Molecular identification of *Salmonella* Infantis isolated from backyard chickens and detection of their resistance genes by PCR

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Summary

This study aims at molecular identification of *Salmonella* Infantis isolated from backyard chickens and the detection of their antibiotic resistance genes. A total of 46 *Salmonella*-suspected samples isolated from backyard chickens of northern Iran were collected. Serotyping was done by the traditional method and then confirmed by PCR. Antimicrobial susceptibility of the isolates against 13 antimicrobial agents was determined by the standard disk diffusion method. There were 44 samples identified as *Salmonella*. Serotyping results showed that all 44 isolates belonged to serogroup C1 and serovar Infantis. The most resistance observed was to tetracycline and doxycycline (100%), chloramphenicol (79%) and florfenicol (72%). The *floR*, *catI*, *tetA* and *tetG* genes were used for the detection of florfenicol chloramphenicol and tetracycline resistance. In order to identify the phenotypic resistance in strains which showed resistance genes by PCR, colony PCR and culture on plates each containing antibiotic was performed simultaneously. All the *Salmonella* Infantis resistant to florfenicol and chloramphenicol harbored *floR* and *catI*. None of the *Salmonella* resistant to tetracycline carried *tetA* or *tetG*. The result of colony PCR and culture in antibiotic medium confirmed the results of PCR and indicated phenotypic resistance in these samples.

Key words: Backyard chickens, Resistance genes, *Salmonella* Infantis

Introduction

Salmonella which can be isolated from numerous animal species is a Gram-negative rod-shaped bacterium in the family of *Entrobacteriacea*. The intestinal tract is the primary reservoir of these zoonotic bacteria where colonisation is favoured by intensive animal production. Poultry products are frequent vehicles in the transmission of *Salmonella* dominating other foods of animal origin as a potential source of infection (Antunes *et al*., 2003; Shahada *et al*., 2006). In addition to its zoonotic importance, the economic loss in poultry industry is also significant (Zahraei Salehi *et al*., 2005a; Nogrady *et al*., 2007). Infected animals are a threat to others and should be identified and separated so as to prevent the disease from spreading. Therefore, rapid identification of this pathogen has to be performed (Zahraei Salehi *et al*., 2005a). The identification of *Salmonella* serovars by slide and tube agglutination tests using O and H antigen-specific anti-sera is both hard and time-consuming (Akiba *et al*., 2011). Molecular methods because of their specificity, rapidity and simplicity compared with traditional serotyping prove strong for this detection.

Salmonella Infantis is a host-unspecific serovar that can infect human and numerous animal species. Salmonellosis by this serovar in human mainly affects children but adults also suffer from it. The major symptom of the disease is septicaemia and the significant feature of this germ is its persistence in hospitals over a long period of time (Ranjbar *et al*., 2012).

In recent years, the incidence of resistance to antimicrobial agents among pathogens has been steadily rising in food as well as in clinical isolates (Van Hoeka *et al*., 2005). Food-producing animals are administered antibiotics for therapeutic, prophylactic, and production purposes to promote animal health, welfare, their growth rate and feed conversion (Schwarz and Chaslus-Dancla, 2001). This widespread use of antimicrobial agents in food animal production has contributed to the occurrence of resistant bacteria in animals, including principle zoonotic pathogens such as *Salmonella* (Shahada *et al*., 2006). Since these pathogens are difficult to treat, antibiotic resistance associated with food-borne diseases has become a major public health issue (Van Hoeka *et al*., 2005).

The occurrence of antibiotic resistance genes is increasing in *Salmonella* Infantis the same as other serovars. There are reports of drug resistance in *Salmonella* Infantis isolates of poultry origin in many countries such as Turkey, Hungary and Japan (Shahada *et al*., 2006; Nogrady *et al*., 2007; Abbasoglu and Akcelik, 2011) but there is little knowledge about this in

poultry of Iran.

