JAPSC
Journal of Animal and Poultry Sciences, 2014, 3 (3): 105-109
Available online at http://www.JAPSC.com

Short communication

Multiplex-PCR Assay for detection of *Salmonella* typhimurium and *Salmonella* enteritidis in poultry feedstuffs

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Abstract

Multiplex polymerase chain reaction (M-PCR), the concurrent amplification of two or more polymerase chain reaction (PCR) products in the same reaction tube, can be applied to the rapid detection of pathogenic microorganisms in animal feedstuffs. Poultry feedstuffs has been implicated as important established source of infection to specific *Salmonella* serovars. Poultry feedstuffs can be an origin for transfer bacteria. In this study, 30 feedstuffs samples were collected from feed stocks of broiler chicken farms in Kermanshah province in the west of Iran. In order to isolate *Salmonella*, conventional cultural methods including pre-enrichment, enrichment and selective agar plating tests were performed. To confirm the identification of isolated colonies as *Salmonella*. and determining as typhimurium and Enteritidisserovars, a M-PCR assay, using three pairs of primers were employed, S141 and S139 for InvA gene, specific for the genus of *Salmonella* spp. Fli15 and Tym for FliC gene, specific for Typhimurium serovar and Sef167 and Sef478 for sefA gene, specific for Enteritidisserovar. M-PCR results indicated that 3.3% of samples were contaminated with *Salmonella* spp. and confirmed that all contaminated samples belong to Salmonella typhimurium serovar and no contamination with *Salmonella* enteritidis has been detected. In conclusion, it is recommended that M-PCR method can be used as a viable alternative to traditional cultural methods for detection of poultry feedstuffs contamination by *Salmonella* serovars.

Key words:*Salmonella* typhimurium, *Salmonella* enteritidis, Multiplex PCR, Poultry feedstuffs

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Introduction

Salmonella species have been considered as one of the most important food borne pathogens all around the world (Gillespie et al.,2003; Malorny et al.,2003a). Poultry are the principal reservoir of this pathogen (Winfield and Groisman, 2003). Food from animal sources such as poultry meat have been proved to carry these pathogens (Gillespie et al., 2003). Poultry products have been recognized as a major source of human illness caused by these pathogens (Amavisit et al., 2001). *Salmonella* enteritidis and typhimuriumserovars are the most frequently isolated serovars from foodborne outbreaks throughout the world (Herikstad et al., 2002). Therefore, it is necessary to discriminate *Salmonella* serovars from poultry origins in order to ensure that if this origins are contaminated (Lim et al., 2003). Conventionally, *Salmonella* was isolated by cultural methods which are based on non-selective pre-enrichment medias followed by selective enrichment and on selective and differential agars (Van Kessel et al., 2003). Generally, these techniques take longer time, since they give only presumptive results after 3-4 days and definitive results after 5-6 days (Malorny et al., 2003b). For this reason, rapid detection methods such as DNA or RNA probing, immune-detection methods and nucleic acid hybridization have been developed, but they do not have enough sensitivity and specificity (Zhu et al., 1996). In vitro amplification of DNA by the PCR methods is a powerful tool in microbiological diagnostics (Malorny et al., 2003b). Several genes have been used to detect *Salmonella* in natural environmental samples. Virulence chromosomal genes including; invA(Malorny et al., 2003a; Malorny et al., 2003b), sefA(Szabo and Mackey, 1999), fliC(Itoh et al., 1997; Soumet et al., 1999) are target genes for PCR amplification of *Salmonella* species. The flagellin gene fliC encodes the major component of the flagellum in *Salmonella* typhimurium(Aldridge et al., 2006). The invAgene of *Salmonella* spp. contain sequence unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications (Rahn et al., 1992). This study was aimed to detect contamination of poultry feedstuffs with *Salmonella*. in poultry farms around the Kermanshah city.

