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Identification of *Salmonella* isolated from poultry by MPCR technique and evaluation of their *hsp gro*EL gene diversity based on the PCR-RFLP analysis

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The aim of this study was to isolate *Salmonella* from poultry and evaluation of their *hsp gro*EL gene diversity by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. In this research 58 strains of 3 different *Salmonella* serogroups (D1, B and C) were isolated from poultry farms of East Azarbayjan province of Iran by bacteriological and biochemical tests. For confirmation of *Salmonella typhimurium* and *Salmonella enteritidis* serovars multiplex polymerase chain reaction (PCR) was applied with four pairs of primers for *S. typhimurium* and three pairs of primers for *Salmonella* Enteritidis. PCR-RFLP analysis was carried out on the 1.6 kb *gro*EL gene for evaluation of their *hsp gro*EL gene diversity. The data generated by multiplex polymerase chain reaction (MPCR) method indicated that strains of *S. enteritidis* (serogroup D1) and *S. typhimurium* (serogroup B) were the most common isolates. Amplification of the *gro*EL gene produced an identical profile for all the 58 *Salmonella* strains. Hae III restriction enzyme was used to restrict the *gro*EL gene for PCR-RFLP analysis. Based on the results of this experiment digested *gro*EL gene of the *S. typhimurium* strains produced four Hae III restricted bands between 150 - 850 bp and serovars belonging to *S. enteritidis* strains produced five Hae III restricted bands between 150 - 630 bp. Strains belonging to serogroup C produced a combination of five and four restricted bands similar to *S. enteritidis* and *S. typhimurium* respectively. This study showed that there were differences in the Hae III restriction sites within the *gro*EL gene of strains belonging to serovars *S. typhimurium* and *S. enteritidis* but clear discrimination between the serovars of different *Salmonella* serogroups was not observed.

Key words: *Salmonella*, poultry, polymerase chain reaction restriction fragment length polymorphism, groEL.

INTRODUCTION

The genus *Salmonella* consist of over 2668 different serotypes. These microorganisms cause disease in both humans and animals. Reptiles, birds, wild animals and even insects are susceptible to *Salmonella* infection. *Salmonella* are potential enteric pathogens and a leading cause of bacterial food borne illness. The transmission of *Salmonella* to a susceptible host usually occurs through consumption of contaminated foods. The most common sources of *Salmonella* include beef, poultry and eggs. Dairy products, vegetables, fruits and shellfish have also been implicated as sources of *Salmonella* (Alena et al., 2009).

Typhoid fever is a life threatening illness cause by *Salmonella* with an annual incidence of 16 million cases and nearly 600,000 deaths caused by *Salmonella enterica* serovar Typhi. Other *Salmonella* serovars, specifically *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Dublin, *Salmonella enterica* serovar *Enteritidis* usually do not cause disseminated, systemic disease in human but clinical manifest as gastroenteritis and diarrhea (Piyush Kumar et al., 2008). Poultry are commonly infected with a wide variety of *S. enterica* serovars. *Salmonella pullorum* and *Salmonella gallinarum* are host specific in poultry but the two serovars that have been of most concern in recent years are *Salmonella enteritidis* and *Salmonella typhimurium*.

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These invasive strains of *Salmonella* may cause disease in young chicks. *Salmonella* is a frequent cause of food borne illness and contaminated poultry products are a major source of infection for humans (Anon, 2001).

Heat shock proteins (HSPs) or stress proteins are synthesized by all cells in response to various types of environmental stress and probably function as molecular chaperones in normal physiological processes. Significantly HSP of microbial pathogens appear to be involved in pathogenesis and host immune response (Seat-Wan tang et al., 1997). Due to this in the present study we have reported diversity of *gro*EL gene of *Salmonella* serovars isolated from poultry based on the Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) analysis.

MATERIALS AND METHODS

Over 634 samples were obtained from spleen, intestine and liver of chickens in poultry farms of East Azarbayjan province of Iran from February, 2009 to August, 2009. Samples were inoculated into selenite F broth (Merck) at 37°C for 24 h and later plated on xylose lysine deoxycholate agar (XLD Merck). Lactose and urease negative bacteria was tested by indole, methyl red, voges-proskauer and citrate (IMViC) biochemical tests. The IMViC test (indole-/MR+/VP- /citrate+) was used for primary diagnosis of *Salmonella.* For determination of serogroups of *Salmonella*, serological screening was performed by using *Salmonella* antisera (Difco). Multiplex Polymerase Chain Reaction (PCR) was applied by the method of Rahn et al. (1992) and Lim et al. (2003) with four pairs of primers for *S. typhimurium* (Table 1) and Pan and Lui (2002) with three pairs of primers for *S. enteritidis* (Table 2). *S. typhimurium* with ATCC 14025 was used as a positive control.

