American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

American Journal of Food Technology 6 (9): 851-856, 2011
ISSN 1557-4571 / DOI: 10.3923/ajft.2011.851.856

Effect of Sample Pre-enrichment and Characters of Food Samples on the Examination for the *Salmonella* by Plate Count Method and Fluorescent *in-situ* Hybridization Technique

R.M.U.S.K. Rathnayaka
Department of Food Science and Technology, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka, Sri Lanka

ABSTRACT

In most of the novel and traditional methods used in the examination of food samples for *Salmonella,* pre-enrichment of samples is used as a means of increasing the sensitivity and reliability. However, the influence of pre-enrichment of sample on some of those methods has not been studied. Furthermore, the effect of the conditions of the sample on the sensitivity of some of those methods are also not been studied. The aim of this research was to study the influence of pre-enrichment and the conditions of the samples on the detection sensitivity of one novel method Fluorescent *in-situ* Hybridization (FISH) and one traditional culture techniques (XLT-4 agar plates). To study the influence of enrichment, 60 pork sausage samples collected from 20 different food outlets were examined for *Salmonella* contamination by both methods with and without pre-enrichment. To study the effect of the conditions of food, collected samples were spiked with 10^7 cfu mL^{-1} *Salmonella enterica* culture and examined with and without pre-enrichment. Detection sensitivity of both methods was higher in pre-enriched fresh samples as well as spiked samples. FISH method was found to be more sensitive and less affected by the conditions of food, compared to culture method.

Key words: *Salmonella,* fluorescent *in-situ* hybridization (FISH), pre-enrichment, plate count method, meat sausages

INTRODUCTION

Salmonella is a major food and water borne pathogenic bacterium which causes intestinal infection, accompanied by fever, abdominal cramps and diarrhea which is commonly known as salmonellosis (Fadel and Ismail, 2009; Jongerius-Gortemaker *et al*., 2002). They are Gram negative, motile, facultative anaerobic bacteria which can be contaminated with food as consequence of many pre-harvest, harvest and post harvest factors. Contaminated egg, meat and poultry products are the main sources of *Salmonella* infections (Seidavi *et al*., 2008; Nowak *et al*., 2007; Wang *et al*., 1996). Contamination of sea foods and drinking water by *Salmonella* is also a major public health concern (Nguendo-Yongsi, 2011; Shabarinath *et al*., 2007).

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 (Selvan *et al*., 2007; Vieira-Pinto *et al*., 2008). In this context, methods based in nucleic acid detection may play an important role (Malkawi and Gharaibeh, 2004). However, the majority of the rapid genetic assays and even traditional techniques, used to detect specific pathogens in foods require an enrichment step, which increases the analytical time (Olsen *et al*., 1995). Although

sample enrichment limits the assay speed, it provides benefits such as, the increase of cell number in to detectable level, the dilution of the detection inhibitors, the differentiation of viable and non-viable cells and the elimination of stressed or injured cells due to food processing (Moreno *et al*., 2001; Piknova *et al*., 2002).

Culture in XLT-4 agar, a *Salmonella* specific culture medium, is traditionally used in the detection of *Salmonella* from food samples. Fluorescence In Situ Hybridization (FISH) using rRNA probes has proved to be a promising tool for detection of microorganism in food (Ercolini *et al*., 2003; Fang *et al*., 2003; Moreno *et al*., 2001; Regnault *et al*., 2002). In both these methods sample pre-enrichment is an essential step. However, effect of sample pre-enrichment on the results of these methods and the effect of different conditions of foods have not been studied. In this study, novel detection method, fluorescent *in situ* hybridization, was comparatively studied with conventional culture based detection for its sensitivity in detection of *Salmonella* from raw and spiked food samples in fresh and pre-enriched conditions.

MATERIALS AND METHODS

Bacterial cultures: *Salmonella enterica* obtained from BIOTECH, NASTDA, Thailand was cultured using nutrient agar broth for 4h at 36°C to mid-exponential growth phase and serially diluted (10^{-1}-10^{-10}) in sterile distilled water for enumeration. Bacteria were enumerated using xylose lysine tergitol-4 (XLT-4) agar plates at 37°C overnight and bacterial concentration was estimated by calculating the average number of colonies on plates containing 30 to 300 colonies. Then a bacterial dilution contained 10^7cfu mL^{-1} was prepared using the same mid-exponential phase bacterial culture.

