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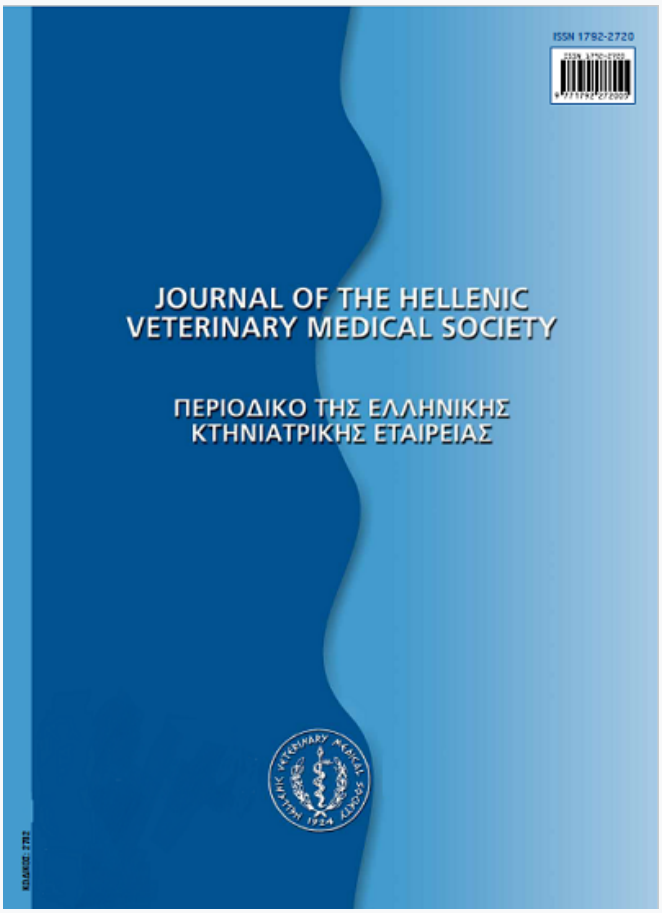

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Detection and Molecular Identification of *Salmonella* Strains Isolated from an Industrial Ostriches farm

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ABSTRACT. Poultry salmonellosis, one of the most prevalent diseases and major source of food-borne infections to humans due to consumption of poultry products is worldwide in distribution. The aim of this study was to investigate the incidence of salmonellosis in ostriches by culture and PCR , determination of antibiotic resistance pattern of the isolates and the infected ostriches antibody level. 87 fecal samples from one industrial ostrich farm with clinical signs of diarrhea, weight loss, mortality and reduced hatchability were collected and evaluated for presence of the *Salmonella*. *Salmonella* was isolated according to standard culture and biochemical tests. The *Salmonella* positive samples were serotyped with O and H antisera based on slide and tube agglutination tests. PCR was done for detection of serovars Infantis and Enteritidis. Then the antibiotic resistance against 14 antimicrobial agents were tested. The antibody level of the infected ostriches were measured by WIDAL agglutination test. Results indicated 9.1% (8 of 87) of ostriches were positive for *Salmonella*. Serotyping results showed 3 samples were serovar Infantis and 5 samples were serovar Enteritidis and PCR confirmed the serotyping results. All 8 samples were resistant to tetracyclin and ampicillin but sensitive to other antibiotics including . ciprofloxacin, kanamycin, sultrim, cephalothin, norfloxacin, chloramphenicol, flumequine, nitrofurantoin, coamoxiclav, gentamicin, enrofloxacin and cefotaxime The results of WIDAL agglutination test indicated that all ostriches were negative except 8 *Salmonella* positive ostriches with the titres 1/80 to 1/360 for the O and 1/80 to 1/640 for the H antigens. To our knowledge this is the first study which reports the peresence of *Salmonella* Infantis in ostriches in Iran and more studies should be done to investigate this pathogen in ostriches herds of Iran.

