Research article

Molecular characterization, antimicrobial sensitivity testing of *Salmonella* Enteritidis and its immune compromising effect on Newcastle disease vaccinated broilers

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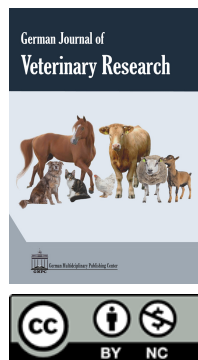
Abstract

Salmonellosis is an important zoonotic disease of chickens that poses a serious threat to the poultry industry in developing countries, especially in Egypt. This study aimed to isolate and identify *Salmonella* (*S.*) from commercial broiler chickens, in addition to serological and molecular evaluation of the immunocompromising effect of *S.* Enteritidis. Out of 246 samples collected from broiler chicken farms, the prevalence of *Salmonella* was 4.1% (8/192), 6.4% (2/31), (0/7), (0/7), (0/5), and (0/4) in the cecum, liver, gallbladder, air sacs, spleen, and pericardium samples, respectively. Those ten isolates were obtained from 88 broiler flocks, representing 11.36% (10/88), and all were characterized as *S.* Enteritidis (1,9,12:g,m). Their *in-vitro* antimicrobial susceptibility testing revealed high resistance (100%) to amoxicillin-clavulanic acid, streptomycin, erythromycin, clindamycin, doxycycline, fosfomycin, and sulfamethoxazole-trimethoprim. All *S.* Enteritidis isolates carried three virulence genes, i.e., *invA*, *sefA*, and *fimH*, while *stn* was detected in 7 isolates. Additionally, all isolates harbored Extended-spectrum β-lactamases (ESBL)-producing (bla_{TEM}) and sul1 antimicrobial resistance genes. Following experimental infection with an *S.* Enteritidis isolate in broilers at seven days old and routine vaccinations using an inactivated and live LaSota, Newcastle vaccines, severe immunocompromising effects were observed in terms of antibody response to vaccination, as well as several immune mediators such as nitric oxide, lysozyme, IFN-γ, IL-6, IL-8, and IL-10 as well as phagocytic count. In conclusion, *S.* Enteritidis carrying several virulence and antimicrobial resistance genes was characterized from cecal and liver samples collected from different broiler flocks. Infection with *S.* Enteritidis induced a prolonged inflammatory response and negatively affected the broilers' immune response to Newcastle disease vaccination.

Keywords: *Salmonella* Enteritidis, Antimicrobial, Immunocompromising, Broiler chickens

Introduction

Chickens are a major source of *Salmonella* infection in humans, causing food poisoning due to inadequate cooking of poultry products (Duc et al., 2019). Chickens could be infected with *Salmonella* serovars through horizontal or vertical routes, colonizing the distal portion of the ileum. The gut-associated lymphoid tissues (GALT) and mucosa-associated lymphoid tissues (MALT) are lymphoid aggregations located along the intestinal tract that are initiators of innate immune response in chickens after *Salmonella* infection through stimulation of pro-inflammatory chemokines (Berndt et al., 2007; Ijaz et al., 2021).

Macrophages play a crucial role in the first line of defense against bacterial infection by detecting, engulfing, and producing microbicidal substances, such as nitric oxide (NO) and lysozyme, to kill the microbial agents (phagocytosis) (He et al., 2013). Previous studies have investigated NO production by mononuclear cells (MNC) and heterophils in Newcastle disease-infected chickens (Ahmed et al., 2007; Tulu, 2020). Cytokines are messengers that are important to the immune response (Wigley and Kaiser, 2003). Interestingly, interleukins are a group of cytokines, and their actions are summarized as chemical signals between leukocytic cells. Interleukin (IL)-6 is the first cytokine to respond

to infection with *Salmonella* enterica serovars in chickens and stimulates the production of mannan-binding lectin by liver cells (Kaiser et al., 2000; Pineda et al., 2021). IL-8 is a chemokine that can mobilize cellular immunity (Borrmann et al., 2007). IL-10 is an anti-inflammatory cytokine produced by monocytic and lymphocytic cells that has an immunoregulatory effect (Flynn and Chan, 2001). Interferon(IFN)-γ is a cytokine with heterogeneous functions in all stages of immune and inflammatory responses (Kogut et al., 2005).

