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RESEARCH ARTICLE

Detection of *Salmonella* spp. in Gastrointestinal Tract of Broiler Chickens by Polymerase Chain Reaction

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Summary

Salmonella spp. cause a number of diseases in broiler, ultimately leading to death or to a decrease in production or condemning of carcasses. Thus, there are needed to rapid, sensitive, and reliable methods for detection of *Salmonella* to reduce the occurrence of salmonellosis. The objective of the present study was to establish a specific, sensitive and rapid PCR protocol for the detection of *Salmonella* at the genus level in Iran poultry industries. *Salmonella* spp. in the duodenum, jejunum, ileum and cecum of broiler chickens were compared by molecular analysis of 16S rRNA genes. Among 96 samples of gastrointestinal segments, a total of 56.25, 12.5 and 6.25% samples were positive for naturally occurring *Salmonella* spp. by PCR method at 4, 14 and 30d of ages respectively. For cecum, a total of 87.5, 12.5 and 12.5% samples were positive for naturally occurring *Salmonella* spp. by PCR method at 4, 14 and 30d of ages respectively. Analysis of the microbial contents of the different small intestine segments examined indicated that *Salmonella* spp. was not consistently detected in all intestinal segments. The results indicated that at 14 and 30 d of ages, *Salmonella* spp. was not detectable in the most of studied segments. In fact, posterior segments exhibited higher levels of *Salmonella* spp. compared with the anterior segments especially cecum. As demonstrated here, the development of new tools for high-throughput analyses will be of key importance for these studies. This technique is simple and rapid and can be used on different chicken samples for routine detection of a large number of samples on a daily basis.

Keywords: *Broiler, Salmonella, Iran, Gut, 16S rDNA*

Broyler Piliçlerin Gastrointestinal Sistemindeki *Salmonella* spp'nin Polimeraz Zincir Reaksiyonu ile Belirlenmesi

Özet

Salmonella spp. broylerlerde ölüm, üretimde düşüş veya karkasın imhasına yol açan birçok hastalığa neden olmaktadır. Dolayısıyla salmonellosisin görülme sıklığının azaltılması için *Salmonella* bakterilerinin hızlı, duyarlı ve güvenli metotlarla tespit edilmesi gerekmetedir. Bu çalışmanın amacı İran tavukçuluk sektöründe *Salmonella* bakterilerinin cins düzeyinde belirlenmesi için özgün, duyarlı ve hızlı PCR protokolunun tespit edilmesidir. Et piliçlerin duodenum, jejenum, ileum ve sekumlarında *Salmonella* spp oranları 16S rDNA geni analizi ile muakeyese edildi. Gastrointestinal segmentlerden alınan 96 ornek içerisinden PCR kullanılarak, 4, 14 ve 30 günlük broylerlerde toplamda sırasıyla %56.25, 12.5 ve 6.25 oranlarında *Salmonella* spp. belirlendi. Sekumda 4, 14 ve 30 günlük broylerlerde PCR metoduyla *Salmonella* spp. sırasıyla % 87.5, 12.5 ve 12.5 oranlarında belirlendi. İnce barsak içeriklerinin mikrobiyel incelemesi *Salmonella* spp. tüm barsak segmentlerinde bulunmadığını göstermiştir. Sonuçlar *Salmonella* spp. 14 ve 30 günlük broylerlerin birçok barsak segmentinde belirlenemedi. Özellikle sekumun posteriyör segmentindeki *Salmonella* spp. oranları anterior segmenttekine göre daha yüksektir. Bu çalışmada gösterildiği üzere çok sayıdaki analizleri yapmak üzere yeni metotların geliştirilmesi bu tür çalışmalarda önemli olacaktır. Bu teknik günlük olarak çok sayıda kanatlı örneğinin rutin teşhisinde kullanılan basit ve hızlı bir tekniktir.

Anahtar sözcükler: *Broyler, Salmonella, İran, Barsak, 16S rDNA*

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INTRODUCTION

Salmonellosis is one of the most frequent foodborne diseases, being an important public health problem in almost all industrialized countries. *Salmonella* is the leading causative agent among nine foodborne bacterial pathogens resulting in 39% of 15600 laboratory-diagnosed infections in 2003 [1]. A majority of cases of human salmonellosis are due to the consumption of contaminated egg, poultry, pork, beef, and milk products. *Salmonella* spp. are found in the gastrointestinal tracts of a wide range of animals, thus contact with animals and foods of animal origin are frequent causes of salmonellosis [2].