Keywords: *Salmonella*, ostriches, PCR, Iran

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INTRODUCTION

Salmonella infection is one of the most important bacterial diseases in animals and human. Despite the continuous monitoring of this disease, it is still the most important food-borne disease worldwide (Oludairo et al., 2013). Three million deaths annually worldwide occur due to *Salmonella* infection. The infection in food-producing animals, could result salmonellosis in human as a zoonotic disease, Because the most common route of transmission of the infection to humans is consumption of contaminated meat products. *Salmonella* infections in humans can lead to various symptoms such as enteritis, enterocolitis and systemic infection, the disease occurs after colonization of the bacteria in the small intestine (Mulvey et al., 2006). The main clinical sings of *Salmonella* infection in animals is reduced production, mortality, intestinal infection, abortions in animals and reduced hatchability in avian species (Ramya et al., 2012). Different serotypes of this bacteria could cause infections in humans and animals, the most common serotypes are *Salmonella* serotype Typhimurium and *Salmonella* serotype Enteritidis. Serotypes Infantis, Typhimurium and Enteritidis well also frequently isolated from poultry (Kaushik et al., 2014; Ramya et al., 2012).

There are several methods available for detection of *Salmonella* infections, such as the isolation of the causative agent by culture or serological tests and polymerase chain reaction (PCR). By using PCR method and different specific primers, various *Salmonella* serotypes can be identified (Malorny et al., 2003). One of the serological methods is the WIDAL agglutination test which is used for diagnosis of typhoid fever and infection to other *Salmonella* serotypes. In WIDAL agglutination test different types of O and H antigen could be used and antibody response to specific type of O and H antigen could be measured (Willke et al., 2002). Many studies have been done on the occurrence of salmonellosis in animals and poultry, but *Salmonella* serotypes causing infection in ostrich has not been well recognized. The aim of this study was to investigate the incidence of salmonellosis in ostriches by Isolation and polymerase chain reaction, determination of antibiotic resistance pattern of the isolates and determination of the infected ostriches antibody level by WIDAL agglutination test.

MATERIALS AND METHODS

Isolation and identification of *Salmonella*

Samples were collected from a farm of industrial ostriches which had the clinical symptoms of diarrhea, weight loss, mortality and reduced hatchability and were previously treated with tetracycline. A total number of 87 fresh fecal samples were collected by cloaca swabs and were transported to the laboratory in isothermal box. The samples were cultured in 10 ml peptone water (Merck, Germany) at 37°C for 24 h. One ml of each culture was then transferred to 10 ml Selenit F and Rappaport broths (Merck, Germany). These samples were incubated at 37°C and 42°C for 24h and then each sample was inoculated onto *Salmonella-Shigella* agar (SS), Brilliant Green agar (BG) (Merck, Germany) and CHROMagar (Paris, France). The plates were incubated at 37°C for 24 h, colonies suspected of *Salmonella* spp. were selected and subjected to preliminary biochemical identification using Triple Sugar Iron Agar (TSI), Urea agar and Lysine Iron agar (Merck, Germany) (Quinn et al., 1994).

Serotyping of the isolated *Salmonella* strains was performed by commercial antisera (Difco, Detroit, Michigan, USA) and the results were interpreted according to the Kaufmann-White scheme (Guibourdenche et al., 2010).

Antibiotic resistance test

Antibiotics susceptibility of the *Salmonella* isolates was determined on Mueller-Hinton agar (Merk, Germany) by Kirby–Bauer (1996) method according to CLSI protocol (2014). Fourteen Antibiotic disks (Padtan Teb, Tehran, Iran) were used including ampicillin (AM: 10 µg), tetracycline (TE: 30 µg), ciprofloxacin (CIP: 5 µg), kanamycin (K: 30 µg), sultrim (SXT: 25 µg), cephalothin (KF: 30 µg), norfloxacin (NOR: 10µg), chloramphenicol (CH: 30 µg), flumequine (FM: 30µg), nitrofurantoin (FD: 30 µg), coamoxiclav (AMC: 30 µg), gentamicin (GM: 10 µg), enrofloxacin (ENF: 5 µg) and cefotaxime (CTX: 30 µg).