The live attenuated *Salmonella* vaccine not only can trigger the body's immune system against *Salmonella* infection, but it also significantly enhances the immune effect of the exogenous gene carried. It was reported that secretion of The hemagglutinin-neuraminidase (HN) protein of Newcastle virus from its recombinant attenuated *S.* Pullorum strain C79-13ΔcrpΔasd (pYA-HN) could provide effective protection against the challenge of *S.* Pullorum and NDV (Ding et al., 2018). The invasive Salmonellae penetrate the mucosal surface, induce local accumulation of heterophils, and then direct the activated heterophils to the site of invasion for phagocytosis (Kogut et al., 1994, 1995).

This study was focused on the isolation, molecular, and sero-

logical characterization of Salmonellae from commercial broilers and the evaluation of the impact of experimental *S*. Enteritidis infection on immune mediators and Newcastle disease vaccination in broilers.

Materials and Methods
Ethical statement
Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Beni-Suef University (BSU-IACUC), Egypt. Every effort was made to minimize the suffering of the chickens. The ethical approval number was BSU-IACUC-022-246.

Sampling
Eighty-eight diseased broiler chicken flocks (capacity = 12000-15000 birds), aged 2-5 weeks, were examined in Beni-Suief and El-Fayoum governorates from October 2022 to January 2023. A total of 246 samples [cecum (n=192), liver (n=31), gallbladder (n=7), air sacs (n=7), spleen (n=5), and pericardium (n=4)] were collected under complete hygienic conditions from 192 sick slaughtered and freshly dead chickens, 2-3 per each flock. These chickens were subjected to clinical and post-mortem examinations at the Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Beni-Suief University, Egypt. The broiler chickens examined suffered from septicemia, enteritis, and cecal core with congested liver with grayish-white necrotic foci and congested spleen, with and without pericarditis.

Bacteriological isolation
Isolation and phenotypic identification of *Salmonella* were performed as described in Collee et al. (1996). For pre-enrichment, 1 g of samples was inoculated into 10 mL buffered peptone water and then incubated aerobically at 37°C for 18-24 h. For selective enrichment, 0.1 mL of the pre-enriched culture was aseptically transferred to 10 mL of Rappaport-Vassiliadis (RV) *Salmonella* enrichment broth and incubated at 41.5°C for 24 hours. A loop of inoculum from RV broth tubes was transferred and plated separately on MacConkey, *Salmonella-Shigella* (SS), and xylose lysine deoxycholate (XLD) selective agar media (Oxoid, Hampshire, UK). Plates were incubated at 37°C for 18-24 hours.

Morphological and biochemical identification of *Salmonella* isolates
All suspected *Salmonella* isolates were stained with Gram stain. They were then identified biochemically according to Koneman (1992); Collee et al. (1996); Quinn et al. (2011) using oxidase, catalase, urease, lysine decarboxylase, triple sugar iron (TSI) agar (Oxoid, Hampshire, UK), and and IMVIC biochemical tests (IMViC) biochemical tests.

Serological identification of *Salmonella* isolates
Serotyping of *Salmonella* isolates was performed by the Kauffmann-White complete plate agglutination test (Kauffmann, 1974) at the Serology Unit, Animal Health Research Institute, Dokki, Giza, according to Grimont and Weill (2007). *Salmonella* isolates initially identified biochemically were tested for the determination of O (somatic antigen) and H (flagellar antigen).

Molecular detection of virulence and resistance genes in *Salmonella* Enteritidis
DNA from 10 *Salmonella* Enteritidis isolates was extracted from overnight tryptone soya broth cultures using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification of *Salmonella* isolates using specific primers (Table 1) was performed using 25 μL volume/reaction mixture including 12.5 μL Emerald Amp GT PCR master mix (2× premix) (Takara, Japan), 5.5 μL PCR grade water, 1 μL forward primer (20 pmol), 1 μL reverse primer (20 pmol), and 5 μL template DNA. The cycling conditions of the PCR protocol were 35 cycles for each: primary denaturation for 5min at 94°C, secondary denaturation for 30 sec at 94°C, annealing for 30-40 sec at 52-60°C (depending on the primer), extension for 30 sec at 72°C and final extension for 7min at 72°C. The PCR products were separated by electrophoresis on 1.5% gel and photographed using a gel documentation system.