Salmonella prevention can be achieved solely by rapid, sensitive and reliable monitoring and screening programs with a proper sampling plan in poultry flocks and in the industry [3]. The primary motivation for controlling *Salmonella* infections in poultry has been to reduce disease incidence, and this has lead to the implementation of extensive testing programs. Since poultry is one of the most important reservoirs of *Salmonella* that can be transmitted to humans through the food-chain, public health concerns have increasingly made the prevention of the foodborne transmission of disease to humans an urgent priority for poultry producers [4].

It is essential that methods for detection of *Salmonella* and other pathogens in foods have the ability to detect low levels of pathogens that are healthy, as well as those that are stressed and injured due to conditions in the food and/or during food processing. Conventional culture-based methods that rely on enrichment of the sample and plating onto selective agar media may not be as sensitive as immunologic- or genetic-based rapid methods [5]. Conventional culture methods used for the detection of *Salmonella* include non-selective pre-enrichment, followed by enrichment and plating on selective and differential agars. Dubious colonies are confirmed biochemically and serologically, probably taking up to 7 days to complete the entire procedure.

Immunologic methods rely on binding of an antigen on the bacterium to a specific monoclonal or polyclonal antibody, and several immunoassay-based systems and devices are commercially available for detection of a variety of food-borne pathogens including *Salmonella* [6]. The DNA of the bacteria are the target for nucleic acid-based systems such as the polymerase chain reaction (PCR). Methods based on the PCR offer the advantages of high specificity and sensitivity, and a number of PCR-based kits are commercially available for testing of samples for the detecting of some pathogens [7]. Some efforts have been made to reduce the time required and to increase the sensitivity and the accuracy of the methods to detect *Salmonella* in poultry samples. PCR has been the most reliable and precise method to detect *Salmonella* from poultry samples within a relatively short time [2]. Now, a number of rapid methods for detection of *Salmonella* have been developed including immuno-assays, nucleic acid hybridization, and polymerase chain reaction (PCR) techniques [3]. PCR assays for the rapid, sensitive, and specific detection of *Salmonella*. have targeted genes like the 16S rRNA gene [8]. Another advantage is that PCR is not dependent on utilization of a substrate or the expression of antigens, thereby circumventing the phenotypic variations in biochemical patterns and lack of detectable antigens.

The main purpose of this study was to optimize a PCR to detect *Salmonella* from gastrointestinal tract, particularly for laboratories with low sample throughput and/or limited budget. Also we studied existence of *Salmonella* in four intestinal segments including duodenum, jejunum, ileum and cecum at three different ages.

MATERIAL and METHODS

Broiler maintenance and sample collection

Commercial broilers (Ross 308) were raised under conditions identical to those found in commercial broiler operations. At 4, 14 and 30 d of ages, eight birds were randomly selected and sacrificed by cervical dislocation. Then duodenum, jejunum, ileum and cecum were removed aseptically Approximately one g of content were collected into a centrifuge tube containing 9 ml of sterile phosphate-buffered saline (PBS) pH 7.4, and homogenized by vortexing with glass beads (4-mm diameter) for 3 min. Debris was removed by centrifugation at 700 g for 1 min, and the supernatant were centrifuged at 13000 g for 5 min. The pellet was washed twice with PBS and stored at -20°C until DNA extraction.

DNA extraction and preparation

Bacterial genomic DNA was isolated by the method of Seidavi et al.[9] with some modifications. Briefly, samples were centrifuged at 14500 g for 2 min and

the cells thoroughly resuspended in 480 µl of 50 mM EDTA. Then 60 µl of 10 mg/ml Lysosyme enzyme was added. The samples were incubated at 37°C for 45 min and centrifuged for 2 min at 14500 g. Then 600 µl Nuclei Lysis Solution was added and incubated at 80°C for 5 min. Three µl of RNase Solution was added and samples were incubated at 37°C for 30 min. Thenceforth 200 µl of Protein Precipitation Solution was added to RNase-treated cell lysate. Samples were incubated on ice for 5 min and centrifuged at 14500 g for 3 min and the supernatant were transferred to 1.5 ml microcentrifuge tubes containing 600 µl of iso-propanol. Tubes were centrifuged at 14500 g for 2 min. The supernatant were poured off and 600 µl of 70% ethanol was added. Tubes were centrifuged at 14500 g for 2 min and carefully was aspirated the ethanol. Then 100 µl of DNA Rehydration Solution were added to the tubes and the DNA was rehydrated by incubating at 65°C for 60 min. Prepared DNA stored at 4°C till PCR amplification.

