Identification of *Salmonella spp*. and *Salmonella typhimurium* by a multiplex PCR-based assay from poultry carcasses in Mashhad- Iran

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(Received 11 March 2008 , Accepted 22 November 2008)

Abstract: **Poultry meat has been identified as one of the principal foodborne sources of Salmonella. In this preliminary study the prevalence of Salmonella spp. and its typhimurium serovar contamination of broiler carcasses, were determined. Using the rinse test method, numbers of 60 samples, representing 20 broiler flocks, were collected from poultry carcasses after the chilling stage in the processing line at a commercial broiler slaughtering facility in Mashhad, Iran. The presence of Salmonella spp and Salmonella typhimurium in collected samples were assessed by performing the pre-enrichment and enrichment culture, followed by multiplex-PCR assay. The primers were selected from the invA and fliC genes, specific for the detection of Salmonella spp. and Salmonella typhimurium, respectively. In this study 8.3% and 1.6% of poultry carcasses were found to be contaminated with Salmonella spp and Salmonella typhimurium respectively. In order to provide a more accurate profile of the prevalence of Salmonella spp and Salmonella typhimurium in broiler carcasses, it is pertinent to use multiplex -PCR method that could be considered as an appropriate alternative to conventional culture method.**

Key words: poultry, *Salmonella spp*, *Salmonella typhimurium*, multiplex PCR.

Introduction

Salmonella species have been considered as one of the most important foodborne pathogens, all around the world (Gillespie *et al*., 2003; Malorny *et al*., 2003a). Animals are the principal reservoir of this pathogen (Winfield *et al*., 2003). Foods from animal sources such as beef, poultry meat, egg and milk 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). It has been reported that in addition to mishandling of poultry product and raw poultry carcasses, uncooked poultry meat is also one of the most frequent causes of human infection by Salmonella species (Panisello *et al*., 2000).

Salmonella enterica serovar Typhimurium and Salmonella enterica serovar Enteritidis are the most frequently isolated serovar from foodborne outbreaks throughout the world (Herikstad *et al*., 2002). According to the antigenic profile of Salmonella species they show different disease syndromes and host specificity.. Therefore, it is necessary and important to discriminate Salmonella

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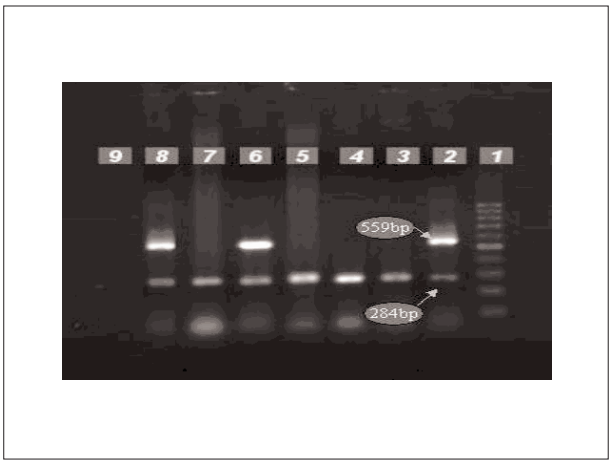

Fig.1: multiplex-PCR assay using two sets of primers. The 284 bp amplified product from invA gene specific for Salmonella spp, and 559 bp from fliC gene specific for S. typhimurium. Lane (1): 100 bp molecular weight marker, Lane (2 and 8) S. typhimurium as positive control, Lane (6) positive sample for S. typhimurium, Lane (3, 4, 5, 7) positive samples for Salmonella spp., Lane (9) negative control.

serovars from each other in order to ensure that each pathogen and epidemiology is correctly recognized (Lim *et al*., 2003).

Salmonella isolation by conventional culture methods, are based on non-selective pre-enrichment followed by selective enrichment and plating on selective and differential agars. Suspected colonies are then confirmed by biochemical and serological methods (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). Rapid detection methods, such as DNA or RNA probing, immuno-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 method is a powerful tool in microbiological diagnostics (Malorny *et al*., 2003b). Several genes have been used to detect Salmonella in natural environmental samples as well as food and feces samples. Virulence chromosomal genes including; invA (Malorny *et al*., 2003a; Malorny *et al*., 2003b), invE (Feder *et al*., 2001), himA (Bej *et al*., 1994), phoP (Way *et al*., 1993), virulence plasmid gene; ipaB (Kong *et al*., 2002), some functional genes; iroB (Soumet *et al*., 1998), lamB (Bej *et al*., 1991), fimbriae genes; fimY (Yeh *et al*., 2002), sefA (Szabo *et al*., 1999), and genes involved in the synthesis of flagellin; Hin (Way *et al*., 1993), fliC (Itoh *et al*., 1997; Soumet *et al*., 1998), H-Li (Marsh *et al*., 1998) are target genes for PCR amplification of Salmonella species. The flagellin gene fliC encodes the major component of the flagellum in Salmonella enterica serovar Typhimurium (Aldridge *et al*., 2006). Due to high variability of its central region the fliC gene has also been used for molecular typing studies on Salmonella (Dauga *et al*., 1998). This structural gene encodes the phase 1 flagellar protein (H_1 antigen), and is expressed alternately with the fljB gene which encodes the phase 2 flagellar protein (H_2 antigen). (MacNab 1996).