Experimental infection
Sixty one-day-old commercial broiler chicks (Cobb-500) were obtained from a local commercial hatchery. After receiving the chicks, 10 pooled cloacal swabs (1 swab/6 chicks) were collected and tested for Salmonellae by conventional polymerase chain reaction (cPCR) using primers targeting *invA* gene (following the protocol mentioned above). All chicks were reared on the floor and were provided with commercial antibiotic-free pelleted feed and clean fresh water *ad libitum*. At seven days of age, the chicks were divided into three equal groups (20 chicks in each). The first group (G1) was non-*Salmonella* infected and housed in a separate room. The second group (G2) was experimentally infected orally with *S*. Enteritidis using 0.5 mL of McFarland 0.5 (1.5×10^8 colony forming unit (CFU)/mL) (Fernández et al., 2001), while the third group (G3) was kept in contact with G2 in the same room one day after*S*. Enteritidis infection. All groups of chickens received the following vaccination program: subcutaneous injection of H5 plus ND inactivated vaccine (Volvac B.E.S.T., Boehringer Ingelheim, Germany) at 10 days old and LaSota live vaccine (Boehringer Ingelheim, Germany) at 18 days of age via the drinking water.

Post-infection observation and sampling
Chicks were observed daily for clinical signs and performance and morbidity and mortality for 18 days post-infection, dpi (7 days post-vaccination with LaSota), or 25 days old.

Bacterial re-isolation
For bacterial re-isolation, cloacal swabs and cecal samples from the five slaughtered chickens in each group on 11 and 18 dpi, pre-enriched in RV broth, incubated at 41°C for 18h, plated on XLD and SS *Salmonella*-selective agar medium and confirmed by biochemical and serological identification using the methods described above (Collee et al., 1996; Quinn et al., 2011).

Immunological studies
Haemagglutination inhibition (HI) test
The HI test was performed on serum samples collected from 5 chickens in each group at 11 dpi and 18 dpi to detect the humoral antibodies against NDV. According to WOAH, the HI test was performed in microtitration using a standard LaSota antigen MEVAC, Cairo, Egypt (WOAH, 2021).

Detection of immune mediators phagocytosis assay, lysozyme, and nitric oxide
Phagocytosis assays using CytoSelectTM, 96-Well with red blood cell substrate (Cell Biolabs Inc., San Diego, CA, USA), and lysozyme and NO levels using colorimetric enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Coon Koon Biotech. Co. Ltd., Shanghai, China) were detected in the collected five serum samples on 11 and 18 dpi according to (Sheela et al., 2003; Yu et al., 2015).

Detection of cytokines using quantitative real-time RT-PCR (qRT-PCR)
Five spleen samples (30mg each) collected at 11 and 18 dpi were individually homogenized with the addition of 1 mL Trizol. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The SYBER green real-time PCR reaction volume was 25 μL, consisting of 2x QuantiTect SYBR Green PCR Master Mix (12.5 μL) (Qiagen, Hilden, Germany), reverse transcriptase enzyme (0.25 μL), forward primer (20 pmol) (0.5μL), reverse primer (20 pmol) (0.5 μL), RNase free water (8.25μl), and template RNA (3 μL). The expression levels of IL-6, IL-8, IL-10, and IFN-γ in the purified RNA samples were determined by quantitative real-time RT-PCR. All primers, probes, and cycling conditions for SYBR green real-time PCR are listed in Table 2. Amplification curves and Ct values were determined by the strata gene MX3005P software. To estimate the gene expression variation on the RNA of the different samples, the Ct of each sample was compared with that of the control group according to the "ΔΔCt" method stated by Yuan et al. (2006) in Table 3, using the following ratio: 2-ΔΔCt, whereas ΔCt = ΔCt reference –ΔCt target, ΔCt target = Ct control – Ct treatment, ΔCt reference = Ct control- Ct treatment and E= efficiency of amplification.

Table 1: Oligonucleotide primer sequences for virulence and resistance genes used in this study.

Gene	Sequence (5´-3´)	Amplified product	Reference
$invA$	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284 bp	Oliveira et al. (2003)
$sefA$	GCAGCGGTTACTATTGCAGC TGTGACAGGGACATTTAGCG	310 bp	Akbarmehr et al. (2010)
$fimH$	TGTGACAGGGACATTTAGCG GTGCCAATTCCTCTTACCGTT	164 bp	Hojati et al. (2015)
stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617 bp	Murugkar et al. (2003)
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	Colom et al. (2003)
$sul1$	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433 bp	Ibekwe et al. (2011)

Table 2: Oligonucleotide primer sequences of qRT-PCR for detection of cytokines.