Materials and methods

In this study, a total of 30 poultry feedstuff samples, were collected from broiler chicken farms located around the Kermanshah. 50 g feed stocks of each farm randomly sampled and reffered to the laboratory and kept at -20º C until analysis. Then, 10 g of each feedstuff samples rinsed in 50cc pepton buffer and incubated at 37°C for 24h. Following then, 10cc of the pre-enrichment rinsed in 100cc tetrationat broth and incubated at 37°C for 48h. 1cc pre-enrichment rinsed in 9cc selenite cystine broth and incubated at 37°C for 24h. The DNA from the enriched culture was obtained by a DNA extraction kit (CinnaGen Co. Tehran-Iran) and the purified DNA was used as a template for the PCR assay. Three primers pairs (CinnaGen Co. Tehran-Iran) were used. The sequence of primers used in this study is shown in Table1.

Table1: Sequences of primer pairs used in the Multiplex-PCR

Primer	Sequence	Target gene	Amplicon fragment(bp)	Reference
S139-F	GTGAAATTATCGCCACGTTCGGGCAA	invA	284 bp	Malorny et
S141-R	TCATCGCACCGTCAAAGGAACC			
Fli15-F	CGGTGTTGCCCAGGTTGGTAAT	fliC	559bp	Soumet et al.
Tym-R	ACTCTTGCTGGCGGTGCGACTT			
Sef167-F	AGGTTCAGGCAGCGGTTACT	sefA	312 bp	Soumet et al.
Sef478-R	GGGACATTTAGCGTTTCTTG			

Reactions were carried out in a 25µl amplification mixture consisting of 12.5 µl mastermix,0.2µl (containing 75 ng) extracted DNA. Amplification was performed in a Bio-Rad gradient thermocycler. The cycling condition was as follows: an initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minutes, elongation at 72°C for 1 minutes, and final extension period for 10 minutes at72°C. Amplified products were run in1 % agarose gel and a 100bp DNA ladder was used as a size reference. Deionized distilled water was used as a template for negative control and S. typhymurium was used as a positive control.

Results

Multiplex-PCR assay for 30 samples of poultry feedstuffs, using three primer pairs (Table 1), showed that 3.33% of samples were contaminated with *Salmonella* typhimurium. No contamination has been detected for *Salmonella* enteritidis serovar (Figure 1).

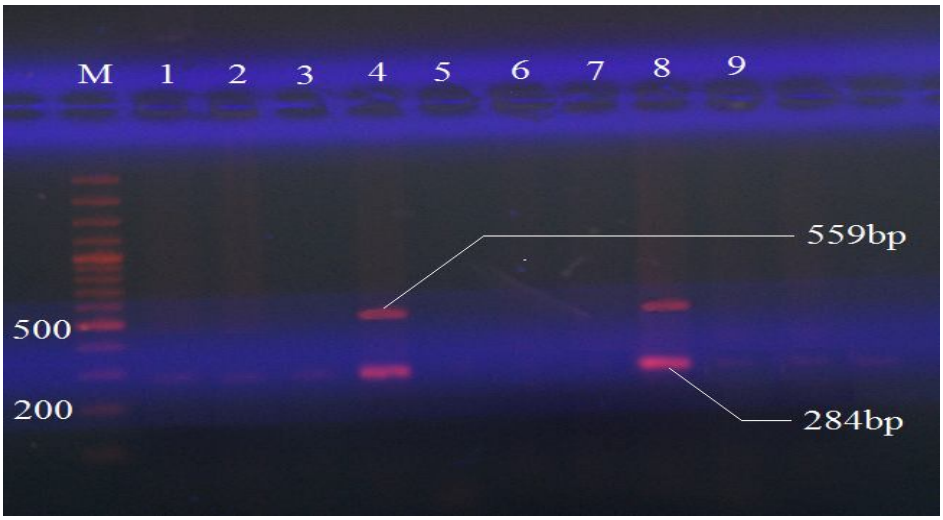

Figure1. Multiplex-PCR was carried out by three sets of primers

The 284 bp amplified product from invA gene specific for *Salmonella* enteritidis, and559 bp from fliC gene specific for *S.* typhimurium. Lane (M): 100bp molecular weight marker, Lane (8) S. typhimurium as positive control, Lane (4) positive sample for S. typhimurium, Lane (4) positive samples for *Salmonella* spp.

