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Evaluation of Multiplex-PCR for Simultaneous Identification of *Salmonella enteritidis, Shigella flexneri,* and *Escherichia coli* O_{157}: H_7 in Poultry

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ABSTRACT

Aims: Foodborne infections caused by bacteria, including *Salmonella enteritidis*, *Shigella flexneri,* and *Escherichia coli* O_{157}:H_7 are one of the most common diseases among poultry and humans. The purpose of this study was the simultaneous and rapid detection of important microorganisms found in fecal samples of poultry and poultry workers.

Materials & Methods: A total of 144 fecal samples were taken from poultry and poultry farms workers. Fecal swabs were cultured on specific media, and biochemical tests were performed for further confirmation of bacterial isolates. Moreover, genomic DNA of fecal swabs was extracted for molecular identification of *S. enteritidis*, *E. coli* O_{157}: H_7 and *S. flexneri* species using multiplex-PCR technique.

Findings: According to the multiplex-PCR technique results, 16.7, 13.9, and 9.5% of the poultry samples were positive for the presence of *S. enteritidis*, *E. coli* O_{157}: H_7 and *S. flexneri* species, respectively; whereas culture method results showed the corresponding prevalence rates of 18.1, 15.2, and 12.5% for the above species. Moreover, regarding the samples collected from the poultry farms workers, multiplex PCR showed the prevalence rates of 6.9, 12.5, and 4.2% for *S. enteritidis*, *E. coli* O_{157}: H_7, and *S. flexneri* species, respectively; whereas culture method showed the corresponding prevalence rates of 8.3, 13.9, and 13.9% for the above species.

Conclusion: In the current study, the sensitivity and specificity of multiplex-PCR in detecting *S. enteritidis*, *E. coli* O_{157}: H_7, and *S. flexneri* species were 74 and 100% for samples taken from the poultry farms workers, and 82.2 and 100% for samples taken from the poultry, respectively, suggesting the possibility of using a designed multiplex-PCR method for rapid detection of infectious agents in poultry farms.

Keywords: *Salmonella enteritidis, Shigella flexneri, Escherichia coli*, Muliplex-PCR, Poultry.

CITATION LINKS

[1] Effect of methionine supplementation in chicken feed on the quality and shelf life of fresh poultry meat. Poult Sci. 2017; 96(8):2853-61. [2] Monitoring of bacterial contamination on chicken meat surface using a novel narrowband spectral index derived from hyperspectral imagery data. Meat Sci. 2016;122:25-31. [3] Isolation and molecular characterization of multidrug-resistant strains of *Escherichia coli* and *Salmonella* from retail chicken meat in Japan. J Food Sci. 2009; 74(7): M405-10. [4] Risk factors associated with *Salmonella enterica* subsp. enterica contamination of chicken carcases in Senegal. Br Poult Sci. 2005; 46(3):293-9. [5] *Campylobacter, Salmonella, Shigella,* and *Escherichia coli* in live and dressed poultry from metropolitan Accra. Int J Food Microbiol. 2001;71(1):21-8. [6] Cloacael carriage and multidrug resistance *Escherichia coli* O157:H7 from poultry farms, eastern Ethiopia. J Vet Med. 2017; 2017:1-9. [7] Prediction of the growth of *Salmonella Enteritidis* in raw ground beef at various combinations of the initial concentration of the pathogen and temperature. Biocontrol Sci. 2015; 20(3):215-20. [8] A novel multiplex PCR for the simultaneous detection of *Salmonella enterica* and *Shigella* species. Braz J Microbiol. 2014; 45(2):667-76. [9] Free-living turtles are a reservoir for *Salmonella* but not for Campylobacter. PLoS One. 2013; 8(8):e72350. [10] Break-ing the resistance of *Escherichia coli*: Antimicrobial activity of Berberis lycium Royle. Microb Pathog. 2017; 102:12-20.

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Introduction

Today, chicken meat is one of the popular products consumed by humans [1] so that in 2016, the worldwide chicken meat production was about 100 million tones[2]. One of the most common problems associated with the consumption of chicken meat is foodborne diseases observed in many countries. Foodborne infections causes by bacteria, including *Salmonella enteritidis, Shigella flexneri,* and *Escherichia coli* O_{157}:H_7 are among the most common diseases between animals and humans [3].

One of the major reservoirs of such infections is the poultry meat. Workers in poultry houses could also be infected by these bacteria, thereby distributing the infection to other society members through oral-fecal routes [4]. The rate of contamination by *S. enteritidis, S. flexneri,* and *E. coli* O_{157}:H_7 strains is considerably high in poultry industry, subsequently leading to a high mortality rate.

Besides, infections caused by these bacteria could impose economic losses to the poultry breeders [4]. Thus, rapid identification of *S. enteritidis, S. flexneri,* and *E. coli* O_{157}:H_7 species using molecular methods such as multiplex PCR-based assay seems necessary. On the other hand, rapid identification of bacteria in poultry farms workers could prevent the spread of *S. enteritidis, S. flexneri,* and *E. coli* O_{157}:H_7 strains to the environment.