Extraction of DNA was performed by boiling method (Zahraei Salehi et al., 2007). PCR was conducted in a volume of 25 µl containing 40 ng of genomic DNA from the *Salmonella* serovar isolates, 1.5 mM $MgCl_2$, 1 µm of primer, 1U of *Taq* DNA polymerase 200 mM dNTPs in 1 x PCR (CinaGen). Amplification was performed in a thermal cycler (Biosystem) programed as follows: initial denaturation 95°C for 5 min, 30 cycles with consisting of 1 min at 95°C, 1 min at 65°C, 30 s at 72°C and a final extension step of 7 min at 72°C (Zahraei Salehi et al., 2007).

Amplified products were resolved in1.2% agarose gel. Following electrophoresis the gel was stained in ethidium bromide and photographed under ultraviolet (UV) light. A 100 bp DNA ladder was used as a marker for determining the molecular weight of PCR products. PCR-RFLP of the *gro*EL gene was performed by using two oligonucleotide PCR primers which amplify the 1.6 kb *gro*EL gene. The sequence of the primers as was as follows:

H1,5'-GATCCATATGGCAGCTAAAAGACGTAAATTCGG
H2,5'-CTAGGTCGACTTACATCATGCGGCCCATGCCAC

PCR was performed in a volume of 25 µl containing 50 mM Tris-HCl, 50mM KCl, 2.5 mM MgCl2, 400 µM dNTPs, 0.2 µM of each primers and 1U of *Taq* DNA polymerase. Amplification was performed in a thermal cycler (Biosystem) Programed as follows: initial danaturation at 95°C for 3 min, followed by 35 cycles 1 min at 93°C, 2 min at 55°C and 1.5 min at 72°C. The PCR amplified *gro*EL gene was digested with 10 U of Hae III (BSU RI) at 37°C for 2 h. Restriction DNA fragments were separated by electrophoresis at 70 V in horizontal gel containing 1.5% agarose (Satheesh Nair, 2002).

RESULTS

Fifty eight (9.1%) out of the 634 samples that was examined by bacteriological test were culture positive for *Salmonella* serovar .The *Salmonella* strains isolated represented three different serogroups of D1,B and C with following frequency: serogroup D1 62.06% (36 of 58), serogroup B 27.58% (16 of 58) and serogroup C 10.34% (6 of 58). 31 serovars of *S. enteritidis* and 13 serovars of *S. typhimurium* were confirmed by MPCR technique among serogroups of D1 and serogroup B respectively (Table 3). MPCR was applied with specific primers only for *S. typhimurium* and *S. enteritidis* (Figures 1 and 2). For this reason other serovars of *Salmonella* isolates were identified only at serogroup level in this study. PCR-RFLP analysis was carried out with 58 strains belonging to three different *Salmonella* sero-groups. Amplification of the groEL gene produced an identical single profile (1.6 kb) for all *Salmonella* strains (Figure 3).

The digested *gro*EL DNA of *S. enteritidis* and other serovars belonging to serogroup D1 produced five Hae III restricted bands between 150 - 630 bp and the digested *gro*EL gene of *S. typhimurium* and other serovars belonging to serogroup B produced four Hae III restricted bands between 150 - 850 bp (Figure 4). Moreover, 2 of 6 strains belonging to *Salmonella* serogroup C produced five Hae III restricted bands similar to *S. enteritidis* and 4 of 6 strains belonging to this serogroup produced four Hae III restricted bands similar to *S. typhimurium* (Table 3).

DISCUSSION

Strains of *Salmonella* are considered important pathogens and a leading cause of bacterial food borne illness. Since genomic variations play an important role in baterial identification it is necessary to use molecular techniques capable of detecting genetic variation in different species of bacteria. Due to this two PCR based techniques (MPCR and PCR -RFLP) were applied for identifying *Salmonella* isolated from poultry farms of East Azarbayjan province of Iran and detecting of polymerphism within the *gro*EL gene for evaluation of their *hsp gro*EL gene diversity. According to (Table 3) *Salmonella* serogroup of D1 with 62.06% frequency and *Salmonella* serogroup B with 27.58% frequency are the most dominant *salmonella* serogroups in poultry farms of Azarbayjan province of Iran. Previous study which was conducted in Shiraz city of Iran (Fars province) showed that *Salmonella* sergroup D1 with 70% frequency was dominant in Shiraz poultry farms (Zahraei Salehi et al., 2006) . These results agree with the results of the present study and revealed that *Salmonella* serogroup D1 is the most common serogroup in poultry farms of most geographical area of Iran. For specific identification of *S. typhimurium* four pairs of primers which selected based

Table 1. Nucleotide sequences used as primers in multiplex PCR for *S. typhimurium* confirmation.

