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Virulence Determination of Three Iranian Isolates of *Salmonella* Enteritidis in Day-Old Layer Chicks

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Abstract

BACKGROUND: *Salmonella* Enteritidis (SE) infection in poultry is one of the most important concerns in poultry. Virulence and pathogenicity of the SE isolates from Iran have not been well studied so far.

OBJECTIVES: In the present study, three *Salmonella* Enteritidis (SE) isolates were compared with a standard SE strain (PT21) for virulence in one-day-old layer chicks. All of the isolates were supposed to be virulent because of carrying a large-sized virulence plasmid.

METHODS: Fifty day-old layer chicks (LSL strain) were divided into five groups of 10 chicks and raised in separate cages until 14 days of age. All three SE isolates were cultured in brain-heart infusion (BHI) broth to reach a concentration of approximately 10^{10} CFU/ml. The challenged groups included three groups inoculated with three SE isolates (A20, S32, S34) and one group inoculated with SE PT21 as positive control. One group was raised as negative control without receiving any bacteria. Any mortality or morbidity observed in any group was recorded. Samples were taken from liver, jejunum and cecum at days 2, 4, 6, 9 and 14 days of age, cultured for SE isolation, colony counting and histopathological examinations.

RESULTS: All challenged groups showed mild to severe diarrhea in all birds and some birds were listless especially in the first week. No signs were seen in the control group. Two mortalities occurred in challenged groups. *Salmonella* Enteritidis was detected in all samples until the end of experiment. The colony count showed less (100 to 1000 times less) SE in liver compared to that of cecal samples. Histopathological findings also were compatible with symptoms and bacteriological results.

CONCLUSIONS: We concluded that all three SE isolates were able to colonize in the digestive system of layer chicks leading to mortality or at least lower performance compared to healthy chicks.

KEYWORDS: Challenge, Chickens, Layer, *Salmonella* Enteritidis, Virulence

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Introduction

More than 2500 serotypes of paratyphoid *Salmonella* have been identified worldwide. *Salmonella* Enteritidis (SE) infection in poultry is one of the most important concerns in poultry industry as well as public health (Gast, 2013; Owen, 2015; Chousalkar and Gole, 2016). This pathogen is an arbitrary intracellular pathogen that can cause local or systemic infections. It is also capable of causing chronic disease without any noticeable signs. Induction of clinical disease depends on the bacterial strain and host characteristics such as age, genetics, route of infection, immune competency, etc. In a specific serotype, virulence of the strains can vary from zero to 100%. Mortality occurs 4 to 10 days post challenge. Clinical disease is observed only in the first week of age. In adult birds, SE is colonized in the reproductive system thus vertical transmission may occur. Birds which recover from SE infection, show growth retardation for a few weeks (Gast, 2013).

Salmonella Enteritidis, especially phage type 4 (PT4), causes gastroenteritis in human (Chousalkar and Gole, 2016). Many disease outbreaks due to SE-infected eggs have been reported from countries such as US, UK and Germany. Recent studies in Iran have shown that SE is the predominant serotype in Iranian poultry flocks (Morshed and Peighambari, 2010; Akbarian et al., 2012; Taheri et al., 2016; Doulatyabi et al., 2017). Virulence and pathogenicity of the relevant isolates have not been studied so far. In this study, three Iranian SE isolates from our previous investigations were compared for their virulence in day-old chicks.

Materials and Methods

Bacteria and inoculum preparation

Three field SE isolates, designated as A20, S32 and S34, from our *Salmonella* isolates collection (Morshed and Peighambari, 2010) and one *S. Enteritidis* PT21 (Morshed and Peighambari, 2009) were used in this study. The bacterial cultures were stored in tryptic soy broth (TSB) with 25% glycerol at -70 °C. Prior to use, a small volume of the frozen stock of each bacterial strain was streaked on the surface of a MacConkey agar plate that was incubated overnight at 37 °C (Waltman et al., 1998). The next day, one colony of each bacterial growth was used to inoculate 5 ml brain-heart infusion (BHI) broth. The BHI cultures were incubated at 37 °C for 18 h with shaking, then the cells were harvested by centrifugation at 4000 x g at 4°C for 10 min, washed three times with PBS, and resuspended in PBS. One-ml samples were taken from each bacterial suspension for determination of the number of bacteria per milliliter (CFU/ml). Bacterial suspensions showed a concentration of 5×109 - 1010 CFU/ml. All media were from Merck, Germany.