Gene	Primer sequence (5´-3´)	Reference
IL-6	GCGAGAACAGCATGGAGATG GTAGGTCTGAAAGGCGAACAG	Jiang et al. (2011)
IL-8	CTGCGGTGCCAGTGCATTAG AGCACACCTCTCTTCCATCC	Kano et al. (2009)
IL-10	CGGGAGCTGAGGGTGAA GTGAAGAAGCGGGTGACAGC	Hong et al. (2006)
IFN-γ	CAAGTCAAAGCCGCACATC CGCTGGATTCTCAAGTCGTT	Adams et al. (2009)

Detection of NDV genotype II using qRT-PCR

The matrix (M) gene primer probe was used for NDV detection to confirm the presence of the LaSota vaccine strain in birds at 7 dpi (25 days old), according to Wise et al. (2004).

Statistical analysis

All data were statistically analyzed using SPSS (IBM SPSS. 20®) utilizing the one-way ANOVA followed by Tukey's multiple range tests. The data of HI assay, phagocytic cells, lysozyme, and nitric oxide in serum samples, and the pro- and anti-inflammatory cytokine profile in spleen samples in chickens of the three groups were analyzed by One-way ANOVA, Tukey's multiple range tests by GraphPad prism 5. The significance was estimated at p values, p ≤0.05.

Results
Prevalence and identification of *Salmonella* isolated from broilers

Ten isolates were recovered from 88 broiler flocks examined, each isolate from each herd, with an overall incidence of 11.36%. They were correctly identified as Salmonellae based on growth on MacConkey (pale colonies), SS (colorless colonies with a black center), and XLD (red colonies with a black center) agars. Suspected colonies were examined microscopically using Gram stain technique and showed Gram-negative medium size and nonsporulated bacilli, then identified biochemically as positive for catalase, lysine decarboxylase, TSI (red slant and yellow butt with H_2S production), methyl red, citrate utilization and negative for oxidase, indole, urease, and Voges-Proskauer. The prevalence of *Salmonella* in the examined internal organ samples was resembling 10/246=4%, detailed as 8/192 (4.1%), 2/31 (6.4%), 0/7, 0/7, 0/5, and 0/4 in the cecum, liver, gallbladder, air sacs, spleen, and pericardium, respectively. Biochemical tests confirmed the serotype of the 10 *Salmonella* isolates as *S.* Enteritidis 1,9,12:g,m.

Antimicrobial susceptibility of *Salmonella* isolates

In-vitro antimicrobial susceptibility testing revealed that the isolated *S.* Enteritidis were highly resistant to amoxicillin-clavulanic acid, streptomycin, erythromycin, clindamycin, doxycycline, fosfomycin, and sulfamethoxazole-trimethoprim with an incidence (100%), followed by 90%, 80%, 70%, and 70% for ofloxacin, apramycin, ceftriaxone, and gentamycin, respectively. At the same time, the isolates were sensitive to amikacin (90%), followed by ciprofloxacin (70%), as shown in Table 4.

Detection of virulence and antimicrobial resistance genes

All *S.* Enteritidis isolates were screened by PCR for four virulence genes: $invA$, $sefA$ $fimH$, and stn. *S.* Enteritidis isolates were found to be positive for the screened virulence genes with an incidence of 100% each for $invA$, $sefA$, and $fimH$, followed by stn (70%) arranged as 8/10 and 1/2 in isolates from cecum and liver, respectively. The isolates were screened for two antimicrobial resistance genes: the ESBL-producing gene (bla_{TEM}) and the $sul1$ gene, with a prevalence of 100% for each.

Post-infection observation and re-isolation from all broiler groups after experimental infection with *S.* Enteritidis

Clinical signs in infected chicks were limited to mild depression, diarrhea, and ruffled feathers; the two chickens that died during the experiment had septicemia, cecal cores, and congested liver and spleen. This study obtained no bacterial re-isolation from all groups at 11 and 18 dpi.
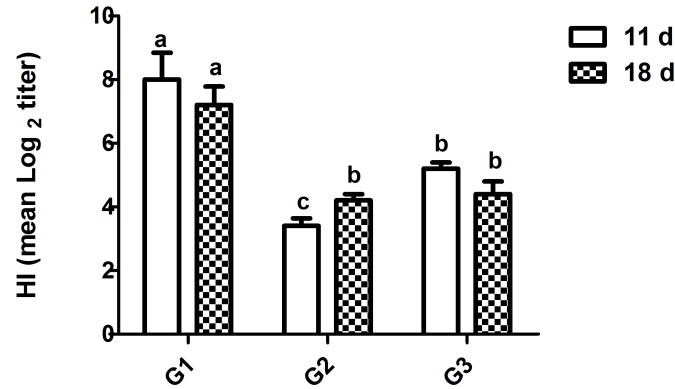
Haemagglutination-inhibition test

The antibody titers of NDV detected by the HI test are summarized in Figure 1. In general, the highest mean antibody level was detected in G1 (control group) at 8 dpi and 7.2 $\log_2$, followed by 5.2 and 4.4 $\log_2$ in G3, while the lowest mean antibody level was detected in G2 at 3.4 and 4.2 $\log_2$ at 11 dpi and 18 dpi, respectively.