Primer	Target gene	Sequence	Amplified fragment size
*rfb*J-s *rfb*J-as	*rfb*J	5'-CCAJCACCAGTTCCAACTTGATAC 5'- GGCTTCCGGCTTTATTGGTAAGCA 5'-	663
*fli*C-s *fli*C -as	*fjlC*	ATAGCCATCTTTACCAGTTCCCCC 5'- GCTGCAACTGTTACAGGATATGCC 5'-	183
*flj*B-s *flj*B -as	*flj*B	ACGAATGGTACGGCTTCTGTAACC 5'- TACCGTCGATAGTAACGACTTCGG 5'-	526
ST 139-s ST141-as	*inv*A	GTGAAATTATCGCCACGTTCGGGCAA 5'-TCATCGCACCGTCAAAGGAACC	284

Primers are from Lim et al. (2003).

Table 2. Nucleotide sequences used as primers in multiplex PCR for *S. enteritidis* confirmation.

Primer	Target gene	Sequence	Amplified fragment size
ST11 ST14	Random[a] Sequence	5'-GCCAACCATTGCTAAATTGGCGCA 5'-GGTAGAAATTCCCAGCGGGTACTGG	429
S1 S4	Spv[b]	5'-GCCGTACACGAGCTTATAGA 5'- ACCTACAGGGGCACAATAAC 5'-	250
SEFA2 SEFA4	sefA[c]	GCAGCGGTTACTATTGCAGC 5'- TGTGACAGGGACATTTAGCG	310

Primers are from Pan and Lui (2002). [a]Randomly cloned sequence specific for the genus *Salmonella*, [b]*Salmonella* plasmid virulent gene, *S. enteritidis* fimbrial antigen gene.

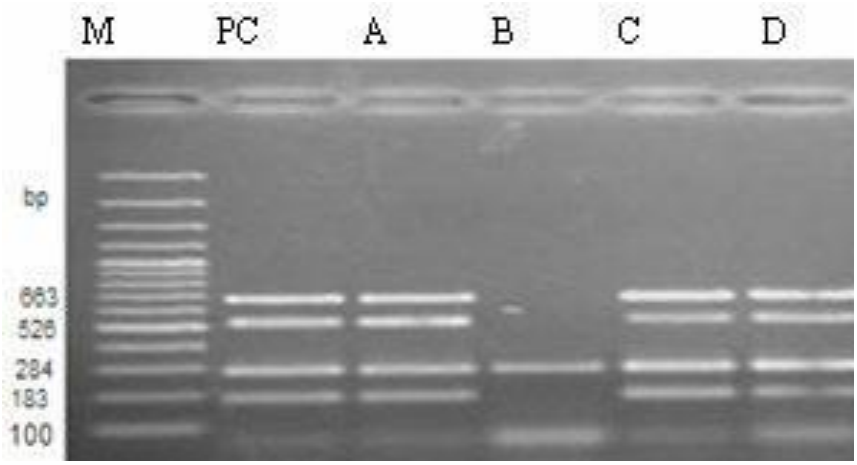

Figure 1. Multiplex PCR with four pairs of primers for *S. typhimurium* isolated from poultry: M marker (100 bp).PC positive control. Lane A, C and D are positive samples for *S. typhimurium*.

on the sequence of the *Salmonella inv*-A, *flj*B and *rfb*j genes were used. The *inv*-A universal primer used for detection of *Salmonella* other than *S. typhimurium*. The *rfb*J, *fli*C and *flj*B genes allowed making a specific identification of O_4, H_1:i and H $_2$:1,2 antigenic properties as only *S. typhimurium* has this antigenic structure combination among 2668 *Salmonella* serovars described (Zahraei Salehi et al., 2006). For specific identification of *S. enteritidis* three pairs of primers which were selected based on the sequence of the *Salmonella* target genes showed in (Table 2). The data generated by MPCR technique indicated that strains of *S. enteritidis* and *S. typhimurium* were the most common isolates in this study Table 3). Poultry are commonly infected with different