Discussion

Culture techniques are universally recognized as the standard methods for the detection of bacterial pathogens, such as *Salmonella* in feedstuffs (Whyte et al., 2002). These techniques generally take longer time (Malorny et al., 2003b) and are less sensitive compared to PCR based methods (Oliveira et al., 2002). In an international research project for the validation and standardization of PCR for the detection of five major foodborne pathogens including *Salmonella* showed that the most selective primer set was found to be invA gene which used in the current study. These specific primer assay, which was validated in that project, showed high selectivity on 242 *Salmonella* strains (sensitivity 99.6%). The amplification of the invA gene has been proposed as an international standard for genus of *Salmonella* detection (Malorny et al., 2003a). The fliC and fliB genes in *Salmonella* spp. encode the phase-1 and phase-2 flagellins, respectively. These genes are found at two different locations (Soumet et al.,1999; Joys 1985), The fimberie encoding gene named sefA. The sefA gene used for *Salmonella* enterieidis (Doran et al., 1996). In this study, specific detection of *Salmonella* typhimurium in M-PCR assay was performed using Fli15 and Tym primers targeting the fliC gene. Oliveira et al. (2002) reported that the Multiplex-PCR assay using invA gene for detection of *Salmonella* and fliC gene for identification of S. typhimurium from poultry-related samples has 100% specificity.

In consistent with present results, other investigations also indicated that PCR methods is a rapid and specific test for detection of Salmonellas in animal feed samples (Rahn et al., 1992; Hoorfar et al., 2000; Lofstrom et al., 2004; Malorny et al., 2008). Maciorowski et al. (2000) showed that PCR based method could be applied to detection of Salmonellas from poultry rations. Jarquin et al. (2009) reported that development of bead-based DNA microarray coupled with flow cytometry and PCR amplification would be ideal for simultaneous detection and differentiation of *Salmonella* serovars commonly associated with poultry breeder feed contamination. It is usefulness to use multiplex-PCR method that could be considered as an appropriate alternative to conventional culture method for detection of *Salmonella* serovars (Jamshidi et al., 2009). This

Selective and/or non-selective enrichment medias combined with PCR have been applied to the detection of many bacterial pathogens (Schrank et al., 2001) to improve sensitivity and dilution of PCR inhibitory substances (Fluit et al., 1993). In this study, tetrationat broth and selenite cystine broth were used for pre-enrichment and enrichment stages and *Salmonella* serovars were diagnosed using M-PCR method. The results of this study showed that M-PCR is a rapid and synchronous method for detection of *Salmonella* SP. from poultry feedstuffs.

References

Aldridge, P., Gnerer, J., Karlinsey, J.E., Hughes, K.T. 2006. Transcriptional and translational control of the *Salmonella* fliC gene. *Journal of bacteriology,* 188: 4487-4496.

Amavisit, P., Browning, G.F., Lightfoot, D., Church, S., Anderson, G.A., Whithear, K.G., Markham, P.F. 2001. Rapid PCR detection of *Salmonella* in horse faecal samples. *Veterinary microbiology,* 79: 63-74.

Doran, J.L., Collinson, S.K., Clouthier, S.C., Cebula, T.A., Koch, W.H., Burian, J., Banser, P.A., Todd, E.C., Kay, W.W. 1996. Diagnostic potential of sefA DNA probes to *Salmonella* enteritidis and certain other O-serogroup D1 *Salmonella* serovars. *Molecular and cellular probes,* 10: 233-246.

Fluit, A.C., Widjojoatmodjo, M.N., Box, A.T., Torensma, R., Verhoef, J. 1993. Rapid detection of *salmonella* in poultry with the magnetic immuno-polymerase chain reaction assay. *Applied and environmental microbiology,* 59: 1342-1346.

Gillespie, B.E., Mathew, A.G., Draughon, F.A., Jayarao, B.M., Oliveri, S.P. 2003. Detection of *Salmonella* enterica somatic groups C1 and E1 by PCR-enzyme-linked immunosorbent assay. *Journal of food protection,* 66: 2367-2370.

Herikstad, H., Motarjemi, Y., Tauxe, R.V. 2002. *Salmonella* surveillance: a global survey of public health serotyping. *Epidemiology and infection,* 129: 1-8.

Hoorfar, J., Ahrens, P., Radstrom, P. 2000. Automated 5' nuclease PCR assay for identification of *Salmonella* enterica. *Journal of clinical microbiology,* 38: 3429-3435.