Considering the importance of *Salmonella* Infantis in backyard chickens and its potential to be transmitted to human via food chain, we tried to identify *Salmonella* Infantis from chicken samples by traditional serotyping and PCR. Besides, the antimicrobial susceptibility test and antibiotic resistance genes detection were carried out.

Materials and Methods

Sample collection and serotyping

46 *Salmonella*-suspected samples isolated from backyard chickens in the north of Iran, Mazandaran province, were collected. They were cultured in McConkey and Salmonella Shigella agar (Merck, Germany). The suspected colonies on these two media were cultured in Chrom agar (Merck, Germany) and then were identified by Urea and TSI medium (Merck, Germany).

For serotyping, the *Salmonella* isolates were first cultured on to TSI slant medium and grown overnight at 37°C, and then were tested using antisera O (B, D, C1 to C4) and H (Difco, USA) based on slide and tube agglutination tests to determine O and H antigens, respectively (Waltman *et al*., 1998).

DNA extraction

A single colony of each isolate on LB agar (Merck, Germany) plate was picked up and suspended in 250 µL of distilled water. After vortexing, the suspension was boiled for 10 min and 100 µL of the supernatant was collected after centrifuging at 6000 × g for 7 min.

PCR amplification of *fljB* gene for the identification of *Salmonella* Infantis

Molecular identification of *Salmonella* Infantis was performed as described previously (Kardos *et al*., 2007). In this study, two primer pairs were used to detect the *fljB* gene in *Salmonella* Infantis (Table 1). The reaction was carried out in a volume of 25 µL which contained 8 mM $MgCl_2$ (Sinaclon, Iran), 200 µM dNTPs (Sinaclon, Iran), 0.2 µM each primer (Sinaclon, Iran), 10 X PCR buffer (Sinaclon, Iran) and 1 U Taq DNA polymerase (Sinaclon, Iran). The amplification program was done by thermocycler (Techne TC-512, UK) as follows: 95°C initial denaturation for 6 min, 35 cycles of 95°C for 1 min, 58°C for 15 s, 72°C for 1 min and a final amplification at 72°C for 4 min. The amplified products were electrophoresed on a 1.2% agarose gel (Sinaclon, Iran) stained with ethidium bromide (Sinaclon, Iran) and visualized under ultraviolet light.

Antibiotic susceptibility testing

Antibiotic susceptibility of the isolates was determined by the disc diffusion method (Kirby-Bauer, 1996) on Mueller-Hinton agar using antibiotic discs (MAST, UK). The following antimicrobial agents were tested: 20 µg ampicillin (AMP), 30 µgcefazolin (CEF), 30 µgcefotaxime (CTX), 5 µgcefixime (CFM), 30 µgceftraxion (CRO), 5 µgenrofloxacin (ENF), 25 µg difloxacin (DIF), 30 µg chloramphenicol (CHL), 30 µgflorfenicol (FFC), 30 µg tetracycline (TE), 30 µgdoxycycline (DXT), 10 µg gentamicin (G), and 25 µgtrimethoperim-sulfamethoxazol (SXT). The inhibition zones were measured and scored as sensitive, intermediate susceptibility or resistant according to the CLSI recommendations. *Escherichia coli* ATCC 25922 was used as a reference strain for antibiotic disc control.

Detection of resistance genes

Antimicrobial resistance genes were characterized by polymerase chain reaction. PCR amplification of *tetA*, *tetG* and *floR* genes was performed as described previously (Randall *et al*., 2004; Abbasoglu and Akcelik, 2011).

For the detection of *cat* gene, primer sequences were based on the sequence in GeneBank (accession No. LK056646.1). Each 25 µL of reaction mixture contained 10 X PCR buffer, 2 mM $MgCl_2$, 0.5 µL of 10 mMdNTPs, 0.4 µM of each of the primer, 1 U of Taq DNA polymerase and 1 µL of DNA template. The PCR amplification involved 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min in a thermocycler (Techne TC 512, UK).