DNA Extraction for PCR Tests

After confirming the isolates as *Salmonella* by biochemical tests, the isolates were sub-cultured on Luria Bertani (LB) Agar; a single colony of each isolate on the LB agar plate was picked and suspended

Table 1: Primers used for the detection of S. Enteritidis and S. Infantis by MPCR

Target sequence	Primer	Sequence (5′ to 3′)	Size (bp)	Reference
Random	ST11 ST14	GCCAACCATTGCTAAATTGGCGCA GGTAGAAATTCCCAGCGGGTACTGG	429	Soumet et al. 1997
Spv	S1 S4	GCCGTACACGAGCTTATAGA ACCTACAGGGGCACAATAAC	250	Soumet et al. 1997
SefA	SEFA2 SEFA4	GCAGCGGTTACTATTGCAGC TGTGACAGGGACATTTAGCG	310	Woodward et al. 1996
fljB	SI-F SI-R	TTGCTTCAGCAGATGCTAAG CCACCTGCGCCAACGCT	413	Kardos et al. 2007

in 200 μl of distilled water. After vortexing, the suspension was boiled for 5 min, and 50 μl of the supernatant was collected after centrifuging for 10 min at 14000 rpm (Rahn et al., 1992).

PCR amplification for detection of *S.* Enteritidis and *S.* Infantis

All the strains which were identified as *Salmonella* were screened by multiplex PCR to confirm *S.* Enteritidis serovar as described by Soumet et al. and Pan and Liu, using ST11 and ST14 specific primers for the genus of *Salmonella* (Random), S1 and S4 primers which is related to virulence (*Spv*) and specific for *S.* Enteritidis and SEFA4 and SEFA2 primers for specificity within *S.* Enteritidis (*SefA*) (Table 1) (Pan and Liu, 2002; Soumet et al. 1999; Woodward et al., 1996). The MPCR reaction were performed in a final volume of 25μl containing 10 μl of extracted DNA, 2.5 μl of 10X PCR buffer (500mM KCl, 200 mMTris-Hcl), 1.5 mM MgCl2, 250 μM dNTP, 0.5 μM of each primer and 1 unit of Taq DNA polymerase in total 25 μl volume. Initial denaturation was at 94°C for for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 56°C for 90 sec, extension at 72°C at 30 sec and final extension at 72°C for 10 min.

Also another PCR reaction was performed to confirm *S.* Infantis serovar as described by Kardos et al. (2007). SI-F and SI-R (Primers was used in this study were which amplify a 413 bp product). The reactions were performed in a final volume of 25 μl containing: 3μl of template DNA, 2.5 μl of reation buffer, 200 μM of dNTPs, 2mM of MgCl2, 1 U of Taq DNA polymerase and 0.4 mM of each primer. Initial denaturation was at 95°c for 60 sec, followed by 35 cycles, of denaturation at 95°c for 60 sec, annealing at 56°c for 15 sec and extension at 72°c for 60 sec, with a final extension at 72°c for 240 sec. The PCR products were electrophoresed on 1% agarose gel for 1 hour at 90V and stained with ethidium bromide.

WIDAL agglutination test

Blood samples were taken from all 87 ostriches. After serum separation, WIDAL agglutination test was performed to detect O and H antibody based on the standard tube test method (Willke et al., 2002).

RESULTS

Salmonella spp. was isolated from 8 of 87 ostriches by culture and biochemical tests, the positive strains were also identified as *Salmonella* spp. by multiplex PCR. Five isolates were determined as *S.* Enteritidis (group D) and 3 isolates were determined as *S.* Infantis (group C1) by serotyping presenting antigenic formula of (O:1, 9, 12, H1: g, m) and (O:6, 7, H1: b, H2: 1, 2) Respectively. PCR Results for detection of *S.* Enteritidis and Infantis serovars was the same with serotyping results (Fig 1 and 2).