Immune mediators in broiler groups

Significantly higher levels of phagocytic cells, lysozyme, and nitric oxide were detected in samples from G2, followed by G3 and G1. At 11 dpi, the titers of the three parameters were 5.6 $\log_{10}$, 7.94, and 6.66 ELISA titer units, respectively. On the 18 dpi, the titers for the three parameters were 4.6, 7.14, and 5.94 $\log_{10}$ ELISA titer units, respectively, in G2. All results are summarized in Figure 2.

Table 3: Cycling conditions for SYBR green real-time PCR used to estimate the gene expression variation.

Genes	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
			Primary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
IL-6 IL-8 IL-10 IFN-γ	50°C 30 min.	94°C 15 min.	94°C 15 sec.	62°C 30 sec.	72°C 30 sec.	94°C 1 min.	62°C 1 min.	94°C 1 min.

Figure 1: Hemagglutination-inhibition (HI) antibody titers for Newcastle disease virus in all broiler chicken groups at 11 and 18 days post-infection. G1: non-*Salmonella* infected. G2: *S.* Enteritidis experimentally infected group using 0.5 mL of 1.5×10^8 CFU/mL. G3: contact group kept with G2 in the same room one day after *S.* Enteritidis infection. Different letters above the bars indicate statistically significant differences at P≤0.05.
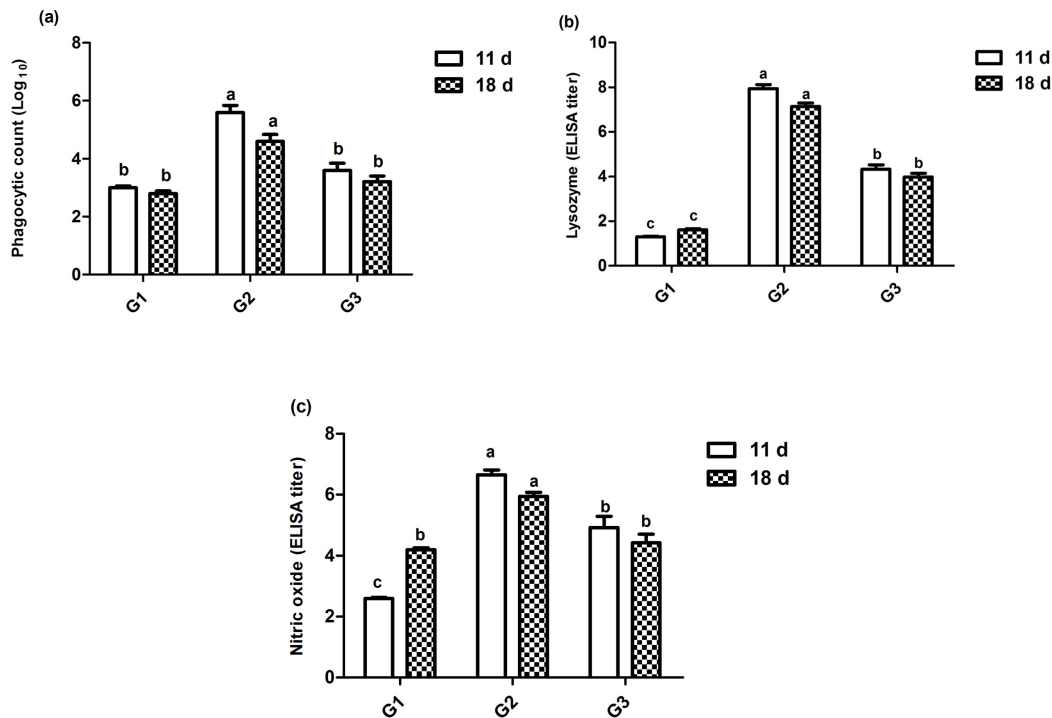
Figure 2: Levels of phagocytic cells, lysozyme, and nitric oxide in serum samples of experimentally infected broilers at 11 and 18 days post-infection. G1: non-*Salmonella* infected. G2: *S.* Enteritidis experimentally infected group using 0.5 mL of 1.5×10^8 CFU/mL. G3: contact group kept with G2 in the same room one day after *S.* Enteritidis infection. Different letters above the bars indicate statistically significant differences at P≤0.05.