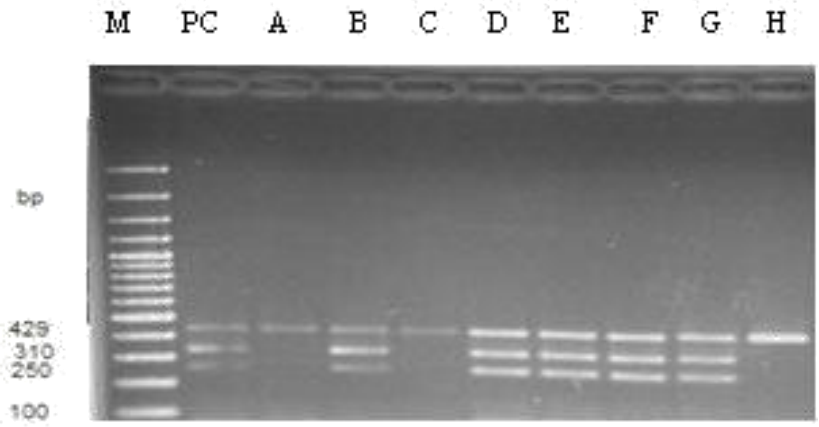

Figure 2. Multiplex PCR with three pairs of primers for *S. enteritidis* isolated from poultry: M marker (100 bp), PC positive control.lane B, D, E, F and G are positive samples for *S. enteritidis.*

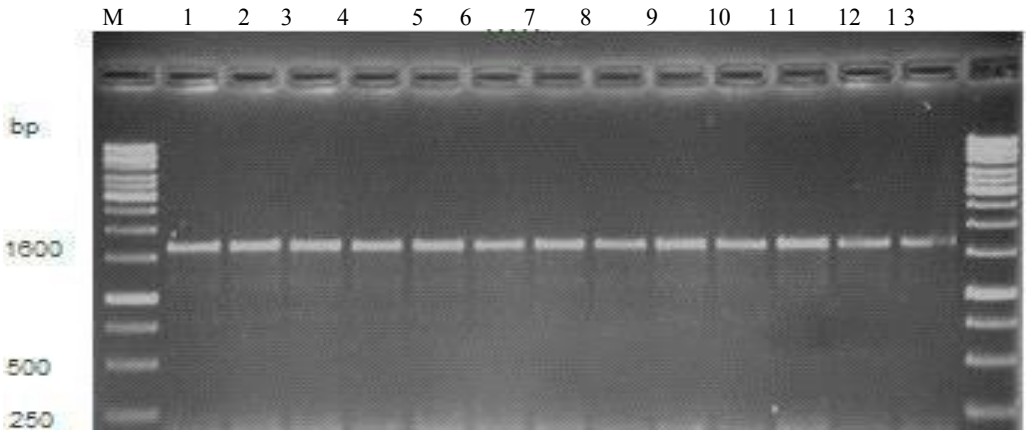

Figure 3. Polymerase chain reaction of *gro*El gene of *Salmonella* Serovars isolated from poultry: M marker (250 bp). Lane 1-13- 1.6 kb *gro*El DNA was observed in all *Salmonella* serovars.

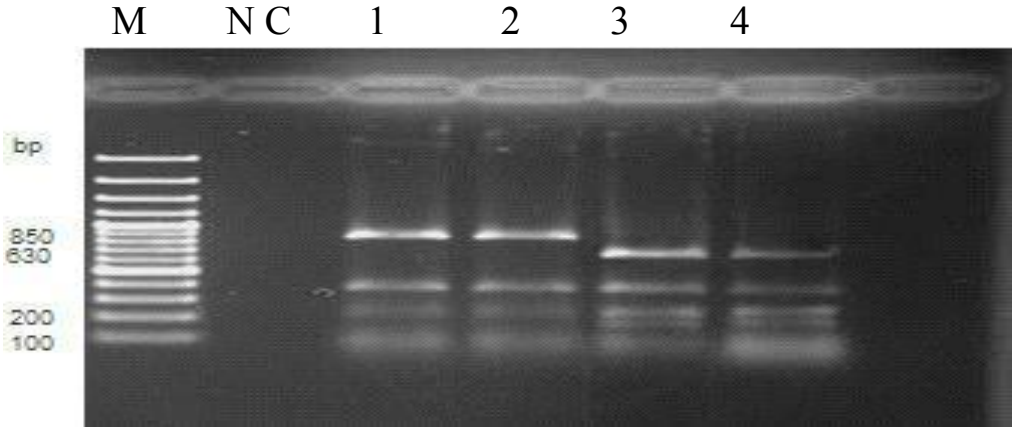

Figure 4. PCR-RFLP profiles of *gro*El gene after Hae III digestion for *Salmonella* isolated from poultry: M marker (100 bp). NC Negative control. Lane 1 *S. typhimurium* (serogroup B). Lane 2 *Salmonella* serogroup C. Lane 3 *S. enteritidis* (serogroup D1). Lane 4 *Salmonella* serogroup C.