The invA gene of Salmonella contains sequences unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications (Rhan *et al*., 1992). Amplification of this gene now has been recognized as an international standard for detection of Salmonella genus (Malorny *et al*., 2003a). This gene encodes a protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host (Darwin and Miller, 1999).

The present preliminary study reports identification of Salmonella genus and its typhimurium serovar from poultry carcasses after the chilling stage in the processing line at a commercial broiler slaughtering facility in Mashhad, Iran, using a multiplex PCR assay.

Materials and Methods

Rinse test sampling: number of 60 samples were randomly collected from chilled broiler carcasses in the processing line, representing 20 broiler flocks (number of 3 samples from each flock). Chicken carcasses were rinsed in 250 ml of 0.1% (w/v) peptone water by shaking for 1 min in sterile plastic containers, followed by filtration through two layers of sterilized cheesecloth. The samples were brought to the laboratory on crush ice and were kept in a refrigerator at 4 °C until testing within 4 hours.

Enrichment: filtrated chicken was rinsed and centrifuged at 16000×g for 10 minutes at 4°C. The

Table 1: Sequence of oligonucleotides used as primers in the multiplex-PCR

Primer	Sequence (5′-3′)	Target gene	Amplicon fragment (bp)	Reference: No
S139-F	GTG AAA TTA TCG CCA CGT TCG GGC AA	Inv A	284	27
S141-R	TCA TCG CAC CGT CAA AGG AAC C			
Fli15-F	CGG TGT TGC CCA GGT TGG TAA T	fliC	559	29, 15
Tym-R	ACT CTT GCT GGC GGT GCG ACT T			

supernatant was discarded and the pellet was suspended in 10ml of lactose broth. After resuspention of the pellet, the samples were incubated at 37°C for 24h as pre-enrichment stage. One milliliter of lactose broth from each tube was then added to 9 ml of selenite cystine broth followed by incubation at 37°C for 24h as enrichment stage.

PCR amplification: The DNA from the enriched culture was obtained by using a DNA extraction kit (Diatom DNA Prep 100) and the purified DNA was used as a template for the PCR assay. For the multiplex PCR, two primer pairs were used. The sequence of primers used in this study is shown in Table1. The S139 and S141 primers are specific for the invA gene of Salmonella spp (Rahn *et al*., 1992) and Fli15 and Tym primers are specific for the fliC gene of Salmonella typhimurium (Soumet *et al*., 1999).

Reactions with these primers were carried out in a 25µl amplification mixture consisting of 2.5µl 10x PCR buffer (500mM KCl, 200mM Tris HCl), 1.25µl dNTPs (10mM), 1.5µl $MgCl_2$ (50mM), 0.5µl of each primer, 0.5µl of Taq DNA polymerase (fermentase) and 2µl of extracted DNA(Soumet *et al*., 1999).

Amplification was performed in a gradient thermocycler (Biorad, icycler). The cycling condition was as follows: an initial incubation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 30 seconds, and final extention priod for 10 minutes at 72°C. Amplified products were electrophoresed in 1.2% agarose gel and a 100bp DNA ladder was used as a size reference. After staining with ethidium bromide the gel was documented with a gel documentation apparatus. Deionized distilled water was used as a template for negative control and *S. typhymurium* (ATCC: 25923) was used as a positive control.

Results

Performing multiplex-PCR assay from 60 samples of chilled broiler carcasses in the processing line, using S139 and S141 primers that amplifies a 284 bp sequence of the invA gene, and Fli15 and Tym primers that amplifies a 559 bp sequence of the fliC gene, showed that number 5 (8.3%) and 1(1.6%) samples were contaminated with Salmonella spp. and Salmonella typhimurium, respectively (fig-1).

Discussion

Studies in other countries have reported on the prevalence of Salmonella in poultry carcasses, with contamination percentages ranging from 3% to 66% (Zhao *et al*., 2001; Uyttendaele *et al*., 1998), although our results were in this range but it should be considered that they have been deduced from a pilot study. The results also depend on the methods applied. The predominant serotypes differ in different countries, but S. typhimurium is not as prevalent as S. enteritidis (Uyttendaele *et al*., 1998), and our results showed a low prevalence (1.6%) of this serotype.