For the identification of phenotypic resistance in strains which showed resistance genes by PCR, colony PCR and culture on plates, each containing antibiotic, was performed simultaneously. PCR from colony and plating bacteria from the same colony on LB agar plates

Table 1: Primers used in this study

Primers	Target gene	Primer sequence (5′ to 3′)	Amplification product (bp)
558f 1275r	*fljB*	AACAACGACAGCTTATGCCG CCACCTGCGCCAACGCT	727
878f 1275r	*fljB*	TTGCTTCAGCAGATGCTAAG CCACCTGCGCCAACGCT	413
tetA	*tetA*	GCTACATCCTGCTTGCCT CATAGATCGCCGTGAAGA	210
tetG	*tetG*	CCGGTCTTATGGGTGCTCTA CCAGAAGAACGAAGCCAGTC	603
floR	*floR*	AACCCGCCCTCTGGATCAAGTCAA CAAATCACGGGCCACGCTGTATC	548
cat1	*Cat1*	CCTATAACCAGACCGTTCAG TGAAACTCACCCAGGGATTG	370

containing chloramphenicol (20 µg/ml) (Sigma, Aldrich, USA) and florfenicol (30 µg/ml) (SERVA, Heidelberg) was performed. The concentration of each antibiotic in culture medium was considered according to CLSI standards (2011).

Results

Forty-four samples were confirmed as *Salmonella* based on the cultural and biochemical methods. The results of serotyping with O and H antisera demonstrated that all the samples belonged to serogroup C1 and serovar Infantis with the antigenic formula of 6,7: r: 1,5.

Both assays which targeted *fljB* gene of *Salmonella* Infantis illustrated products of the expected size in all 44 *S.* Infantis isolates (Fig. 1).

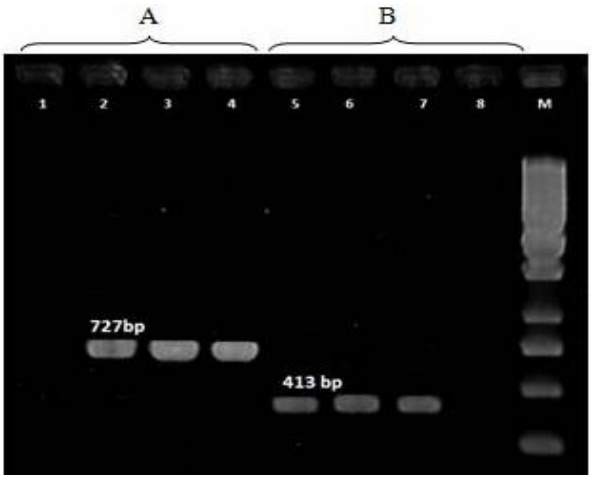

Fig. 1: Results of PCR assays used to identify *S.* Infantis with the primer pairs 558f-1275r (**A**) and 878f-1275r (**B**). M: 250 bp ladder. Lane 1: *S.* Paratyphi C as negative control, Lane 2: Positive control, Lanes 3-4: Positive detection, Lane 5: Positive control, Lanes 6-7: Positive detection, and Lane 8: *S.* Paratyphi C as negative control

The most observed resistance resistance was to tetracycline (100%), doxycycline (100%), chloramphenicol (79%) and florfenicol (72%) respectively. All the isolates were sensitive to other antibiotics with two exceptions. Of those two exceptions, one was resistant to gentamicin and both of them were resistant to enrofloxacin.

All the *S.* Infantis isolates resistant to florfenicol contained *floR* gene (Fig. 2) and the chloramphenicol-resistant isolates showed resistance gene *cat1* with the expected bands (Fig. 3).

None of the tetracycline and doxycycline-resistant strains carried *tetA* or *tetG* gene.

For the detection of phenotypic resistance, PCR and culture in antibiotic medium was performed at the same time. Their outcome confirmed the PCR results and demonstrated phenotypic resistance in these samples.

