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Optimizing Multiplex Polymerase Chain Reaction Method for Specific, Sensitive and Rapid Detection of *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. in Chick Gastrointestinal Tract

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Abstract: In the present study, a multiplex Polymerase Chain Reaction (mPCR)-based assay was developed for the simultaneous identification of *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp., that isolated from broiler gastrointestinal in Iran. In order to detect these bacteria species, we were used a set of specific primers that produce different fragment size. Our results showed that all tested gastrointestinal segments were contaminated by these species and therefore the mPCR method is a appropriate method to identify these species directly from poultry gastrointestinal samples. There was no difference in the sensitivity of this method between samples of different gastrointestinal segments. This method has the potential to save considerable time and effort within the laboratory. Our developed mPCR is fast, sensitive, specific and can be used as a reliable choice for routine detection method of these bacteria species in chick gastrointestinal contents.

Key words: Multiplex PCR, *Salmonella*, *Escherichia coli*, *Bifidobacterium*, chick

INTRODUCTION

The three most commonly reported bacterial species in poultry industry are *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. It is known that *Salmonella* sp. 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 (D'Aoust *et al*., 2001; Fratamico, 2003). Because of relatively high prevalence of *Salmonella* sp. in broiler meat (D'Aoust, 2000; Fratamico, 2003), as well as the high incidence of disease caused by these organisms, rapid, sensitive and reliable methods for detection of *Salmonella* sp. are needed to reduce the occurrence of salmonellosis.

Escherichia coli infections are responsible for significant economic losses in the poultry industry world-wide. The pathogenesis and the role of virulence factors are not yet fully elucidated, although considerable progress has been made in recent years to establish the mechanisms of pathogenesis. In poultry, *Escherichia coli* infections include egg peritonitis, omphalitis, coligranuloma, swollen head syndrome, cellulitis and colisepticaemia, of which the latter is the most severe (La Ragione and Woodward, 2002).

Bifidobacterium sp. are Gram-positive anaerobic bacteria that are major components of the broiler gastrointestinal tract (Rada and Petr, 2000). The assumed health and nutritional benefits ascribed to

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these bacteria are many and varied, including anticancerogenic and anticholes terolemic effects and immunocompetence. From this reason, there has been an increasing interest in the enumeration of *bifidobacteria* sp. in intestinal samples.

Regular monitoring of poultry industry pathogens is required to protect broiler and consumer health. Traditionally, detection and enumeration of bacterial pathogens have been largely based on the use of selective culture and standard biochemical methods. Such methods suffer from a number of drawbacks. First, pathogenic bacteria which normally occur in low numbers tend to incur large errors in sampling and enumeration (Kong *et al*., 2002). Second, culture-based methods are time-consuming, tedious, invariably mono-specific (i.e., detecting only one type of pathogen) and low throughput. Third, many pathogenic organisms in the environment, although viable, are either difficult to culture or non-culturable (Kong *et al*., 2002).

In recent years, PCR-based methods increasingly used to identify several bacterial species from samples (Jofre *et al*., 2005). Although PCR methods have been developed for the characterization of these bacteria groups separately, none are capable of identifying and differentiating all these groups directly from broiler gastrointestinal samples in a single reaction. It is desirable to develop a single mPCR system for detection and differentiation of these microorganisms. Multiplex PCR allows the simultaneous amplification of more than one target sequence in a single PCR reaction, saving considerable time and effort and decreasing the number of reactions to be performed in order to assess the possible presence of microorganisms in sample (Jofre *et al*., 2005).

The current study is aimed at investigating the applicability of the mPCR in simultaneously detecting *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. present in duodenum, jejunum, ileum and cecum of broilers.

MATERIALS AND METHODS

Bacterial Samples

This study was conducted in Islamic Azad University, Science and Research Branch and Genomics Laboratory of Agriculture Biotechnology Research Institute of North Region of Iran (ABRINRI) during January till September 2007. The three bacteria that used in this study are included *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. genera. Broiler chicks were raised under conditions identical to those found in commercial broiler operations. The broilers were fed a diet of commercial feed (NRC, 1994). At the age 30 day, eight birds were randomly selected and sacrificed by cervical dislocation. Then duodenum, jejunum, ileum and cecum were removed aseptically, clamped with forceps and placed in sterile plastic bags on ice. In the laboratory each section were inverted onto sterile glass rods. Approximately one g of content were collected into a 25 mL 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

Briefly, samples centrifuged at 14500 g for 2 min and resuspend the cells thoroughly in 480 µL of 50 mM EDTA. Thenceforth 60 µL of 10 mg mL^{-1} 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. Then they were added 3 µL of RNase Solution and samples 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 were transferred the supernatant to clean 1.5 mL microcentrifuge tubes containing 600 µL of isopropanol. Tubes were centrifuged at 14500 g for 2 min and carefully the