Inflammatory cytokines in broiler groups

The results showed a significant upregulation of pro-inflammatory cytokines (IL-6 and IL-8) and IFN-γ on 11 and 18 dpi in G2, followed by G3, compared to G1 with higher signifi-cance at 11 dpi (these results were indicated by lower Ct values). For the anti-inflammatory cytokine, IL-10, a significant increase occurred in G2 birds at 11 dpi (Ct: 32.2) compared to G1 (34.61) and G3 (33.9), which increased significantly in both G2 (17.69)
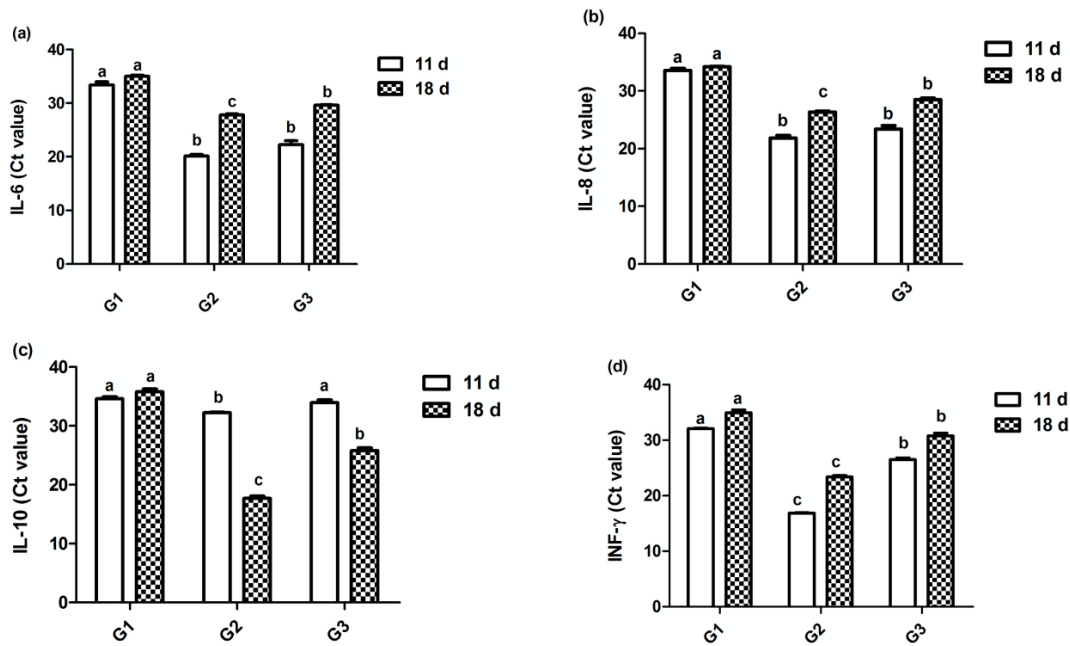
Figure 3: The pro- and anti-inflammatory cytokine profile in all broiler chicken groups at 11 and 18 days post-infection. G1: non-*Salmonella* infected. G2: *S.* Enteritidis experimentally infected group using 0.5 mL of 1.5×10^8 CFU/mL. G3: contact group kept with G2 in the same room one day after *S.* Enteritidis infection. Different letters above the bars indicate statistically significant differences at P≤0.05.

Table 4: Antimicrobial results of 10 *S.* Enteritidis isolates.

Antimicrobial agent	*Salmonella* isolates			
	Resistance		Susceptible	
	No.	%	No.	%
Amoxicillin-Clavulanic acid (AMC)	10	100	0	0
Ceftriaxone (CTR)	7	70	3	30
Amikacin (AK)	1	10	9	90
Apramycin (APR)	8	80	2	20
Gentamycin (Gen)	7	70	3	30
Streptomycin (S)	10	100	0	0
Ciprofloxacin (Cip)	3	30	7	70
Ofloxacin (OF)	9	90	1	10
Erythromycin (E)	10	100	0	0
Clindamycin (DA)	10	100	0	0
Doxycycline (Do)	10	100	0	0
Colistin sulfate (CL)	5	50	5	50
Fosfomycin (Fo)	10	100	0	0
Sulfamethoxazole- Trimethoprim (sxt)	10	100	0	0

and G3 (25.8) compared to G1 (35.8) at 18 dpi (Figure 3).