Itoh, Y., Hirose, K., Miyake, M., Khan, A.Q., Hashimoto, Y., Ezaki, T. 1997. Amplification of rfbE and fliC genes by polymerase chain reaction for identification and detection of *Salmonella* serovar Enteritidis, Dublin and Gallinarum-Pullorum. *Microbiology and immunology,* 41: 791-794.

Jamshidi, A., Bassami, M.R., Afshari-Nic, S. 2009. Identification of *Salmonella* spp. and *Salmonella* typhimurium by a multiplex PCR-based assay from poultry carcasses in Mashhad- Iran. *Int J Vet Res,* 3: 43-48.

Jarquin, R., Hanning, I., Ahn, S., Ricke, S.C. 2009. Development of rapid detection and genetic characterization of *salmonella* in poultry breeder feeds. *Sensors (Basel),* 9: 5308-5323.

Lim, Y.H., Hirose, K., Izumiya, H., Arakawa, E., Takahashi, H., Terajima, J., Itoh, K., Tamura, K., Kim, S.I., Watanabe, H. 2003. Multiplex polymerase chain reaction assay for selective detection of *Salmonella* enterica serovar typhimurium. *Japanese journal of infectious diseases,* 56: 151-155.

Lofstrom, C., Knutsson, R., Axelsson, C.E., Radstrom, P. 2004. Rapid and specific detection of *Salmonella* spp. in animal feed samples by PCR after culture enrichment. *Applied and environmental microbiology,* 70: 69-75.

Maciorowski, K.G., Pillai, S.D., Ricke, S.C. 2000. Efficacy of a commercial polymerase chain reaction-based assay for detection of *Salmonella* spp. in animal feeds. *Journal of applied microbiology,* 89: 710-718.

Malorny, B., Hoorfar, J., Bunge, C., Helmuth, R. 2003a. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and environmental microbiology,* 69: 290-296.

Malorny, B., Hoorfar, J., Hugas, M., Heuvelink, A., Fach, P., Ellerbroek, L., Bunge, C., Dorn, C., Helmuth, R. 2003b. Interlaboratory diagnostic accuracy of a Salmonella specific PCR-based method. *International journal of food microbiology,* 89: 241-249.

Malorny, B., Lofstrom, C., Wagner, M., Kramer, N., Hoorfar, J. 2008. Enumeration of *salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. *Applied and environmental microbiology,* 74: 1299-1304.

Oliveira, S.D., Santos, L.R., Schuch, D.M., Silva, A.B., Salle, C.T., Canal, C.W. 2002. Detection and identification of salmonellas from poultry-related samples by PCR. *Veterinary microbiology,* 87: 25-35.

Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galan, J.E., Ginocchio, C., Curtiss, R., 3rd, Gyles, C.L. 1992. Amplification of an invA gene sequence of *Salmonella* typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and cellular probes,* 6: 271-279.

Schrank, I.S., Mores, M.A., Costa, J.L., Frazzon, A.P., Soncini, R., Schrank, A., Vainstein, M.H., Silva, S.C. 2001. Influence of enrichment media and application of a PCR based method to detect *Salmonella* in poultry industry products and clinical samples. *Veterinary microbiology,* 82: 45-53.

Soumet, C., Ermel, G., Rose, V., Rose, N., Drouin, P., Salvat, G., Colin, P. 1999. Identification by a multiplex PCR-based assay of *Salmonella* typhimurium and *Salmonella* enteritidis strains from environmental swabs of poultry houses. *Letters in applied microbiology,* 29: 1-6.

Szabo, E.A., Mackey, B.M. 1999. Detection of *Salmonella* enteritidis by reverse transcription-polymerase chain reaction (PCR). *International journal of food microbiology,* 51: 113-122.

Van Kessel, J.S., Karns, J.S., Perdue, M.L. 2003. Using a portable real-time PCR assay to detect *Salmonella* in raw milk. *Journal of food protection,* 66: 1762-1767.

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.

Winfield, M.D., Groisman, E.A. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and Escherichia coli. *Applied and environmental microbiology,* 69: 3687-3694.

Zhu, Q., Lim, C.K., Chan, Y.N. 1996. Detection of *Salmonella* typhi by polymerase chain reaction. *The Journal of applied bacteriology,* 80: 244-251.