Table 1: Primer sequences and size of PCR-amplified gene targets of three types of studied bacteria

Bacteria	Primer	Primer Sequence (5′→3′)	Expected product size (bp)	Reference
Escherichia coli	ECO-f	GACCTCGGTTTAGTTCACAGA	585	Candrian *et al.* (1991)
	ECO-r	CACACGCTGACGCTGACCA		
Bifidobacterium sp.	Bif164-f	GGGTGGTAATGCCGGATG	510	Langendijk *et al.* (1995)
	Bif662-r	CCACCGTTACACCGGGAA		
Salmonella sp.	Sal201-f	CGGGCCTCTTGCCATCAGGTG	396	Amit-Romach *et al.* (2004)
	Sal597-r	CACATCCGACTTGACAGACCG		

supernatant were pour off and 600 µL of 70% ethanol was added and 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 until PCR amplification.

Oligonucleotide Primers

All primer sequences that used in this study are shown in Table 1.

Optimized mPCR Reaction and Parameters and Analysis of PCR Products

The PCR amplification mixture (25 µL) consisted of 1 µL of 25 ng DNA sample, 0.08 mM of each dNTP, 1.2 mM $MgCl_2$, 1xPCR buffer, 0.28 ρM of each primer (ECO-f, ECO-r, Bif164-f, Bif662-r, Sal201-f, Sal597-r and LAA-r), 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 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 70 sec, with a final extension at 72°C for 5 min. *Escherichia coli*, *Bifidobacterium angulatum* and *Salmonella typhimurium* prepared using defined culture medium were used as positive controls. The positive control strains of all bacteria used in this study were prepared from the bacterial isolate archives of the Agriculture Biotechnology Research Institute of Iran and Scientific and Industrial Research Organization of Iran. Products of amplification were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

A mPCR assay was developed for the simultaneous identification of *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. from chick gastrointestinal samples (Fig. 1). The nucleotide sequences used in the design of mPCR primers were retrieved from the Candrian *et al*. (1991), Langendijk *et al*. (1995) and Amit-Romach *et al*. (2004). The applicability of the ECO-f, ECO-r, Bif164-f, Bif662-r, Sal201-f and Sal597-r primers, shown in Table 1, has been demonstrated in these previous studies. Also primer homology analysis was carried out using the BLAST web interface (3http://www.ncbi.nlm.nih.gov/BLAST). Detection by PCR has provided an effective identification for these groups. Thus, a set of oligonucleotide primer pairs was selected to distinguish these three bacteria groups by production of three subtype-specific fragments (Table 1), which could be easily differentiated on the basis of size.

This mPCR assay was specific for the three studied bacteria groups. All gastrointestinal segments tested including duodenum, jejunum, ileum and cecum were positive in the mPCR assay (Fig. 1, lanes 1-4). Each species specific primers did not amplify other likely species in the PCR reaction. There was no difference in the sensitivity of this method between samples of different gastrointestinal segments.

PCR products were obtained specifically only when DNA from the corresponding genus was present in the reaction either in isolation or in combination. The positive controls showed the positive

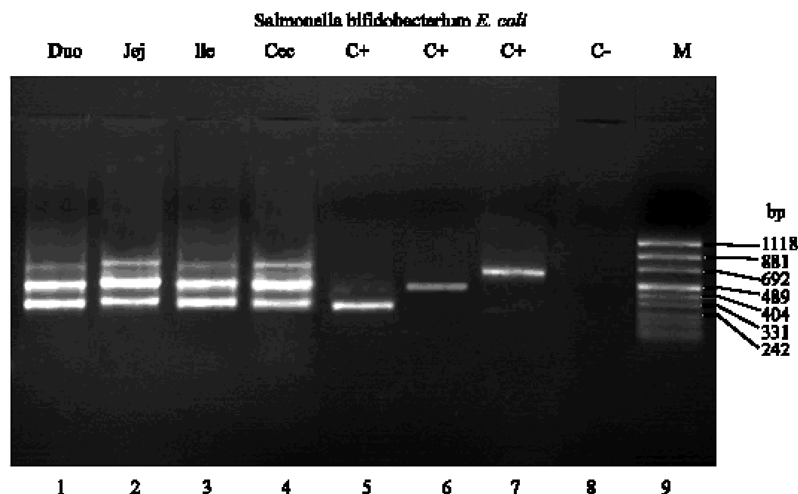

Fig. 1: Electrophoresis of multiplex PCR products on 2% agarose gel stained with ethidium bromide. Intestine samples = lanes 1-4: Amplification products from DNA of *Escherichia coli*, *Bifidobacterium* sp. and *Salmonella* sp., with primers ECO-f, ECO-r, Bif164-f, Bif662-r, Sal201-f and Sal597-r, C+ (*Salmonella typhimurium*) = lane 5: Positive control amplified DNA, C+ (*Bifidobacterium angulatum*) = lane 6: Positive control amplified DNA, C+ (*Escherichia coli*) = lane 7: Positive control amplified DNA, C- = lane 8: Negative control, M=lane 9: Molecular weight marker