Detection of NDV genotype II using qRT-PCR

The M gene detection of NDV-II was detected only in the tracheal G1, G2, and G3 swabs examined birds at 18 dpi with Ct values of 33.5, 28.8, and 31.4, respectively.

Discussion

Although Salmonellosis is an old problem, it is very interesting and worth pursuing. *Salmonella* is the main cause of worldwide economic and public health losses in developing countries due to its relevant impact on the poultry industry and humans (Abd El-Mohsen et al., 2022). In the elderly and immunocompromised individuals, *S.* infection is a significant health problem that threatens human life and requires antibiotic therapy for control (Caffrey et al., 2021). *Salmonella* Enteritidis is the major cause of human illness due to egg contamination (Ishola, 2010).

In the current study, out of 88 broiler farms tested for *Salmonella*, 10 were positive (11.36%), corresponding to 10/246 (4%) of the samples tested, including 4.1% (8/192) from cecum and 6.4% (2/31) from the liver. Radwan et al. (2022) recovered *S.* Enteritidis with a prevalence of 3.3% from sick broilers.

In contrast, our result was inconsistent with the results obtained by Abd El-Mohsen et al. (2022), who isolated *S.* Enteritidis from diseased and freshly dead chickens with an incidence of 22.22% in Assiut, Egypt. Furthermore, Akeila et al. (2013) reported that 18.5% of broilers were carriers of *S.* Enteritidis. However, a lower infection rate of 2.4% *S.* Enteritidis among the *Salmonella* tested was reported by Sedeik et al. (2019).

Salmonella isolates were serotyped according to the Kauffman-White scheme using a complete plate agglutination test, and *S.* Enteritidis 1,9,12:g,m was the predominant serotype in all isolates tested. This result agreed with Awad et al. (2020), who reported *S.* Enteritidis 1,9,12:g,m in chicken samples. The most isolated serotype from different organs of newly hatched chicks was *S.* Enteritidis 1,9,12:g.m 1,7 (Sedeik et al., 2019). The virulence of *Salmonella* serotypes is related to the sequences of genes responsible for invasion, colonization, and dissemination within the susceptible host (Awad et al., 2020). In the current study, the isolates harbored the virulence-associated genes with a percentage of 100% each for *invA*, *sefA*, and *fimH*, followed by *stn* (70%). It has been suggested that the *invA* primer is specific and a marker for molecular detection of *Salmonella* serotypes using PCR assay, while the *stn* gene is responsible for the produc-

tion of enterotoxin (Malorny et al., 2003; Ahmed et al., 2016). According to the resistance genes, 100% of all isolates carried the ESBL-producing gene (bla_{TEM}) and $sul1$ gene, similar to the previously reported prevalence of 97.3% and 97.8%, respectively (Zhu et al., 2017).

Treatment with antimicrobial agents is the first choice for bacterial diseases, but their overuse in both humans and animals has led to the emergence of antimicrobial resistance, which has become a triggering issue for the One-Health approach. *Salmonella* can acquire resistance to antimicrobials by protecting their target site or by mutating target genes such as (topoisomerase IV and DNA gyrase). Resistance can be acquired through the over expression of efflux pumps; Salmonellae have the ability to pump out the antibiotic after it enters into the cell or through other mechanisms that contribute to resistance, including quorum sensing and biofilm formation (Martins et al., 2011; Castro-Vargas et al., 2020). In the current study, the antibiogram results of *S.* Enteritidis isolates showed the highest resistance to the antimicrobials amoxicillin/clavulanic acid, sulfa/trimethoprim, streptomycin, erythromycin, doxycycline, and fosfomycin. On the other hand, they were highly susceptible to amikacin and ciprofloxacin. This finding agreed with Abed et al. (2020), who reported higher resistance of *Salmonella* isolates to trimethoprim-sulphamethoxazole and streptomycin. Higher antimicrobial resistance to erythromycin, sulfamethoxazole-trimethoprim, streptomycin, and doxycycline was reported by Awad et al. (2020). Susceptibility to amikacin of 75% was reported by (Radwan et al., 2022).