Objectives: The purpose of this study was the simultaneous and rapid detection of important microorganisms found in fecal samples of poultry and poultry workers.

Materials and Methods

Standard strains preparation: All standard strains, including *E. coli* O_{157}:H_7 *ATCC 35150, S.enteritidis ATCC 13076*, and *S. flexneri ATCC 12022* were purchased from the Genetic Reserve Center, Tehran, Iran. All the strains were cultured on LB agar medium, and their genomes were extracted using the genomic DNA kit®(QIAamp, GmbH, Germany). In the present study, specific bacterial genes were selected, and specific primers were designed (Table 1).

Detection of specific genes: The annealing temperature of each specific primer was determined using the gradient Uniplex-PCR. Amplification of each target DNA was carried out in a total volume of 50 µL. The reaction mixture contained 5 µL 10× amplification buffer ,1 µL of 25 mM $MgCl_2$, 0.6 µL of 2.5 mM dNTPs (Fermentas, GmbH, Germany), 1 µL of each primer (forward and reverse; 10 pmol), 0.4 µL Taq DNA polymerase (5 U/µL), and 80pg of the extracted DNA. PCR conditions were as follows: initial denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 56±6 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 3 min. Amplified products were separated by agarose (1.2%) gel electrophoresis in 1× TBE and stained by gel red. The PCR products were extracted from the vial using PCR Purification Kit® (QIAquick, GmbH, Germany) and sequenced by Macrogen Inc. (Seoul, Korea).

Multiplex-PCR set up: Multiplex-PCR amplification of each target DNA was carried out in a total volume of 50 µL. The reaction mixture contained 5 µL 10× amplification buffer ,1.2 µL 25 mM $MgCl_2$, 0.8 µL of each of 2.5 mM dNTPs (Fermentas, GmbH, Germany), 1 µL of each primer (forward and reverse, 10 pmol), 0.6 µL Taq DNA polymerase (5 U/µL), and 80pg of the extracted DNA. PCR conditions were as follows: initial denaturation at 94 °C for 5 min; followed

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Table 1) Sequences of specific primers used for the detection of each bacterium

Bacteria	Gene	Sequence 5'→ 3'	Amplification Product (bp)	Reference
E. coli O_{157}:H_7	*eaeA*	GTG GTG TAG AGC ATT ACG AAG TGT GGG TCA ATA ATC AG	250	This study
S. flexneri	*ipaH*	ATG GTT AGT TGG TTG TTA TCT AC TGA GAG GCG GTG TAA GTG	1088	This study
S. enteritidis	*invA*	TAT TAC TTG TGC CGA AGA G CCC TTT GCG AAT AAC ATC C	725	This study

by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 12 min.

Sample collection: A total of 144 samples were randomly collected from the stool of poultry (72 samples) and poultry farms workers (72 samples). Inclusion criteria included poultry farm workers taking no antibiotic in the past six months. A fecal swab was taken from each worker and poultry and cultured on MacConkey, sorbitol MacConkey, and Hektoen enteric agar media. To confirm the bacteria identification, further biochemical assays were performed, including IMViC, TSI, and API-20. Another fecal swab was also prepared for molecular purposes, including DNA extraction and multiplex PCR-based assay.

The genomes of the fecal swabs taken from the workers and poultry were extracted using DNA Stool Mini Kit® (QIAamp, GmbH, Germany). Multiplex-PCR was performed according to the above mentioned protocol for genomes extracted from the fecal samples of poultry and workers. To determine the sensitivity and specificity of the presented method in this study to identify bacteria in the fecal samples of poultry and poultry farms workers, the results of multiplex-PCR and culture techniques were compared.

Findings

Identification of bacteria using culture method and molecular techniques: In the present study, after culturing poultry fecal samples and performing biochemical tests, as the gold standard method, the prevalence rates of *S. enteritidis, E. coli* O_{157}:H_7, and *S. flexneri* species were reported as 13 (18.1%), 11 (15.2%), and 9 (12.5%), respectively. However, multiplex-PCR results showed the corresponding prevalence rates of 12 (16.7%), 10 (13.9%), and 7 (9.7%) for the above species.

Furthermore, the culture of poultry farms workers' fecal samples showed the prevalence rates of 6 (8.3%), 10 (13.9%), and 4 (5.6%) for *S. enteritidis, E. coli* O_{157}:H_7, and *S. flexneri* species; whereas the multiplex-PCR assay showed the corresponding prevalence rates of 5 (6.9%), 9 (12.5%), and 3 (4.2%) for the above species.

According to the culture method results, the total positive fecal samples of both workers and poultry for the presence of *S. enteritidis, E. coli* O_{157}:H_7, and *S. flexneri* species were 19 (26.4%), 21 (29.2%), and 13 (18.1%), respectively; whereas according to the multiplex-PCR results, the corresponding prevalence rates were 17 (23.6%), 19 (26.4%), and 10

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(13.9%) for the above species. The band patterns of all amplified genes following the multiplex-PCR reaction are shown in Fig. 1.