Experiment

Fifty LSL female chicks at day one of age were obtained from a commercial layer breeder flock and transferred to the animal isolation facility of the Veterinary Medical Research and Teaching Hospital (VMRTH) of the University of Tehran. On the day of arrival, the chicks were randomly assigned to 5 experimental groups of 10 chicks and placed in 5 well-separated battery cages. One cage (control group) was kept in a separate room. All chicks were kept under routine conditions and had free access to feed and water. Feed samples from supplier breeder flock and meconium of chicks were examined to be free from *Salmonella* (Waltman et al., 1998). Feeds for experimental chicks were

obtained from a feed mill as pellet form and were also examined to be free from *Salmonella* (Waltman et al., 1998). All negative samples were run for delayed secondary enrichment test. The five experimental groups were assigned as 1 to 5. Group 1 (control) were not challenged but groups 2, 3, 4, and 5 were challenged with SE strains A20, S32, S34, and PT21, respectively, at day one of age.

For groups 2 to 5, a small catheter was used to inoculate each chick directly into the crop with 0.5 ml of assigned bacterial suspension. At days 2, 4, 6, and 9 post challenge (PC), two chicks from each of five groups were randomly selected, euthanized by cervical dislocation, necropsied, and sampled for bacterio- and histopathologic examinations. At day 14 PC (end of experiment), all remaining chicks in all groups were euthanized and treated in the same manner as previous ones. Liver, jejunum, and ceca of each necropsied chick were removed aseptically. Fecal content of jejunum and ceca was washed with sterile PBS and discarded. Then, parts of liver, jejunum, and ceca were cut and sent for histopathologic examination. The rest of liver and ceca belonging to two chicks were separately pooled and homogenized for serial dilution in PBS. A volume of 0.1 ml of each diluted sample was cultured on XLD agar to determine the number of colonized bacteria in the above-mentioned organs. Suspected colonies were examined using biochemical tests (triple sugar iron and urea) for *Salmonella* identification.

Results

Clinical observations

In this experiment, only one chick from group 4 (S34) died four days PC. All chicks in challenged groups showed mild to intermediate diarrhea from day 2 to day 9 PC. These chicks were not alert and conscious as much as chicks in control group during the experiment. Challenged chicks appeared to be healthy at day 14 (end of the experiment) but compared to those of control group, they had lower weight.

Bacteriologic examination

No bacteria were isolated from chicks in control group throughout the experiment (Table 1). Two days PC, the highest number of SE was found in liver of groups 3 (S32) and 5 (PT21) and in ceca of group 5 (PT21). At day 4 PC, load of SE in all liver samples decreased significantly. The number of bacteria in cecal samples was also reduced but not as much as that of in livers. Ceca of the chicks from group 3 (S32) showed more severe infection compared to that of in other groups. At day 6 PC, bacterial counts in both liver and cecal samples showed a reduction compared to those at day 4 PC. At day 9 PC, the reduction trend in bacterial count in all examined tissues was slowly continued. At day 14 PC, most of the liver and cecal samples also showed a decrease in bacterial counts. The reduction trend was especially noticeable in liver samples and it appeared that the livers will be cleared from SE faster.

Gross and histopathological examinations

At necropsy, no lesions were found in chicks of control groups but all chicks in challenged groups showed enteritis and pale foci on liver until 4 days PC. Some birds in groups 2, 3, and 4 were somehow emaciated 4 days PC but not at 6 days PC. Unabsorbed yolk sac and typhilitis were observed more or less in birds of challenged groups.

In histopathology, all samples prepared from control chicks were normal but samples from challenged birds demonstrated variable lesion scores (Tables 2, 3 and 4) during the

Table 1. Bacteriological results of liver and cecum colony counts after challenge.