Table 3. *Salmonella* strains isolated from poultry and used in the PCR-RFLP study.

Strain no.	*Salmonella* serogroup	*Salmonella* serovar (Comfirmed by MPCR assay)	No.of PCR-RFLP bands (groEL HaeIII)	Strain no.	*Salmonella* serogroup	*Salmonella* serovar (Comfirmed by MPCR assay)	No.of PCR-RFLP bands (groEL HaeIII)
1	D_1	*Enteritidis*	5	30	D_1	*Enteritidis*	5
2	D_1	*Enteritidis*	5	31	D_1	*Enteritidis*	5
3	D_1	*Enteritidis*	5	32	D_1	U*	5
4	D_1	*Enteritidis*	5	33	D_1	U	5
5	D_1	*Enteritidis*	5	34	D_1	U	5
6	D_1	*Enteritidis*	5	35	D_1	U	5
7	D_1	*Enteritidis*	5	36	D_1	U	5
8	D_1	*Enteritidis*	5	37	B	*Typhimurium*	4
9	D_1	*Enteritidis*	5	38	B	*Typhimurium*	4
10	D_1	*Enteritidis*	5	39	B	*Typhimurium*	4
11	D_1	*Enteritidis*	5	40	B	*Typhimurium*	4
12	D_1	*Enteritidis*	5	41	B	*Typhimurium*	4
13	D_1	*Enteritidis*	5	42	B	*Typhimurium*	4
14	D_1	*Enteritidis*	5	43	B	*Typhimurium*	4
15	D_1	*Enteritidis*	5	44	B	*Typhimurium*	4
16	D_1	*Enteritidis*	5	45	B	*Typhimurium*	4
17	D_1	*Enteritidis*	5	46	B	*Typhimurium*	4
18	D_1	*Enteritidis*	5	47	B	*Typhimurium*	4
19	D_1	*Enteritidis*	5	48	B	*Typhimurium*	4
20	D_1	*Enteritidis*	5	49	B	*Typhimurium*	4
21	D_1	*Enteritidis*	5	50	B	U	4
22	D_1	*Enteritidis*	5	51	B	U	4
23	D_1	*Enteritidis*	5	52	B	U	4
24	D_1	*Enteritidis*	5	53	C	U	5
25	D_1	*Enteritidis*	5	54	C	U	5
26	D_1	*Enteritidis*	5	55	C	U	4
27	D_1	*Enteritidis*	5	56	C	U	4
28	D_1	*Enteritidis*	5	57	C	U	4
29	D_1	*Enteritidis*	5	58	C	U	4

(U*=unknown serovar).

Salmonella serovars. Infections are generally sub-clinical and one serovar may be a predominant isolate in a country for several years before it is replaced by another serovar (Wray et al., 1996). *S. enteritidis* first emerged as a frequent infection of poultry in Great Britain in 1987 (Brien, 1988).

The *gro*EL gene encodes a heat shock protein (HSP) which is a member of the stress response protein family. Heat shock proteins are induced

and expressed in various stressful conditions and function to stabilize essential and virulence related protein in bacteria during exposure to environmental stress. HSP proteins affect virulence regulation in pathogens (Piyush et al., 2008; Seat-Wan tang, 1997). This study ledus to investigate within the 1.6 kb *gro*EL gene between *Salmonella* serovars isolated from poultry. Based on the results of PCR-RFLP analysis there are differences in the Hae III restriction sites within the *gro*EL gene of strains belonging to serovars *S. typhimurium* (four Hae III restricted bands) and strains belonging to serovars *S. enteritidis* (five Hae III restricted bands) . These results agree with the work of Satheesh Nair et al. (2002) . Other strains belonging to *Salmonella* serogroup B and *Salmonella* serogroup D1 had results similar to *S. typhimurium* and *Salmonella Entertidis* respectively. But strains of serogroup C produced a combination of five and four Hae III restricted bands similar to *S. enteritidis* and *S. typhimurium* in PCR- RFLP analysis. Therefore this study show that there are differences in the Hae III restriction sites within the *gro*EL gene among serovars *S. typhimurium* (serogroup B) and *S. enteritidis* (serogroup D1) but there are no clear discrimination between different *Salmonella* serogroups and serovars in PCR-RFLP analysis of the *gro*EL gene. Further studies have to be carried out using more *Salmonella* strains from various serovers in different geographical area to obtain more information about genetic variation within the *gro*EL genes of *Salmonella.*

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