Culture techniques are universally recognized as the standard methods for the detection of bacterial pathogens, such as Salmonella in food stuffs (White *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, the most selective primer set was found to be 139-141, which targets the invA gene. This specific PCR assay, which was validated

in that project, showed high selectivity on 242 Salmonella strains (sensitivity 99.6%) and 122 non-Salmonella strains (specificity 100%). Thus, the amplification of the invA gene has been proposed as an international standard for genus of Salmonella detection (Malorny *et al*., 2003a).

In the present study we used S138 and S141 primers for specific detection of Salmonella at the genus level.

Although Rahn *et al*., (1992) reported that S. Litchfield and S. senftenberg could not be detected by S139 and S141 primers (Rahn *et al*., 1992), but Malorny could detect these serotypes with the same primers by modification in thermal cycling conditions and using hot start PCR (Malorny *et al*., 2003a).

The fliC and fljB genes in Salmonella spp. encode the phase-1 and phase-2 flagellins, respectively. These genes are found at two different locations on the chromosome (Lim *et al*., 2003). The analysis of first-phase alleles encoding different H antigens showed that high degree of variability and fliC sequences were variable enough to allow the design of primers specific for each antigen (Herrera-Leon *et al*., 2004).

According to Joys and Soumet (Soumet *et al*., 1999; Joys 1985), In this study, specific detection of Salmonella typhimurium in m-PCR assay was performed using Fli15 and Tym primers targeting the fliC gene.

Oliveira 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 was 100% specific (Oliveira *et al*., 2002).

In this study we proccesed 60 samples from 20 broiler flocks, and the results of this pilot study do not determine the prevalence of Salmonella spp. and Salmonella typhimurium in broiler flocks, slaughtered in Mashhad abattoirs.

Selective and/or non-selective enrichment 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, lactose broth was used for pre-enrichment stage and selenite cystine broth for enrichment stage.

The results of this study highlight the usefulness of the m-PCR for concurrent and rapid detection of Salmonella spp and Salmonella typhimurium from poultry carcasses.

The results also indicate the need to improve hygiene and sanitary standards in poultry slaughter lines, besides the information to consumers.

Acknowledgement

This work was funded by research council of Ferdowsi University of Mashhad. ?The authors wish to thank Mrs Samira Khajehnasiri for her excellent technical assistance.

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مجله بین المللی تحقیقات دامپزشکی، ۱۳۸۸، دوره ۳، شماره ۱، ۴۸-۴۳

کاربرد روش PCR چند گانه در شناسائی باکتریهای جنس سالمونلا و گونه تایفی موریوم در لاشه طیور کشتار شده در کشتارگاه صنعتی شهرستان مشهد- ایران

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(دریافت مقاله: ۲۰ اسفند ماه ۱۳۸۶، پذیرش نهایی: ۱ آذر ماه ۱۳۸۷)

چکیده

گوشت طیور به عنوان یکی از مهمترین عوامل انتقال عفونت سالمونلائی در انسان شناخته شده است. در این مطالعه جهت تعیین میزان آلودگی لاشه طیور گوشتی به باکتری جنس سالمونلا و سروار تایفی موریوم، تعداد ۶۰ نمونه از ۲۰ گله گوشتی با استفاده از روش تست شستشو از لاشه طیور، پس از مرحله سرد کردن و قبل از مرحله بسته بندی در یکی از کشتارگاههای صنعتی طیور در اطراف شهرستان مشهد برداشت گردید. در آزمایشگاه ابتدا مراحل پیش غنی سازی و غنی سازی و سپس مرحله استخراج DNA انجام گردید. جهت انجام تست m-PCR از پرایمرهائی که قسمتی از ژن *invA* و ژن *fliC* را تکثیر می کنند و به ترتیب مشخص کننده باکتریهای جنس سالمونلا و گونه تایفی موریوم می باشند مورد استفاده قرار گرفت. در این مطالعه مقدماتی میزان آلودگی لاشه طیور به جنس سالمونلا ۸/۳ درصد و میزان آلودگی به سروار تایفی موریوم ۱/۶ درصد تعیین گردید. جهت تعیین میزان آلودگی لاشه طیور به باکتریهای جنس سالمونلا و سروار سالمونلا تافی موریوم استفاده از روش m-PCR توصیه می گردد که می تواند جایگزین مناسبی برای روش کشت باشد.

واژه‌های کلیدی: طیور، جنس سالمونلا، سالمونلا تایفی موریوم، PCR چند گانه.

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