Groups	Organ	Days Post Challenge				
		2	4	6	9	14
		Bacterial Concentration (CFU/ml)				
(1) Control		0	0	0	0	0
(2) A2O		2×10	6×10^2	1.38×10^3	10^3	10^7
(3) S32	Liver	2.1×10	5×10^2	6×10^3	7×10^3	2×10^8
(4) S34		1.3×10^3	1.2×10^3	4×10^2	6×10^3	2×10^7
(5) PT21		5.8×10^2	1.2×10^3	2×10^3	2×10^3	2×10^8
(1) Control		0	0	0	0	0
(2) A2O		1.73×10^4	2.1×10^5	2.2×10^6	2.1×10^7	2.2×10^8
(3) S32	Cecum	8.5×10^4	2×10^5	2×10^6	6×10^7	3×10^8
(4) S34		1.52×10^5	6.2×10^5	2.4×10^6	5×10^6	1.3×10^9
(5) PT21		4.3×10^4	3×10^5	4.1×10^6	9×10^6	8.7×10^9

experiment. Two days PC, liver samples of all challenged groups showed moderate to severe hyperemia in sinusoids and around central veins of sinusoids. Jejunal samples of challenged groups seemed to be normal but some heterophil infiltration and epithelial hyperplasia were found in some samples. All cecal samples from challenged-birds samples demonstrated inflammatory cell infiltration. In groups 3 (S32) and 5 (PT21), epithelial necrosis was obvious in cecal samples. At day 4 PC, livers of challenged birds were moderate to severe hyperemic together with increasing inflammatory cells in the figure of typhoid nodule. In liver samples of group 5 (PT21), bacteria were observed in abscess- es (liquification necrosis). In all jejunal and cecal samples, heterophil infiltration and epithelial necrosis were occurred. However, jejunal samples from group 4 (S34) appeared to be normal. At day 6 PC, liver samples of challenged birds were hyperemic with fibrotic tissue around the sinosuids. Jejunal and cecal samples also showed inflammatory cell infiltration with epithelial necrosis. Jejunal samples from group 5 (PT21) were normal but in other groups showed inflammatory cell infiltration together with epithelium and submucosal glands necrosis. At day 9 PC, liver and cecal samples did not show significant change in lesion score. Although, damage and necrosis of epithelial cells were decreased and crypts appeared to be more hyperplastic. At day 14 PC, majority of livers from challenged groups were normal but a few samples were involved with mild to moderate hyperemia. Jejunal samples were also normal but some showed hyperplasia. Necrosis and destruction of epithelium together with inflammatory cells infiltration were observed in the submocusa and lamina propria of all chicks in challenged groups. In general, lesion scores of liver, jejunum and cecum in histopathology, except in a few occasions, did not differ significantly ($P \leq 0.05$) among four *Salmonella* strains A20, S32, S34 and PT21 of this study.

Table 2. Liver lesions score. Differences in lowercase superscript letters in each column indicate statistical significance ($P \leq 0.05$). DPC=Days post challenge, 0= normal, 1= mild, 2= moderate, 3= intensive.

Group/ DPC	Score of liver parameters																								
	Typhoid nodules					Diffuse leukocyte infiltration					+ Fibrosis Biliary hyperplasia					Necrosis					Sinusoidal congestion				
	2	4	6	9	14	2	4	6	9	14	2	4	6	9	14	2	4	6	9	14	2	4	6	9	14
1 (Ctrl)	0	0.5	0[ac]	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0.5	0[b]	1
2 (A2O)	0	0	1.5	0.5	0	0	1.5	0.5	0.5	0.5	0	0	0	0.5	0	0	0	0	0	0	0.5	2	1.5	1	0.5
3 (S32)	0	1	0[a]	1	1	0	0.5	0	1	0	0	0	0	0.5	0	0	0.5	0	0	0	2.5	1.5	1	1.5[a]	2
4 (S34)	0	1	2.5[b]	1	0.5	0	0.5	1.5	0.5	0	0	0	1	0	0	0	0	0	0.5	0	0.5	2	1.5	1	0.5
5 (PT21)	0.5	0	0.5	0.5	0	0	2	1	0.5	0	0	0	0	0.5	0	0	1	0.5	0	0	2	0.5	1.5	1	0.5

Table 3. Jejunum lesions Score. Differences in lowercase superscript letters in each column indicate statistical significance ($P \leq 0.05$). DPC=Days post challenge, 0= normal, 1= mild, 2= moderate, 3= intensive.