Discussion

Salmonella enterica is one of the most important

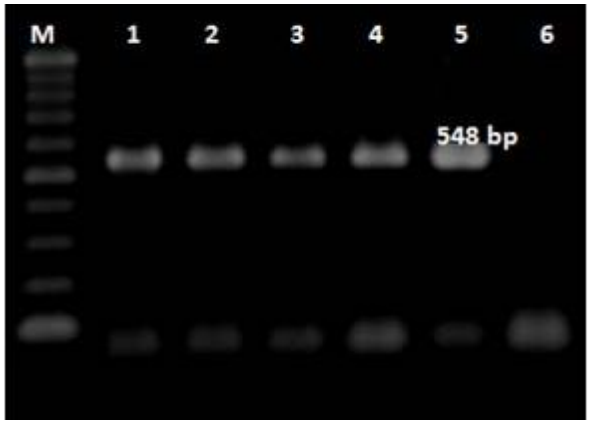

Fig. 2: Results of PCR assays for identification of *floR* gene. M: 100 bp ladder. Lane 1: Positive control, Lanes 2-5: Positive detection, and Lane 6: Negative control

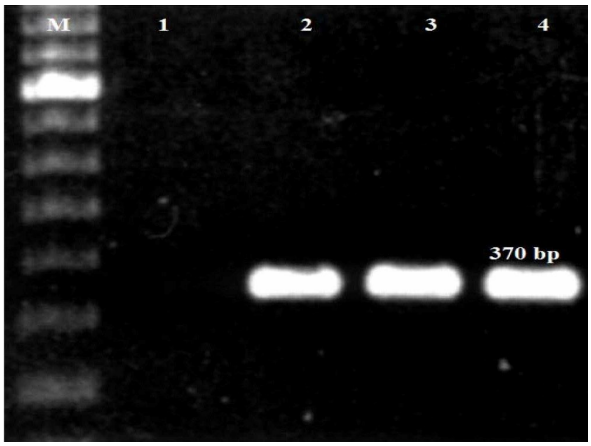

Fig. 3: Results of PCR assays for identification of *cat1* gene. M: 100 bp ladder. Lane 1: Negative control, Lane 2: Positive control, and Lanes 3-4: Positive detection

food-borne pathogens throughout the world (Nogrady *et al*., 2008). The prevalence of each serovar of *Salmonella* in human and animal is changing and one may replace another at any time (Zahraei Salehi, 1999). In this research, all the *Salmonella* isolates belonged to serovar Infantis. Previous studies showed that the most predominant serogroups isolated from poultry in Iran were of D and B and the main serovars were Enteritidis and Typhimurium (Zahraei Salehi *et al*., 2005a; Jaffari *et al*., 2007; Emaddi Chashni *et al*., 2009; Mirzaei *et al*., 2010). However, the present investigation indicates a higher prevalence of serogroup C1 and serovar Infantis. In European countries, the prevalence of *S.* Enteritidis and *S.* Typhimurium has decreased due to their vaccination programs but the serovar Infantis is still increasing and is a major problem in some countries such as Hungary (Miller *et al*., 2010).

The survey of *Salmonella* Infantis in hospitals of Iran indicates an increase in the prevalence of this serovar. Naghoni *et al*. (2010) and Ranjbar *et al*. (2011) found the serovar Infantis as the second most prevalent in the human cases of salmonellosis. In the survey conducted in the years 2007-2010, 19% of the isolates were *S.* Infantis (Ranjbar *et al*., 2012). Since the late 1970s, the incidence

of the human cases of serovar Infantis has been increasingly recorded worldwide in countries like Argentina, Australia, Brazil, The Netherlands, Finland, Canada, Hungary, Japan, New Zealand and Russia (Miller *et al*., 2010). In recent years, the rate of *Salmonella* infections with this serovar among poultry has been increasing in some countries such as Hungary and Japan and is the main cause of salmonellosis in their poultry (Kardos *et al*., 2010; Miller *et al*., 2010). The reason for such a significant increase in humans is that this pathogen is food-borne and can be transmitted through poultry products.