Antibiogram showed that all the *Salmonella* strains were sensitive to ciprofloxacin, kanamycin, sultrim, cephalothin, norfloxacin, chloramphenicol, flumequine, nitrofurantoin, coamoxiclav, gentamicin,

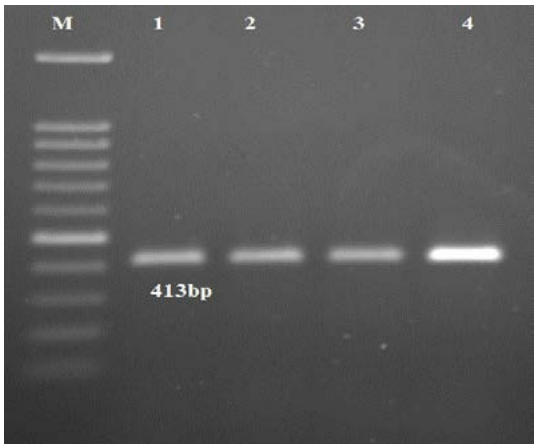

Fig 1: PCR results for identification of *Salmonella* Infantis, M: Marker, 1: positive control, 2-4: positive detection.

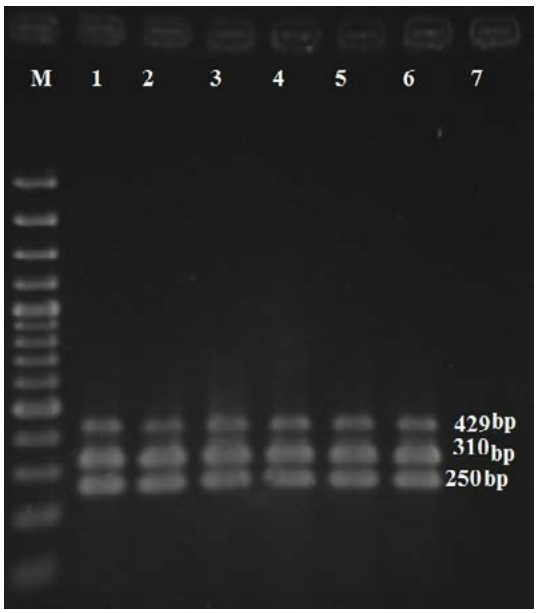

Fig 2: PCR results for detection of *Salmonella* Enteritidis. M:100 bp marker, 1: positive control 2-6: positive detection 7: negative control.

enrofloxacin and cefotaxime and resistant to ampicillin and tetracycline (table 2).

The results of WIDAL agglutination test indicated that all ostriches were negative except 8 *Salmonella* positive ostriches, the antibody level against O and H for three *Salmonella* Infantis positive samples were 1/320-1/80, 1/320-1/160 , 1/320-1/160 and in five *Salmonella* Enteritidis infected ostriches the titer was 1/320-1/160, 1/320-1/80, 1/80-1/640, 1/80-1/640 and 1/80-1/320.

DISCUSSION

Salmonella could cause infection in human beings if the bacteria are contracted either from the wild-life directly or by consuming the meat products . Therefore, monitoring of the farms to control and prevent the transmission of *Salmonella* to humans is crucial, especially in the case of ostrich industry, that there is not enough information about common *Salmonella* serotypes (Oludairo et al., 2013). In current study, fecal samples were collected from one industrial ostrich farm with clinical signs of diarrhea, weight loss, mortality, and reduced hatchability. *Salmonella* was isolated from 9.1% (8 0f 87) of ostriches. Many studies have been conducted on the presence of *Salmonella* in poultry and avian species but there are few studies on the isolation and identification of *Salmonella* from ostrich and some studies indicated that *Salmonella* infection could increase chicks mortality (Arshad et al., 2006; Chiari et al., 2013; Silva-Hidalgo et al., 2012).