Group/ DPC	Epithelial hyperplasia					Leukocyte infiltration to lamina propria					Epithelial necrosis					Glandular epithelial necrosis				
	2	4	6	9	14	2	4	6	9	14	2	4	6	9	14	2	4	6	9	14
1 (Ctrl)	0	0	0	0	0	0	0	1.5	0	0[b]	0	0	1.5	0	0	0	0	1.5	0	0
2 (A2O)	0	0	0	1	1	0.5	1	1.5	0	1.5[a]	0	0	1.5	0	0	0	0	1	0	0
3 (S32)	0.5	0	2	1	0.5	0	0.5	1	0	0[b]	0	0	0	0	0	0	0	0	0	0
4 (S34)	0	0	0	2	0	0	0	1	1	1	0	0	1.5	0.5	0	0	0	1	0	0
5 (PT21)	0	0	0	0	1	0.5	1.5	0	0.5	0[b]	0	1.5	0	0	0	0	1.5	0	0	0

Table 4. Cecum lesion score. Differences in lowercase superscript letters in each column indicate statistical significance ($P \leq 0.05$). DPC=Days post challenge, 0= normal, 1= mild, 2= moderate, 3= intensive.

Group/ DPC	Epithelial hyperplasia					leukocyte infiltration to lamina propria					Epithelial necrosis					Glandular epithelial necrosis				
	2	4	6	9	14	2	4	6	9	14	2	4	6	9	14	2	4	6	9	14
1 (Ctrl)	0	0	0	0	0	1.5	1.5	0[b]	1	0	0	0	0	0	0	0	0	0	0.5	0[c]
2 (A2O)	2	0	0	0	3	1.5	3	3[a]	3	1.5	0	1.5	1.5	1.5	0	0	1	2.5	1	0.5[ac]
3 (S32)	0	0.5	1	2	0	3	1.5	3[a]	3	1.5	0.5	0	0	1	1.5	0	0	2	0	3[b]
4 (S34)	0	0	1	0.5	0	0	3	3[a]	0	2.5	0	1	3	1.5	2.5	0	2.5	1	1.5	2[b]
5 (PT21)	0	0	1	0	1.5	1.5	1.5	2.5[a]	2	2	1.5	1.5	0	0	1.5	0	1.5	2.5	2	0[c]

Discussion

This study demonstrated the relative virulence of three Iranian field SE isolates (A20, S32, S34) and the known strain of SE PT21 for day-old layer chicks.

As it was expected, the peak of SE recovery was seen 48 h post challenge (PC) in all groups which is compatible with Bohez et al.'s (2006) trial results. In our study, day-old chicks infected with a high dose of wild type SE strain demonstrated an efficient initial colonization of the ceca 2 days post-challenge (PC). These results are in accordance with results of Morgan et al (2004), Bohez et al. (2006), Rychlik et al. (2014) and Barbosa et al. (2017) indicating the SE ability to colonize the ceca of chickens in the first days PC. In chickens, the ceca is the main colonization site for *Salmonella* (Desmidt et al., 1996; Rychlik et al., 2014; Moreau et al., 2016). Differences in virulence between SE isolates have also been reported (Gast, and Benson, 1995; Ben Salem et al., 2017). These researchers also reported that laying-type single comb white leghorns are more sensitive than broiler-type white plymouth rocks to the virulence of various phage types of SE. However, in another study, broiler chicks experimentally infected with the specific phage types experienced slightly higher mortality (Dhillon et al., 1999). Previous reports (Shivaprasad, 1990; Barrow, 1991; Gast, and Benson, 1995) indicate variation in the virulence between the isolates of different SE phage types.

Following ingestion, SE localize in the intestine and then enter the blood stream, giving rise to bacteremia that results in hepatitis, splenitis and omphalitis together with reduced body weight gains even though no mortality may be seen in chicks inoculated with SE as we observed in our study (Dhillon et al., 1999; Alisantosa et al., 2000). At the end of this study, chicks in control group (uninoculated) had approximately 15% more average body weight than those of challenged group. These findings were comparable with those of Dhillon et al (1999) on commercial broiler chicks. They concluded that subclinical infections following *Salmonella* outbreak in young broiler chicks or pullets are probably responsible for reduced body weight gains and lack of uniformity in broilers.