Little is known about the presence of this serovar in the poultry of Iran and we have screened the backyard chickens for its the occurrence. Also, molecular methods have been used for rapid identification of *Salmonella* Infantis for the reasons mentioned above. The results of PCR confirmed the serotyping and all the *Salmonella* isolates were recognized as *Salmonella* Infantis.

Our study depicts a high percentage of antibiotic resistance in *Salmonella* Infantis isolated from poultry showing extensive usage of antibiotic in them while backyard flocks are not treated with antibiotics like the industrial poultry. Such a high percentage of antibiotic resistance observed in this research could be due to the transmission of resistant bacteria from industrial poultry by different vehicles such as human, free flying birds and poultry products (Emaddi Chashni *et al*., 2009).

One the common resistance observed in *S*. Infantis was to tetracycline and doxycycline. Tetracycline has been one of the most commonly used antibiotics for the production of animals. As a result, the very frequent occurrence of resistance among almost all bacterial species is probably a consequence of this (Gebreyes and Altier, 2002). The incidence of tetracycline resistance has been described recently by other authors in *Salmonella* isolates of poultry origin in Iran (Zahraei Salehi *et al*., 2005b; Jafari *et al*., 2007; Mirzaei *et al*., 2010; Morshed *et al*., 2010).

The resistance to tetracycline is associated with *tet* genes. Six classes of genes including *tetA*, *B*, *C*, *D*, *E*, and *G* were identified responsible for resistance to tetracyclines. We have selected *tetA* and *tetG* genes for the identification of resistance to tetracycline. The isolates carried neither *tetA* nor *tetG*. These isolates may carry other *tet* genes that have not been studied here. *TetA* was the most common gene detected in poultry responsible for resistance to this antibiotic (Shahada *et al*., 2006; Nogrady *et al*., 2007; Abbasoglu and Akcelik, 2011). *Salmonella* Infantis isolated from human cases in Iran carried both *tetA* and *tetB* genes (Tajbakhsh *et al*., 2012). The study of antibiotic resistance genes in Brazil showed the presence of *tetD* gene in *Salmonella* Infantis isolated from human cases (Fonseca *et al*., 2006). The *tetG* gene was mostly detected in *Salmonella* Typhimurium in previous studies (Walker *et al*., 2001; Randall *et al*., 2004).

Resistance to chloramphenicol and florfenicol as antibiotics of the same family was seen in more than 70% of the isolates. Chloramphenicol used to be applied for the treatment of salmonellosis in animals but due to becoming resistant to this antibiotic, florfenicol was introduced. However, the resistance to this antibiotic soon appeared (Nogrady *et al*., 2005). In the previous studies, there was antibiotic resistance to these two antibiotics in poultry *Salmonella* isolates (Zahraei Salehi *et al*., 2005b; Emaddi Chashni *et al*., 2009; Morshed and Peighambari, 2010) but it seems these days this resistance is on the increase. The florfenicol resistant strains can show cross resistance to chloramphenicol (Nogrady *et al*., 2005).

Cat1 gene encoding chloramphenicol acetyl transferase was detected in all the chloramphenicol resistant isolates and the florfenicol resistant strains harbored *floR* gene. In other studies on *Salmonella* Infantis, *cat1* gene was predominately observed in chloramphenicol resistant isolates (Fonseca *et al*., 2006; Dionisi *et al*., 2011). The *floR* gene was also identified in numerous *Salmonella* serovars such as *S*. Infantis, *S*. Typhimurium (Bolton *et al*., 1999), *S*. Agona (Cloeckaert *et al*., 2000), *S.* Paratyphi B (Meunier *et al*., 2002), *S.* Albany (Doublet *et al*., 2003) and many other serovars (Randall *et al*., 2004).

In conclusion, this study shows the high incidence of antibiotic resistance in *Salmonella* Infantis isolated from backyard chickens. In order to prevent antimicrobial resistance, antibiotic administration in animals has to be closely supervised. However, this is a short report about the occurrence of drug resistant *Salmonella* Infantis and there should be more studies concerning the prevalence of this pathogen in both poultry and humans.

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Conflict of interest

We declare no conflict of interest.

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