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Detection of *InvA* Gene in Isolated *Salmonella* from Broilers by PCR Method

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Abstract: The presence of *Salmonella* was detected in 192 samples of poultry carcasses from poultry farms in Shiraz province (Iran). A total of 30 *Salmonella* isolates were found in chicken samples (15.6%), by conventional culturing and confirmed by PCR and serology methods. Strains of serogroup D1 were the most prevalent strains, followed by serogroups C1, B and C2. All strains were subjected to *Salmonella*-specific gene (*invA*) and were confirmed as *Salmonella* positive by the predicted product a 284-bp DNA fragment.

Key words: *Salmonella*, broiler, polymerase chain reaction (PCR), *invA* Gene, Iran

Introduction

The genus of *Salmonella* is a gram-negative rod shaped bacteria in the family of *Entrobacteriacea*. Poultry and poultry products have been implicated as a major source of *Salmonella* infections in human. *Salmonella* infections still occur at high frequencies in industrialized nations and developing countries as well. Infections due to *Salmonella* serotypes continue to be a major health problem (Ferretti *et al*., 2001; Lampel *et al*., 2000; Malorny *et al*., 2003; Peplow *et al*., 1999). In the other hand they are very important bacterial pathogens of poultry in the all of the world caused an important loss in poultry rearing and food industries. So that prevention of *Salmonella* infection is important for poultry health and for food processing industries (Carli *et al*., 2001). Infected animals in farms must be quickly identified and be isolated from other ones to prevent and control of spreading of infection. Therefore, efficient control measurements to reduce *Salmonella* contamination of poultry should begin at the farm (Ferretti *et al*., 2001; Weeks *et al*., 2002). Established conventional methods to detect and identify *Salmonella* are time consuming and include selective enrichment and plating followed by biochemical tests (Bennasar *et al*., 2000; Burtscher *et al*., 1999; Chiu and Jonathan, 1996). Several techniques for improving the detection of *Salmonella* serovars, such as the use of selective culture medium and enzyme-linked immunosorbent assay have been developed. However, because of controversy in interpreting of results and low sensitivity and specificity of these methods, it needs to modify and improve them (Chiu and Jonathan, 1996). The Polymerase Chain Reaction (PCR) is a rapid and reliable method for detection and identification of food- born pathogens such as *Salmonella*.

In this research samples from broiler chickens were tested, for isolation of *Salmonella*, by culturing and biochemical method and then they were confirmed by serogrouping and PCR methods.

Materials and Methods

Samples from broiler carcasses and culture methods: One hundred and ninety two samples were collected from chickens in Shiraz province. Samples were aseptically cultured into selenite F broth (Merck) and incubated at 37°C for 24 hours. Subsequently, a loopful of each broth was streaked on surface of *Salmonella-Shigella* agar plates (Merck) and Xylose Lysine Desoxycholate agar (Merck) for further incubation at 37°C for 24 hrs. The biochemical characters of bacteria from non-lactose fermenting determined using triple sugar iron agar (Merck) and urease test (Merck). Colonies that show biochemical reactions like *Salmonella* were transferred to nutrient agar slant (Merck) and incubated at 37°C for 24 hrs. Then they serogrouped by commercial anti *Salmonella* antisera (Difco). Citrobacter freundii, *E. coli* O_2 K_{12}, Proteous mirabilis, Shigella sonnei, Shigella boydi and *Arizona* were cultured at 37° C for 24 h in Mac Conkey agar (Merck) and used as negative controls.

DNA extraction of isolated *Salmonella*: Bacteria were cultured on LB agar for 24 hrs at 37°C. Extraction of DNA was performed by boiling for 10 min and centrifuged at 6000 rpm for 5min. The supernatant were used for amplification by PCR with *Salmonella* specific primers.

Primers set and PCR amplification program: *Salmonella* specific primers, S139 and S141 (Rahn *et al*., 1992) have respectively the following nucleotide sequence based on the *invA* gene of *Salmonella* 5′ - GTG AAA TTA TCG CCA CGT TCG GGC AA - 3′and 5′ - TCA TCG CAC CGT CAA AGG AAC C -3′. Reaction with these primers were carried out in a 25 micro liters

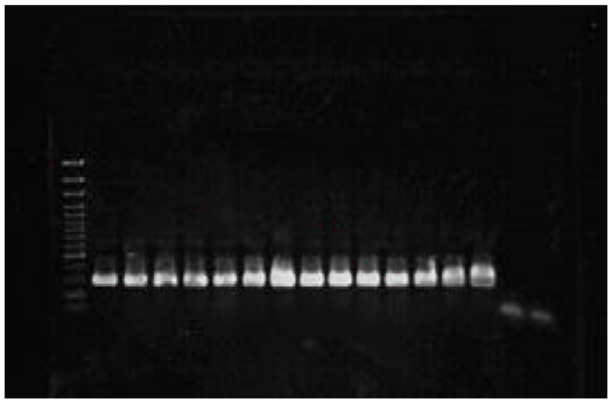

Fig 1: *Salmonella* - specific PCR of *Salmonella* isolates using primer set *invA*. Lane M: 100bp Marker (fermentase). Lane 1, *Salmonella typhimurium* as positive control; lanes 2 and 3: strains of serogroup C1 of *Salmonella*; lane 4: strain of serogroup C2 of *Salmonella*; Lanes 5 to 12: strains of serogroup D1 of *Salmonella*; Lane 13: strains of serogroup B of *Salmonella*; lane 14: *E. coli O2 K12* (A bacterium as negative control); Lane 15: Negative control of PCR processes (Water) and lane 16: Negative control of PCR reagents.

amplification mixture consisting of 2.5 micro liters 10X PCR buffer (500 mM KCl, 200 mM Tris HCl), 1.25 micro liters dNTPs (10mM), 1.6 micro liters $MgCl_2$, 0.5 micro liter of each primer 0.5 micro liter of Taq DNA polymerase (Fermentas) and 1.5 micro liters of extraction for each isolate were used in the reaction. Amplification was conducted in Master-gradient Thermocycler (Eppendorf). The cycle conditions were as follow: An initial incubation at 94°C for 60 sec. Followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 64°C for 30 sec and elongation at 72°C for 30 sec, followed by 7 min final extension period at 72°C.