In the present study, we recovered SE from all samples. Gast and Holt (1998) reported that liver and spleen were usually cleared within 8 weeks after inoculation. Guillot et al (1995) and Barbosa et al (2017) indicated that there is a difference in the frequency of *Salmonella* colonization in the spleen and liver but not in the cecum. Asheg et al. (2001) showed the difference in the frequency of colonization in the cecum and liver in the low and high dose challenges. They reported rapid clearance of SE challenged groups (high and low doses), was observed at 28 DPC. Several researchers have shown the rapid elimination of *Salmonella* in birds infected experimentally (Humphrey et al., 1989; Timoney et al., 1989; Barbosa et al., 2017). In contrast, SE was isolated from the visceral organs of naturally infected birds several months after presumed outbreak (Chart et al., 1990; Rychlik et al., 2014; Kogut et al., 2016). In this study, all cecum samples showed large decline in bacterial cecal count from 9 DPC to 14 DPC. The susceptibility of chicks to persistent intestinal colonization and organ invasion by *Salmonella* decreases sharply during the 1st week after hatch (Gast and Beard, 1990; Gorham et al., 1991; Foley et al., 2013). In the present study, all liver samples also demonstrated a gradual de-

crease in count from 2 days to 14 DPC.

Some researchers have reported that colonization of organs with *Salmonella* might be merely dependent on host factors (Brownell et al., 1970; Fanelli et al., 1971; Barrow et al., 1988). Also, Asheg et al. (2003) and Upadhyaya et al. (2013) showed that ability of SE to adhere and colonize to the intestinal tract can be dose dependent. Differences in lesions might be due to differences in experimental design or differences between strains or SE phage types (Poppe et al., 1993).

As it has been demonstrated in Table 1, considerable decrease in colony count in all groups for liver and cecal samples are seen. Chicks hatch with immature T lymphocytes and become fully responsive when the chicks are around 4 days old. Therefore, it is likely that cellular immunity and mucosal responses in the gut would develop significantly only after approximately 4 days of age, which would leave the chick relatively unprotected for the first few days of its life (Milanez et al., 2018).

Knowing phage types of the trial isolates cannot help us to determine its invasiveness. Several researchers reported that the difference in virulence of the two SE PT4 strains in their test, showed that phage type alone is not the most important criterion for virulence (Poppe et al., 1993; Raspoet et al., 2014; and Bertelloni et al., 2017). Investigations on SE with different phage types have also noted substantial differences in virulence among those strains when inoculated to young chickens or adult hens by oral route (Humphery et al., 1989; Timoney et al., 1989; Gast and Beard, 1990).

Histopathologic lesions due to *Salmonella* infection have been reported in various investigations (Desmidt et al., 1996; Alisantosa et al., 2000; Rychlik et al., 2014; Barbosa et al., 2017). In the present experimental study, samples from challenged birds showed histopathologic lesions in different visceral organs with variable lesion scores. Liver samples were hyperemic together with increasing inflammatory cells in the figure of typhoid nodule. Liver lesions in this study were compatible with those of Desmidt et al. (1996) that reported the presence of small foci of inflammatory cells in the liver from 4 days post infection. In our study, diffuse leukocyte infiltration, fibrosis, biliary hyperplasia, necrosis and sinusoidal congestion were observed in liver samples with various severity. Similar liver lesions were described by Alisantosa et al. (2000) including acute multifocal necrosis of hepatocytes with infiltration of heterophils. Barbosa et al. (2017) also noticed the presence of numerous small pale foci (necrotic foci) in the liver after SE infection of chicks. In the present study, histopathologic examination of jejunum and cecum also demonstrated epithelial hyperplasia, leukocyte infiltration to lamina propria, epithelial necrosis and glandular epithelial necrosis at various degrees. Other investigations have found compatible findings. Rychlik et al. (2014) reported thickened appearance (hyperplasia) of ceca associated with an influx of leukocytes. Alisantosa et al. (2000) reported increased cellularity of the lamina propria of the ceca due to infiltration of heterophils and lymphocytes in the mucosa. Desmidt et al. (1996) observed many heterophils infiltrating in the lamina propria and emigrating between epithelial cells. They reported granulomatous nodules in lamina propria of cecum that were similar to glandular epithelial necrosis of our study. Diffuse and moderate lymphocyte infiltration in cecal lamina propria observed in this study, have also been reported by Barbosa et al. (2017).