Sample collection and preparation: A total number of 60 pork sausage samples were obtained from 20 different food outlets and two 25 g sub-samples were taken from each sample and grinded. One grinded sub-sample taken from a sample was used to inoculate 225 mL of nutrient broth and mixed by stomacher and divided in to two containing approximately 125 mL in each. Then one of those broths was used to enumerate *Salmonella* on XLT-4 agar plates and to detect by fluorescent *in-situ* hybridization (FISH). The other broth was used to enrich at 37°C for 12 h and performed the same tests. The other sub-sample was spiked with 5 mL of 10^7 *Salmonella enterica* culture and then inoculated into 225 mL of nutrient broth and mixed by stomacher. The inoculated broth was divided in to two as above and performed the same tests as above.

Culturing in XLT4 agar: Prepared samples were serially diluted (10^{-1}-10^{-10}) in sterile distilled water and enumerated in *Salmonella* specific culture medium, XLT4 agar, as describe by Fratamico (2003). Plates were incubated at 37°C over night and plates with red colonies were considered as positive for *Salmonella* and others as negative.

Detection by Fluorescent *in-situ* Hybridization (FISH): 1 mL of each prepared sample was 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°C. Fixed cells were then washed twice with 1 mL of 1×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°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°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, pH 7.2, 0.1% SDS) containing 5 ng μL^{-1} 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°C in humid chamber. After incubation slides were washed by buffer solution (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.1% SDS) at 46°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 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 and photographs were taken.

RESULTS AND DISCUSSION

A comparative analysis between the culture method and the FISH method, for the detection of *Salmonella,* was performed for 60 naturally contaminated and spiked pork sausage samples in fresh and pre-enrich conditions. Formation of read colonies in XLT4 agar (Fig. 1) for culture method and fluorescent microscopic visualization of cells hybridized with Sla3 probe (Fig. 2) for FISH technique were used as indicators to identify positive samples for *Salmonella* contamination.

From fresh samples, Culture method identified 2 samples as contaminated by *Salmonella* while FISH identified 4 samples. Enrichment step increased these numbers up to 7 for culture method and 12 for FISH method (Table 1). As per these results, the detection sensitivity of culture method was lower than the FISH method. As previously described by Flowers *et al.* (1987), Bottari *et al.* (1995) and Fang *et al.* (2003), reasons for higher number of positive results by hybridization methods compared to culture method could be, detection of dead or viable but not culturable cells by hybridization method or detection of other bacteria rather than *Salmonella* by those methods. However, the possibility of the detection of dead cells by FISH technique is very low since rRNA in dead cells are rapidly degrade. Since Sal3 probe has been used in number of studies and proven its ability to detect *Salmonella* from mixed bacterial population, there is no possibility to detect other bacteria rather than *Salmonella* by Sal3 probe. However, by hybridization methods, there is a possibility for the detection of viable but non-culturable cells that exhibit a metabolic activity level

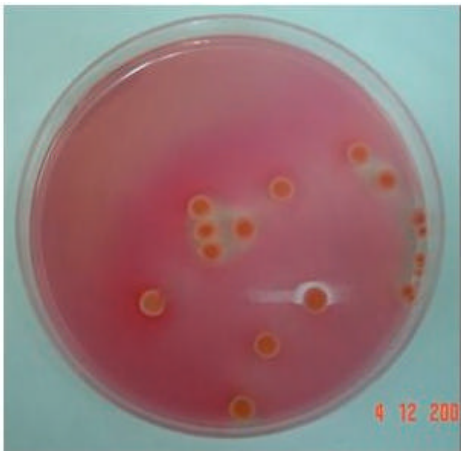

Fig. 1: *Salmonella* colonies on XL T4 agar plate

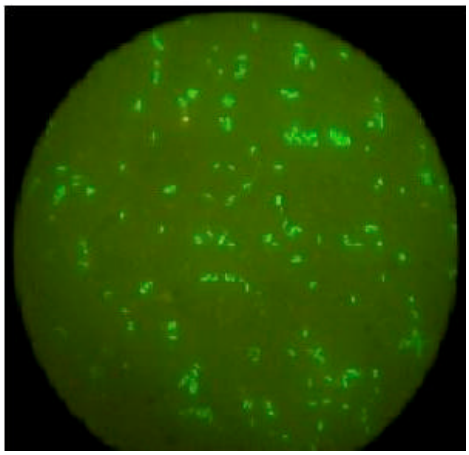

Fig. 2: Fluorescent microscopic view of *Salmonella* cells hybridized with Sal3 probe