PCR amplification

The PCR amplification mixtures (25 µl) were consisted of 1 µl of 25 ng DNA sample (DNA concentration was quantified by using a Beckman spectrophotometer), 0.08 mM of each dNTP, 1.2 mM $MgCl_2$, 1× PCR buffer, 0.24 ρM (0.6 µl) of each primer (forward and reverse), 1 U of Taq DNA polymerase and 18.6 µl ddH_2O. Amplification was performed on a thermocycler (ABI 9700) which initial denaturation was at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 60 s, with a final extension at 72°C for 3 min. Amplified products were electrophoresed in 2% agarose gels containing ethidium bromide (1 µg/ml). A pUC Mix Marker 8 was used as molecular size marker. Two types of samples were used in the PCR amplification including 96 individual samples (comprising of 4 gastrointestinal segments × 3 different ages × 8 replications or chicks; *Table 1*) and 12 mixed samples (comprising of 4 gastrointestinal segments × 3 different ages; *Fig 1*). In 12 mixed samples, all eight similar replications or chicks' samples were mixed together. Unless otherwise indicated, chemical reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

The specific detection of the *Salmonella* spp. was based on PCR amplification of the 16S rRNA gene using oligonucleotide primers Sal201-f CGGGCCTCTTGCCATCAGGTG and Sal597-r CACATCCGACTTGACAGACCG. Also PCR product size was 396 bp. The GenBank program BLAST was used to ensure that the applied primers were complementary with the target species but not with other species. Primers were compared with sequences in the GenBank, and none were found to have the exact same sequence as the non-targeted sequence.

In both individual and mixed sample approaches, negative and positive controls were used for *Salmonella* spp. confirmation and detection by PCR technique. ddH_2O was used as negative control to confirm the absence of contamination of material and facilities and removal of experimental errors and to prove the exclusion of non-target DNA. The *Salmonella typhimurium* strain as positive control used in this study was prepared from the bacterial isolate archive of the Razi Vaccine and Serum Research Institute (RVSRI) in Iran. Double-stranded DNA extracted from each isolate was examined to confirm the presence of PCR-compatible DNA.

RESULTS

In total 96 gastrointestinal tract samples including 24 duodenum (4, 14 and 30 d of ages), 24 jejunum (4, 14 and 30 d of ages), 24 ileum (4, 14 and 30 d of ages) and 24 cecum (4, 14 and 30 d of ages), which were examined by PCR method for *Salmonella* spp. detection (see *Table 1* and *Fig 1*).

For total intestine segments, 56.25, 12.5 and 6.25% samples were positive for naturally occurring *Salmonella* spp. by PCR method at 4, 14 and 30 d respectively *(Table 1)*. Also *Salmonella* spp. was not detected in the oldest chickens. Detailed data regarding number and percentages of naturally occurring *Salmonella* spp. in duodenum, jejunum, ileum and cecum are shown in *Table 1*.

In positive control sample, an unambiguous band corresponding to a bacterial chromosome on agarose gels was established. Subsequent PCRs performed with negative samples did not show any amplification. However, 396-bp fragments were amplified when DNA from a *Salmonella* spp.-positive control was added to the negative samples *(Fig 1)*. The robustness of the method was based on positive and negative control samples processed throughout the entire protocol in each series of samples analyzed.

Analysis of the microbial contents of the different small intestine segments examined indicated that *Salmonella* spp. was not consistently detected as same in all intestinal segments. The results indicated

Table 1. *Summary of the results of PCR for the detection of Salmonella spp. in gastrointestinal tract of broilers*
Tablo 1*. Broyler gastrointestinal sisteminde Salmonella türlerinin PCR ile belirlenmesi*

Number of positive samples /Number of assayed samples (Ratio) and its Percentage (%)

Segment of broiler gut ►		**Total**		**Duodenum**		**Jejunum**		**Ileum**		**Cecum**	
Number and percentage ►		**Ratio**	**%**	**Ratio**	**%**	**Ratio**	**%**	**Ratio**	**%**	**Ratio**	**%**
Age (day) ►	**4**	18/32	56.25	3/8	37.5	3/8	37.5	5/8	62.5	7/8	87.5
	14	4/32	12.5	1/8	12.5	1/8	12.5	1/8	12.5	1/8	12.5
	30	2/32	6.25	0/8	0.0	0/8	0.0	1/8	12.5	1/8	12.5