In conclusion, the virulence of four *Salmonella* strains A20, S32, S34 and PT21 used in this experimental study was shown in day-old chick model. All strains were able to produce gross and histopathologic lesions in challenged chicks; although, except for in a few occasions, the virulence of strains did not differ significantly. Further studies are required to compare the virulence of strains in adult layer chickens..

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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تعیین حدت سه جدایه سالمونلا انتریتیدیس ایرانی در جوجه های تخمگذار یک روزه

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چکیده

زمینه مطالعه: عفونت با سالمونلا انتریتیدیس یکی از مهمترین معضلات کنونی صنعت طیور در دنیاست. حدت و پاتوژنیسیته جدایه های ایران تاکنون مورد بررسی دقیق واقع نشدهاند.

هدف: در مقاله پیش رو حدت و بیماریزایی سه جدایه سالمونلا انتریتیدیس ایران با سویه استاندارد PT۲۱ در جوجههای یکروزه تخمگذار مورد مطالعه قرار گرفتند. در تمامی سه جدایه مورد مطالعه بدلیل حضور پلاسمید بزرگ مسبب حدت، پیش فرض چنین بود که همگی آنها حدت و بیماریزایی بالایی برای جوجهها داشته باشند.

روش کار: پنجاه عدد جوجه یکروزه تخمگذار (نژاد LSL) به پنج گروه دهتایی تقسیم شده و هر گروه در قفسهای جداگانه تا سن ۱۴ روزگی نگهداری شدند. هر سه جدایه سالمونلا انتریتیدیس ابتدائا" در محیط آبگوشت BHI کشت داده شدند تا مایع مورد استفاده جهت چالش در هر سه گروه به غلظت ۱۰^۱۰ CFU در هر میلی لیتر برسد. گروههای چالش عبارت بودند از سه گروهی که با جدایههای ایران تلقیح شدند (با نامهای S۳۲, A۲۰ و S۳۴) و یک گروه هم با سویه استاندارد PT۲۱ بعنوان گروه کنترل مثبت مورد تلقیح واقع شد. گروه آخر نیز بعنوان کنترل منفی درنظر گرفته شده و هیچگونه چالشی را تجربه ننمود. هر گونه تلفات و مشاهدات ناخوشی در کلیه گروهها ثبت گردید. نمونه های بافتی از کبد، ژژنوم و سکوم در روزهای ۲، ۴، ۶، ۹ و۱۴ روزگی جوجههای فوقالذکر برداشته شده و جهت جداسازی سالمونلا انتریتیدیس، شمارش کلنی و هیستوپاتولوژی مورد بررسی واقع گردیدند.

نتایج: تمامی گروههایی که مورد چالش واقع شده بودند، کلیه پرنگان دچار اسهال خفیف تا متوسط شدند، بعضی از آنها نیز خصوصا در هفته اول دچار بیحالی و بی رمقی بودند. در گروه کنترل منفی هیچگونه علایم خاصی دیده نشد. دو عدد تلفات در گروههای چالش بوجود آمد. در تمامی گروههای چالشی، سالمونلا انتریتیدیس تا آخر دوره جدا شد. در آزمایش شمارش کلنی بافتها، باکتری جدا شده از نمونههای کبدی حدود ۱۰۰ تا ۱۰۰۰ برابر کمتر از نمونههای سکومی بود. نتایج آزمایشات هیستوپاتولوژی با علایم بالینی و یافتههای باکتریولوژیک تطابق داشتند.

نتیجه گیری نهایی: پس از بررسی نتایج بدست آمده از آزمایشات مزبور چنین نتیجهگیری میشود که تمامی سه جدایه مذکور با حدتی که از خود نشان دادند قابلیت کلونیزه شدن در داخل دستگاه گوارش جوجههای تخمگذار را داشته و نهایتا" موجبات تلف شدن یا حداقل عملکرد ضعیف پرنده را متعاقب عفونت در مقایسه با پرندگان سالم را در پی خواهند داشت.

واژههای کلیدی:

سالمونلا انتریتیدیس، چالش، مرغان، حدت، تخمگذار

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