Table 1: Detection of *Salmonella* from naturally contaminated and spiked pork sausage by culture and FISH methods

	FISH technique											
	Naturally contaminated samples						Spiked samples					
	Fresh samples			Pre-enriched samples			Fresh samples			Pre-enriched samples		
Culture method	(+)	(-)	Total	(+)	(-)	Total	(+)	(-)	Total	(+)	(-)	Total
(+)	2	-	2	7	-	7	55	-	55	60	-	60
(-)	2	56	58	5	48	53	3	2	5	-	-	-
Total	4	56	60	12	48	60	58	2	60	60	-	60

(+): *Salmonella* detected, (-): *Salmonella* not detected

with a sufficient number of ribosomes detectable by hybridization with Sal3 probe but that are not viable enough to express their ability to multiply during the culture procedure as explained by Vieira-Pinto *et al.* (2008). The other reason for these results could be, physical and chemical properties of pork sausage (salt concentration, preservative concentration, pH etc.) which could stress *Salmonella* cells so that they are unable to form colonies in culture plates but exhibit a metabolic activity level with a sufficient number of ribosomes detectable by FISH method. However, the increase of sensitivity of both method by pre-enrichment indicated the inability of both method to detect some of the naturally contaminate samples with low number of bacterial cells.

The results were further confirmed by the analysis of pork sausage samples spiked with *Salmonella* culture (Table 1). Five samples were identified as negative for *Salmonella* by culture technique, although they were spiked with *Salmonella* culture, from fresh samples. This may be due to chemical and physical properties of the food as explained above. Two negative results obtained by FISH test for spiked fresh samples may be due to deficient microscopic visualization (fluorescent background) or because a small number or insufficient accessibility of the target molecules is present as explained by Amann *et al.* (1995).

In this experiment, higher positive results were observed for both methods for both raw and spiked samples after enrichment indicating the ability of pre-enrichment to increase the detection sensitivity. Reason for these results may be small number or injured or inactive microorganisms

present in food samples as explained by Niederhauser *et al.* (1992) and Fang *et al.* (2003). Due to this reason, false negative results could be obtained by both culture and hybridization methods for raw samples. Hence, pre-enrichment before testing food for microbial contamination is important. However, on the other hand, extensive enrichment could cause the degradation of rRNA target which leads to false negative results. So, further research should be carried out to find the best enrichment period for each test method.

CONCLUSION

Pre-enrichment step found to be effective to increase the detection sensitivity of both methods tested in this study. FISH technique shown high detection sensitivity compared to culture based method and was minimally affected by chemical and physical properties of the food. Hence, in conclusion, FISH method is recommended over culture based method for the detection of *Salmonella* from food samples with an enrichment step.

REFERENCES

Amann, R.I., W. Ludwig and K.H. Schleifer, 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev., 59: 143-169.

Bottari, D.A., C.D. Emmet, C.E. Nichols, K.D. Whippie, D. Rodriguez and G.W. Durbin, 1995. Comparative study of a colorimetric DNA hybridization method and conventional culture procedure for the detection of *Listeria* sp. in foods. J. Food Protect., 58: 1083-1090.

Ercolini, D., J.H. Philip and E.R.D. Christine, 2003. Development of a fluorescence *in situ* hybridization method for cheese using a 16S rRNA probe. J. Microbiolog. Methods, 52: 267-271.

Fadel, H.M. and J. Ismail, 2009. Prevalence and significance of *Staphylococcus aureus* and *Enterobacteriaceae* species in selected dairy products and handlers. Int. J. Dairy Sci., 4: 100-108.

Fang, Q., S. Brockmann, K. Botzenhart and A. Wiedenmann, 2003. Improved detection of *Salmonella* sp. in foods by fluorescent *in situ* hybridization with 23S rRNA probes: A comparison with conventional culture methods. J. Food Protect., 66: 723-731.