band for all three controls specifically for the respective bacteria (Fig. 1, lanes 5-7). This was confirmed from the amplified fragments of 396, 510 and 585 bp specific for *Salmonella* sp., *Bifidobacterium* sp. and *Escherichia coli*, respectively. In this reaction, ddH_2O have been used as negative control and have been tested to explain the contamination factor (Fig. 1, lane 8).

Traditional methods for the identification of *Escherichia coli* O157 and other serogroups involve enrichment cultures, selection of bacterial colonies, biochemical analysis of the isolates and determination of the main virulence markers (Tarr, 1995; Clifton-Hadley, 2000; Settanni and Corsetti, 2007). Gilbert *et al.* (2003) established a useful mPCR assay in order to detect *Campylobacter jejuni*, *Salmonella* sp. and *Escherichia coli* in a variety of raw and ready-to-eat food products. The primers amplified a single product from each target bacterium. Furthermore, *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus* and *Listeria monocytogenes* were also simultaneously detected in kimchi (Park *et al.*, 2006; Settanni and Corsetti, 2007). Recently, Mukhopadhyay and Mukhopadhyay (2007) used specific primers to establish an mPCR test for simultaneous detection of *Escherichia coli* and *L. monocytogenes*. Furthermore this mPCR method was used to identify arcobacters isolated from broiler carcasses at three sites along the processing line: pre-scald, pre-chill and post-chill (Settanni and Corsetti, 2007; Son *et al.*, 2007).

This study addresses the need for development of a rapid, accurate and economical molecular diagnostic method for large scale screening of important bacteria groups in poultry gastrointestinal. The assay developed was appropriate due to its ability to carry out amplification of three targets simultaneously in a multiplex format using conventional PCR. As such, this unique characteristic could serve as a reference for the development of assays for detection and differentiation of other useful bacteria and pathogens.

Technical and economic burdens have been placed on clinical microbiology laboratories to detecting *Salmonella* sp. and *Escherichia coli* using conventional microbiological tests, which can take up to 5-7 days and are prone to misdiagnoses. Hence, an effort is underway to replace conventional testing with rapid and accurate molecular-based methods, including mPCR. The development of mPCR

has obviated the need to manipulate post-amplification PCR products, thereby reducing the risk of false-positive results generated by amplicon contamination. From negative control results (Fig. 1, lane 8), no false-positive results due to probable contamination of laboratory equipment were detected. These results, representative of three experiments, did not show variability and highlighted a high reproducibility.

As expected, three bands for mPCR were simultaneously amplified, confirming the specificity of the primer pairs chosen for each genus. Low homology regions selected as specific inner primers. The amplicons being of different sizes, allowed a simple and specific detection of each genus. Although PCR methods have been developed for the detection of *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. from poultry samples such as Li and Drake (2001), Mangin *et al.* (2006) and Patela *et al.* (2006), none have been capable of detecting *Salmonella* sp., *Escherichia coli* and *Bifidobacterium* sp. in a single reaction. Here, we describe a mPCR assay capable of identifying these genera in a single reaction, directly from poultry gastrointestinal samples.

This method has the potential to save considerable time and effort within the laboratory. Up until now, sequence analyses or serological investigations were the only methods able to discriminate between these groups. Sequence analysis is expensive and time consuming and serological investigation also has many limitations such as cross-reactions with other groups, possible false positive etc. (Ruzek *et al.*, 2007).

In conclusion, we demonstrated that our mPCR is a simple, sensitive, rapid and inexpensive method. Moreover, we believe that use of this concept will enable laboratories to perform bacteria diagnosis easily without the need for expensive instrumentation. Today mPCR and multiplex real-time PCR provides rapid and sensitive laboratory detection and quantification of multiple specific targets in one test tube while reducing the number of experimental steps. This minimizes the risk of contamination, ensuring a better understanding of the epidemiology of an outbreak (Kim *et al.*, 2007).

Finally, developed mPCR is reliable and offers more advantages for routine laboratory screening of these three bacteria groups. It has been demonstrated that mPCR has a series of advantages over common PCR including sensitivity, cost-saving, efficiency and reproducibility. Therefore, this method may solve the problem to rapid detect of these microorganisms at gut.

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