Electrophoresis of PCR products: The amplified DNA products from *Salmonella* specific-PCR were analyses with electrophoresis on 1.2% agarose w/v gels stained with ethidium bromide and visualized by UV illumination. A current of 120 V was applied to each gel. Eight micro liter of PCR product mixed with 3 micro liters of 6 X-loading dye were loaded on to agarose gel. A 100 bp DNA ladder was used as a marker for PCR products.

Specificity of PCR method: *Citrobacter freundii*, *E. coli O2 K12*, *Proteous mirabilis*, *Shigella sonnei*, *Shigella boydi* and *Arizona* were cultured at 37°C for 24 h in Mac Conkey agar (Merck). The DNA extraction and PCR method used were the same as described for *Salmonella*.

Results

Four different serogroups were found among 30 *Salmonella* isolates. Strains of serogroup D1, which accounted for 70% of total isolates, were the most common strains. Strains in serogroup C1 were the second most common serotypes (20%) and other strains were belonged to serogroup C2 (6.6%) and serogroup B (3.3%) (Fig. 2). Thirty *Salmonella* strains were isolated from broiler specimens, by culturing in selenite F and then transferring to *Salmonella-Shigella* agar, when subjected to *Salmonella* specific-PCR using primers S139 and S141 belong to *inv*A, all isolates including positive control and *Arizona* generated a single 284 bp amplified DNA fragment, on 1.2% agarose gel. Other PCR products of bacteria include *Citobacter freundii*, *Shigella boydii*, *Shigella sonnei*, *E. coli* O_2 K_{12}, and *Proteous mirabilis* did not showed any amplified DNA fragment (Fig. 1).

Discussion

In recent years most investigators try to establish a method, which can reduce the periods of *Salmonella* identification procedures from various samples. Guo *et al.* (1999) use primer set *invA/invE* for confirmation of isolated *Salmonella* from turkeys. Ferretti *et al.* (2001) propose a rapid method with primers salm 4 and salm for *invA* gene, which allows the detection of *Salmonella* serotypes within a maximum of 12 hrs. Schneder *et al.* (2002) design a lightcycler *Salmonella* detection kit that enables the user to detect one single colony forming unit *Salmonella* in 25g sample in less than 24 hrs. *Salmonella* specific PCR with primers for *invA* is rapid, sensitive, and specific for detection of *Salmonella* in many clinical samples (Lampel *et al.*, 2000). The present study supports the ability of these specific primer sets to confirm the isolates as *Salmonella*. All PCR products of isolates include positive control, screened by PCR, resulted in 284 bp amplified fragment. No amplified DNA fragments were obtained from non-*Salmonella* species. The ability of *Salmonella* specific primers to detect *Salmonella* species rapidly and accurately is primarily due to the primer sequences that are selected from the gene *invA* of *S. typhimurium*. *The inv*A gene codes for protein in inner membrane of bacteria, which is necessary for invasion to epithelial cells (Darwin and Miller, 1999).

In conclusion, with attention to high level of *Salmonella* infections in poultry farms, it is necessary to consider control programs to prevention of economically loss resulting from mortality and spreading of infection. Rapid detection of *Salmonella* in poultry farms has an effective role in these programs. PCR based methods with genus-specific primers belong to *invA*, according to its quick, specificity and sensitivity is a reliable technique for this proposes. In other hand this technique provides a tool, in lieu of using culturing and polyvalent antisera, for

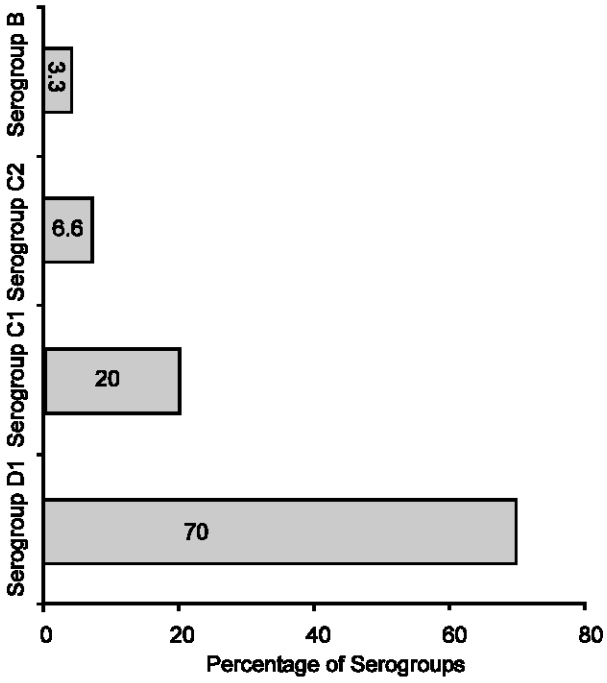

Fig. 2: Relative frequency of *Salmonella* serogroups isolated from chicks.

confirmation of isolates as *Salmonella*. Although techniques which in recent years proposed for rapid and reliable detection and confirmation of *Salmonella* are very progress, but the PCR method which we use in our study, yet is a certain technique in identification and confirmation of *Salmonella*. The use of this method in most diagnostic and research laboratories is possible and through the molecular basis *Salmonella* identification techniques, this method is the simplest and has a less expensive.

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