.

Moter, A., Gobel, U. B. (2000). Fluoresece in situ hybridization (FISH) for direct visualization of microorganisms. J. Microbiol. Methods 41. 85-112.

Nam, H. M., Srinivasan, V., Gillespie, B. E., Murinda, S. E., & Oliver, S. P. (2005). Application of SYBR green real-time PCR assay for specific detection of Salmonella spp. In dairy farm environmental samples. *International Journal of Food Microbiology*, 102, 161– 171.

Nowak, B., Muffling, T. V., Chaunchom, S., & Hartung, J. (2007). Salmonella contamination in pigs at slaughter and on the farm: A field study using an antibody ELISA test and a PCR technique. *International Journal of Food Microbiology*, 115, 259 – 267.

Peng, H., & Shelef, L. A., (2001). Automated simultaneous detection of low levels of listeriae and salmonellae in foods. *International Journal of Food Microbiology*, 63, 225–233.

Regnault, B., Martin-Delautre, S., & Grimont, P.A.D. (2000). Problems associated with the direct viable count procedure applied to gram-positive bacteria. International Journal of Food Microbiology, 55, 281–284.

Vieira-Pinto, M., Oliveira, M., Aranha, J., Martins, C., & Bernardo, F. (2007). Influence of an enrichment step on Salmonella sp. detection by fluorescent in situ hybridization on pork samples. Food Control, doi:10.1016/j. foodcont.2007.04.005.

Wang, H., Blais, B. W., Brooks, B. W., & Yamazaki, H. (1996). *Salmonella* detection by the polymyxin-cloth enzyme immunoassay using polyclonal and monoclonal detector antibodies. *International Journal of Food Microbiology*, 29, 31-40.

Whyte, P., Mc Gill, K., Collins, J. D., & Gormley, E. (2002). The prevalence and PCR detection of Salmonella contamination in raw poultry. *Veterinary Microbiology*, 89, 53–60.