Serotyping and PCR results showed that 3 and 5 of the detected *Salmonella* strains was *S.* Infantis (O:6, 7, H1: b, H2: 1, 2) and *S.* Enteritidis (O:1, 9, 12, H1: g, m) respectively. This result could be due to high prevalence of *S.* Enteritidis (belonging to serogroup D) in livestock in Iran (14). While in Arshad et al. (2006), Silva-Hidalgo et al. (2012) and Chiari et al. (2013) studies the most predominant *Salmonella* serogroups isolated from wild animals were D, B and C1 Respectively. Many studies indicate high prevalence of *S.* Infantis in poultry. Infection of ostriches with *S.* Infantis could be result of the transmission of this serovar from poultry to them.

WIDAL agglutination test results in ostriches indicated that all of infected ostriches to *S.* Infantis had an active infection but in case of *S.* Enteritidis higher titer were observed for H antigen which may occur in past infection or in immunized response. Validity of WIDAL test were examined for diagnosis of typhoid fever in many studies (Pang et al., 1983; Silva-Hidalgo et al., 2012), but there is not enough information about diagnosis of other *Salmonella* serotypes infection by WIDAL test.

In This study *spv* gene were detected in all of the *S.* Enteritidis isolates respectively. The plasmid-encoded *spv* locus was shown to be required for progressive systemic infection by strains pathogenic for mice and

Table 2: Results of antimicrobial susceptibility testing in *Salmonella* isolates.

Antibiotics	Sensitive (n)		Resistant(n)	
	Enteritidis	Infantis	Enteritidis	Infantis
Tetracycline	0	0	5	3
Enrofloxacin	5	3	0	0
Ciprofloxacin	5	3	0	0
Norfloxacin	5	3	0	0
Kanamycin	5	3	0	0
Sultrim	5	3	0	0
Flumequine	5	3	0	0
Nitrofurantoin	5	3	0	0
Gentamicin	5	3	0	0
Chloramphenicol	5	3	0	0
Ampicillin	0	0	5	3
Cephalothin	5	3	0	0
Coamoxiclav	5	3	0	0
Cefotaxime	5	3	0	0

for the virulence of host-adapted serovars in their corresponding animal hosts (Bacci et al., 2006; Chikami et al., 1985; Gulig et al., 1987; Heffernan et al., 1987; Libby et al., 1997). The gene may not always be present in all *Salmonella* spp. It was shown that *Salmonella* virulence effector locus, the *spv* operon, is required for the induction of cytopathology during infections of human monocyte derived macrophages. Absence of the gene in the confirmed *Salmonella* isolates can lead to lack of invasiveness by those isolates (Bacci et al., 2006). The implication of the presence of *spv* gene in *Salmonella* isolates is that the organisms are actually able to cause infection in host, especially if host immunity is suppressed.

Antibiotic usage is possibly the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Neu, 1992; witte, 1998). In this study antibiotic susceptibility of the *Salmonella* strains were determined, all the isolates were resistant to ampicillin and tetracycline. Some studies also indicates that multiple antibiotic-resistant strains of *Salmonella* were isolated from wild and domestic animals (Murugkar et al., 2005; Shetty et al., 2012). Our results indicated that the strains were sensitive to most tested antibiotic, while in Shetty et al. (2012) and Murugkar et al. (2005) studies the most effective antibacterial drugs were ceftriaxon and ciprofloxacin respectively. This difference could be due to the variety of antibacterial drugs which were used in different area.

CONCLUSIONS

There is little information available regarding for *Salmonella* infection in ostriches. Due to the presence of *Salmonella* infection in ostrich, the results may indicate the possibility that ostriches have an important role for carriage of *Salmonella* strains. On the other hand, continuous monitoring of farm animals for early treatment of *Salmonella* infection and prevention of transmission to other animals and humans is necessary. To conclude more precisely about the excretion status of *Salmonella* strains in ostriches in Iran, a more perfect study with more samples in different parts of Iran should be conducted. To the best of our knowledge, this appears to be the first report of *S.* Infantis detection from ostriches.

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