Flowers, R.S., M.J. Klatt, M.A. Mozola, M.S. Curiale, D.A. Gabis and J.H. Silliker, 1987. DNA hybridisation assay for detection of Salmonella in foods: Collaborative study. J. Associat. Official Analyt. Chemists, 70: 521-529.

Fratamico, P.M., 2003. Comparison of culture, Polymerase Chain Reaction (PCR), TaqMan *Salmonella* and transia card salmonella assays for detection of *Salmonella* sp. in naturally-contaminated ground chicken, ground turkey and ground beef. Mol. Cell. Probes., 17: 215-221.

Jongerius-Gortemaker, B.G.M., R.L.J. Goverde, F.V. Knapen and A.A. Bergwerff, 2002. Surface plasmon resonance (BIACORE) detection of serum antibodies against *Salmonella enteritidis* and *Salmonella typhimurium*. J. Immunolog. Methods, 266: 33-44.

Malkawi, H.I. and R. Gharaibeh, 2004. Rapid and simultaneous identification of two *Salmonella enterica* serotypes, enteritidis and typhimurium from chicken and meat products by multiplex PCR. Biotechnology, 3: 44-48.

Moreno, Y., M. Hernandez, M.A. Ferrus, J.L. Alonso, S. Botella and R. Montes, 2001. Direct detection of thermotolerant Campylobacter in chicken products by PCR and *in situ* hybridization. Res. Microbiol., 152: 577-582.

Nguendo-Yongsi, H.B., 2011. Microbiological evaluation of drinking water in a sub-saharan urban community (yaounde). Am. J. Biochem. Mol. Biol., 1: 68-81.

Niederhauser, C., U. Candrian, C. Hofelein, M. Jermini, H.P. Buhler and J. Luthy, 1992. Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. Applied Environ. Microbiol., 58: 1564-1568.

Nowak, B., T.V. Muffling, S. Chaunchom and J. Hartung, 2007. Salmonella contamination in pigs at slaughter and on the farm: A field study using an antibody ELISA test and a PCR technique. Int. J. Food Microbiol., 115: 259-267.

Olsen, J.E., S. Aabo, W. Hill, S. Notermans, K. Wernars and P.E. Granum, 1995. Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. Int. J. Food Microbiol., 28: 1-78.

Piknova, L., A. Stefanovicova, H. Drahovska, M. Sasik and T. Kuchta, 2002. Detection of Salmonella in food, equivalent to ISO 6579, by a three-days polymerase chain reaction-based method. Food Control, 13: 191-194.

Regnault, B., S. Martin-Delautre, M. Lejay-Colin, M. Lefevre and P. Grimont, 2002. Oligonucleotide probe for the visualization of *Escherichia coli*/*Escherichia fergusonii* cells by in situ hybridization: Specificity and potential applications. Res. Microbiol., 151: 521-533.

Seidavi, A.R., S.Z. Mirhosseini, M. Shivazad, M. Chamani and A.A. Sadeghi, 2008. Optimizing multiplex polymerase chain reaction method for specific, sensitive and rapid detection of *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. in Chick gastrointestinal tract. AJAVA., 3: 230-235.

Selvan, P., R.N. Babu, S. Sureshkumar and V. Venkataramanujam, 2007. Microbial quality of retail meat products available in Chennai city. Am. J. Food Technol., 2: 55-59.

Shabarinath, S., S.H. Kumar, R. Khushiramani, I. Karunasagar and I. Karunasagar, 2007. Detection and characterization of Salmonella associated with tropical seafood. Int. J. Food Microbiol., 114: 227-233.

Vieira-Pinto, M., M. Oliveira, J. Aranha, C. Martins and F. Bernardo, 2008. Influence of an enrichment step on *Salmonella* sp. detection by fluorescent in situ hybridization on pork samples. Food Control, 19: 286-290.

Wang, H., B.W. Blais, B.W. Brooks and H. Yamazaki, 1996. *Salmonella* detection by the polymyxin-cloth enzyme immunoassay using polyclonal and monoclonal detector antibodies. Int. J. Food Microbiol., 29: 31-40.