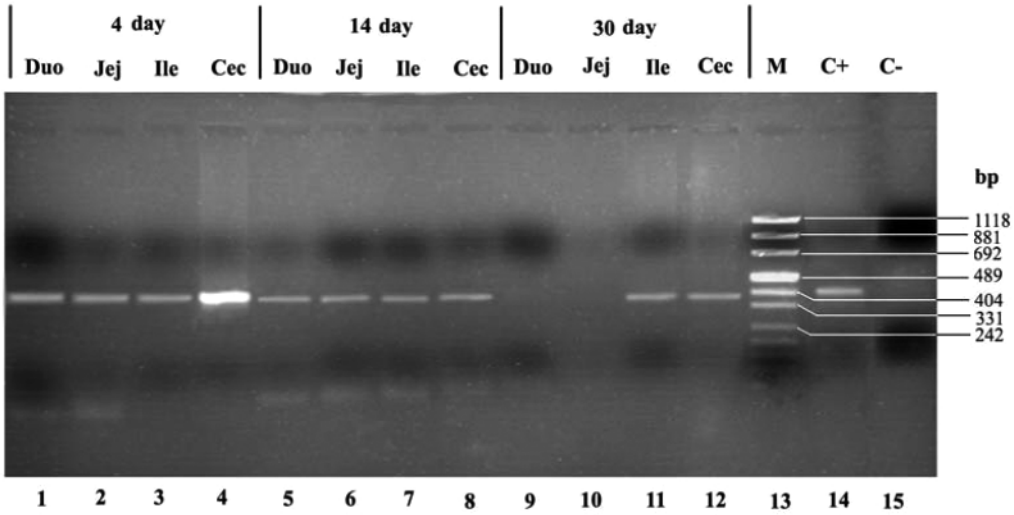

Fig. 1. Electrophoresis of PCR products (combined) on 2% agarose gel stained with ethidium bromide. Gastrointestinal tract samples = **Lanes 1-12:** Amplification products from DNA of *Salmonella* spp. with primers Sal201-f and Sal597-r, M = **Lane 13:** Molecular weight marker, C+ = **Lane 14:** Positive control amplified DNA, C- = **Lane 15:** Negative control. **Lanes 1-4:** 4 day old; **Lanes 5-8:** 14 day old; **Lanes 9-12:** 30 day old. ***Duo:*** Duodenum; ***Jej:*** Jejunum; ***Ile:*** Ileum; ***Cec:*** Cecum

Şekil 1. PCR ürünlerinin ethidium bromide ile boyanmış %2'lik agaroz jelde elektroforezisi. Gastrointestinal sistem örnekleri = **Sıra 1-12:** Primer Sal201-f ve Sal597-r ile *Salmonella* spp. DNA amplifikasyonu, M= **Sıra 13:** moleküler markır, C+ = **Sıra 14:** pozitif control, C- = **Sıra 15:** Negatif control **Sıra 1-4:** 4 günlük; **Sıra 5-8:** 14 günlük; **Sıra 9-12:** 30 günlük, ***Duo:*** Duodenum; ***Jej:*** Jejunum; ***Ile:*** Ileum; ***Cec:*** Cecum

that at 30 d of ages, *Salmonella* spp. was not detectable in the duodenum and jejunum. In fact, posterior segments exhibited higher levels of *Salmonella* spp. compared with the anterior segments especially cecum.

DISCUSSION

The findings presented herein showed that there were *Salmonella* spp. in all four gastrointestinal segments of chickens including duodenum, jejunum, ileum and cecum of broilers. *Salmonella* spp. reported that there are in poultry gut [10] and support the results observed in the present study. Meanwhile, obtained values and percentages for naturally occurring *Salmonella* spp. in duodenum, jejunum, ileum and cecum are in agreement with the other reports regarding frequency of *Salmonella* spp. among the intestinal commensals present in poultry at different ages [10].

Conventional methods like culture-based approaches for *Salmonella* spp. detection present serious difficulties in laboratories. There is no general agreement concerning determination of the gold standard for the detection of this foodborne pathogen [11]. To date, culture techniques are sometimes recognized as the appropriate method for the detection of bacterial groups such as *Salmonella* spp. in broilers gastro-intestinal tract. In theory, these methods are capable of detecting as few as one viable cell in a sample following pre- and selective enrichment stages. However, increased sensitivity of PCR methods,

compared to culture techniques, has been reported for the detection of *Salmonella* spp. and was attributed to the fact that PCR can detect target sequences, irrespective of the growth potential, of target cells.