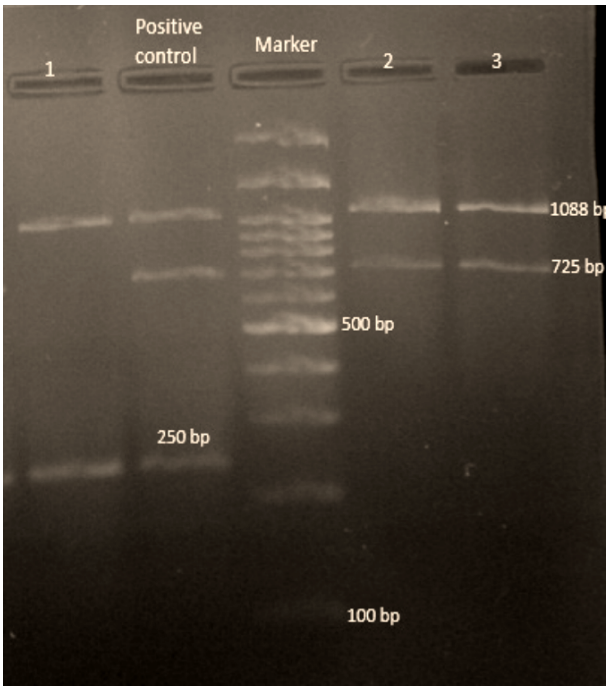

Figure 1) Multiplex-PCR for the detection of each bacterium. Line 1; *S. flexneri* and *E. coli*. Line 2-3; *S. flexneri* and *S. enteritidis*

Comparing the results obtained by culturing bacteria on the specific media and performing biochemical tests with the results of multiplex-PCR, the sensitivity, specificity, and positive predictive value of the multiplex-PCR test were determined as 79.5, 100, and 100%, respectively. All statistical analyzes were performed using SPSS software Version 21.

Discussion

The most important pathogens in poultry could be transmitted directly and indirectly to humans, including *S. enteritidis*, *E. coli* O_{157}:H_7, and *S. flexneri*. The poultry farms workers and staff could be exposed to the infected poultry or their contaminated products, thereby acting as bacterial carriers in the community [5]. Notably, poultry meat could contaminate the kitchen, leading to the spread of *S. enteritidis*, *E. coli* O_{157}:$H_{7,}$ and *S. flexneri.*

Shecho et al. (2017) were able to isolate 13.4% *E. coli* O_{157}:H_7 strains from a total of 194 fecal specimens which were randomly collected from two poultry farms. They used biochemical tests and specific media for bacteria identification[6]. Their results were compatible with the results of the current study.

In another study, Fujikawa et al. (2015) reported the prevalence rates of *E. coli* O_{157}:H_7 and *S. enteritidis* as 21 and 16%, respectively [7].Their results were similar to those obtained in the current study.

In 2015, Radhika et al. (2014) used *ipaH* gene for the detection of *S. flexneri*, and *invA* gene for the detection of *S. enteritidis* in 50 poultry fecal samples using multiplex-PCR. According to their results, the prevalence rates of *S. enteritidis* and *S. flexneri* strains were reported as 20 and 11%, respectively [8], which is similar to the current study results.

In addition, in 2013, Marin et al. reported a high prevalence rate for *S. enteritidis* strains (66.7%) in 65 samples obtained in two years [9]. Given the high prevalence of influenza virus in 2013 and the contribution of this viral infection to bacterial infections in poultry, the high incidence of *S. enteritidis* infection in this study could be attributed to the high prevalence of influenza virus.

Malika et al. (2017) also reported the prevalence rates of 45 and 17% for *E. coli* O_{157}:H_7 and *S. enteritidis* species (in 150 poultry samples in Iraq), respectively using the multiplex-PCR technique. The high prevalence rate of *E. coli* O_{157}:H_7 seems to be due to the poor health status in Iraq and the simple transmission chain of this bacterium [10].

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Conclusion

The sensitivity, specificity, and positive predictive value of multiplex-PCR method designed in this study to identify *S. enteritidis, S .flexneri,* and *E. coli* O_{157}:H_7 species were higher than that reported by other studies in this area, suggesting it as a precise system for the detection of these bacteria. In fact, since poultry contamination with these bacteria could result in economic losses, adopting this molecular method seems to be an essential strategy for their rapid and precise identification in order to immediately take the necessary measures.

On the other hand, the use of antimicrobial agents in poultry diet has led to the decreased intestinal normal flora, increased pathogenicity, and increased antibacterial resistance, leading to the ease of entry of such organisms into the human food chain. Hence, rapid detection of pathogenic organisms could help us reduce poultry industry contamination. The limitation of this project includes the lack of easy access to poultry farms.

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Ethical Permissions: Since we did not use any animal model, we used isolates which were previously obtained from the clinical samples.

Conflict of interests: The authors declare no conflicts of interest..

Authors' Contribution: Sara Sadeqi: original researcher, Seyed Mahmoud Amin Marashi, Parvin Heidarieh, and Farhad Nikkhahi: Methodologist/ introduction author/ discussion author, Mostafa Qorbani: statistical analyst/ Methodologist.

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