The genus-specific PCR amplified 16S rDNA of the correct predicted size from the appropriate *Salmonella* spp. control strain tested, but not from the other bacterial strains. An adequate strategy to assess the validity of the PCR results is the use of positive and negative controls which are co-amplified with the target sequence by the same set of primers. Therefore one positive controls and one negative control were PCR tested to prove the specificity of the primers and the reaction condition. The novel set of primers found to work properly and gave optimum yield of product in samples that were used as positive control *(Fig 1, lane 14)*. PCR products were obtained specifically only when DNA from the corresponding genus was present in the reaction. This was confirmed from the amplified fragment of 396 bp specific for *Salmonella* spp. In this reaction, ddH_2O have been used as negative control and have been tested with positive samples *(Salmonella typhimurium)* to explain the contamination factor *(Fig 1, lane 15)*. Meanwhile PCR primers of the bacteria genus were search using NCBI BLAST search option, which showed high homology with the gene bank sequence from which the primers were applied.

Also a multiplex PCR test could be developed, which would allow the analysis of a complex microflora in a single reaction. The PCR protocol used in this work detected *Salmonella* spp. in intestinal samples as efficiently. Furthermore this reduction in detection time represents a true advantage compared to the traditional culture and other rapid methods in food and gut microbiology.

Furthermore the full effect of dietary manipulations like antibiotics on gut microflora cannot be fully evaluated using selective plate culture-based methods, since these do not reflect the complex microbial community within the gastrointestinal tract. Therefore molecular methods are essential to evaluate this flora.

The frequency of *Salmonella* spp. in poultry gut samples was high, but similar to that reported by authors from other countries such as Sackey et al.[12] and Schrank et al.[11]. These high incidences are not surprising if we consider the spread of microorganisms in environment, diet, water, litter, slaughtering etc.

The cost of multiple-drug resistance in terms of money, mortality and disability is enormous and the emergence of drug resistant bacteria via the food chain requires urgent attention. There is therefore the need for international coordination in the development of appropriate monitoring programs like PCR [12].

This reduction in detection time represents a true advantage compared to the traditional culture and other rapid methods in food and gut microbiology. In fact, as a result of the fastidiousness and diversity of *Salmonella* spp., it is clear that no one medium will provide an accurate measure of their occurrence [13].

In this study, the *Salmonella* spp. existence in the contents of chicken duodenum, jejunum, ileum and cecum were determined. Our data may have significant implications for the monitoring of *Salmonella* spp. in chickens samples.

In the present study, duodenum, jejunum, ileum and cecum samples were collected from 24 chickens. Since the samples were from the same chickens, the bacterial populations represented the gut microflora in the duodenum, jejunum, ileum and cecum of these birds as a whole source.

The minimum dose of some bacteria in broiler gastrointestinal tract is believed to be extremely small, while that of other bacteria quantity are extremely huge [14]. This emphasizes the need for high sensitivity of detection methods for bacteria. Some times enrichment of samples with defined medium can increase the number of target organisms and reduce the risk of false-positive results due to amplification from non-target cells which have similar needs for nutrients and growth conditions. Thus, it is possible to enrich these bacteria simultaneously and then detect them at one time in the same PCR system. Therefore, it must be noticed that caution needs to be taken when reporting negative results of the duplex PCR assay for samples with notably high microbial background levels [15]. Using a selective medium instead of non-selective medium for enrichment might reduce competition from indigenous flora and improve detection sensitivity of the PCR assay. In fact, some samples required for selective enrichment of cells may influence the effectiveness of the PCR and cause inhibitory effects. Thus, the implementation of PCR-based assays as routine microbiology diagnostic

tools, especially in testing laboratories with quality assurance programs, requires proper controls to verify the accuracy of the results obtained.

The differences of *Salmonella* spp. existence in these four segments can resulted from their different functions. The function of the ileum (the lower end of the small intestine) is mainly nutrient absorption, while the cecum is the site where extensive bacterial fermentation occurs, resulting in further nutrient absorption and detoxification of substances that are harmful to the host [16]. Since these segments have different functions, thus provide different environments, it is expected that different bacteria would colonize and distinct microflora would develop [17]. In this study, we found posterior segments of gastrointestinal tract exhibited higher levels of *Salmonella* spp. compared with the anterior segments especially cecum.

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