NDV is a highly contagious disease in chickens that causes economic losses in the poultry sector (Kumar et al., 2011). In the present study, oral inoculation of *S.* Enteritidis 1,9,12:g,m in the broilers resulted in mild clinical signs at 18 dpi. There was no re-isolation of *S.* Enteritidis in the cloaca or ceca at 11 and 18 dpi. The use of a lower dose of infection (0.5 ml McFarland 0.5, 1.5×10^8 CFU/mL) and the delayed timing of re-isolation could be the cause of the nil cloacal shedding, which should be highly positive during the first-week post-infection as several previous reports (Berndt et al., 2007; Attia et al., 2012), but here we aimed to test the shedding for a longer period to see if the hidden effect of infection on immunity was correlated with bacterial shedding or not. This hidden effect of immunocompromising *Salmonella* was evident, particularly HI antibody titers. HI is one of the most commonly used methods to assess the immune response induced by ND vaccines, as it directly corresponds to the level of protection. Therefore, it could be a strong indicator of the immune response of birds, especially those exposed to *Salmonella* infection (Capua and Alexander, 2009; Yang et al., 2017). Here, HI titers were significantly higher in G1 chickens (non-*Salmonella* infected and ND vaccinated) at 11 and 18 dpi as 8 and 7.2 log$_2$, respectively, while significantly lower titers were recorded in G2 and G3, indicating the very weak immune response due to *Salmonella* infection.

Avian macrophages produce a significant amount of NO in response to *Salmonella* serotypes, and bacteria killing is not proportional to the level of NO produced (Balan and Babu, 2017). This was evident in our results, as significantly higher levels of phagocytic cells, lysozyme, and NO were detected in G2, followed by G3 compared to the control G1, which persisted until 18 dpi. The increase in macrophages' expression of lysosomal and phagosomal proteins after infection with *S.* Enteritidis can be attributed to the inactivation of *S.* Enteritidis and the ability of macrophages to present antigens (Sekelova et al., 2017).

Cytokines also play an important role in regulating the immune system by determining the type and magnitude of the immune response produced following pathogenic infection or vaccination. Depending on the combination of cytokines produced, a protective immune response can be generated as either humoral immunity (Th2) or cellular immunity (Th1) (Lowenthal, 2000). Acquired immunity in birds is dependent on CD4+ T cells, and IFN-γ is produced after specific recognition of antigen with the effector T cell receptor (TCR); IFN-γ produced by T helper 1 (Th1) cells plays a crucial role in the activation of cellular immunity and is important in the surveillance of infection (Harrington et al., 2008; Kaiser and Stäheli, 2014). qRT-PCR results in this study showed upregulation of IFN-γ, IL-6, and IL-8 in the spleen of G2 birds at 11 dpi. The expression of these interleukins

may be attributed to the ability of chicks at 11 dpi to control primary infection with *S.* Enteritidis through increased antigen presentation and pro-inflammatory response (Penha Filho et al., 2012). This was followed by a significant upregulation of the anti-inflammatory cytokine IL-10 in G2 at 18 dpi.

Matulova et al. (2013) reported prolonged inflammation for two weeks after oral infection with *S.* Enteritidis through the high upregulation of IFN-γ, IL-17, and IL-22 genes' expressed by T-lymphocytes in cecal tissue of ISA Brown chickens. In Fayoumi chickens, a significant upregulation of IL-6 mRNA was found in the *S.* Enteritidis challenged group compared to the un-challenged group (Coble et al., 2011). IL-6 is a pro-inflammatory cytokine that stimulates B cells to produce specific antibodies, is involved in natural and acquired immunity, and controls tissue homeostasis (Liu et al., 2016). Coble et al. (2011) mentioned that the differences in gene expression could be attributed to the variation in experimental designs that could lead to the alteration of gene expression, including the *Salmonella* serotype used, the age of the chickens at the time of infection, the number of days post-infection at which the spleen samples were collected.

Conclusions

Salmonella Enteritidis was characterized from cecal and liver samples collected from broilers. It carried several virulence and antimicrobial resistance genes. Experimental infection with *S.* Enteritidis produced a prolonged inflammatory response and immunocompromising effect on Newcastle disease vaccination in broilers.

References

Abd El-Mohsen, A., El-Sherry, S., Soliman, M.A., Amen, O., 2022. Serological and antibacterial characteristics of *Salmonella* isolates from chickens in Assiut, Egypt. Benha Veterinary Medical Journal 41, 93–99. 10.21608/bvmj.2021.93816.1468.

Abed, A., Hasan, W., Abd El-Latif, M., 2020. Bacteriological studies on ascites in broiler chickens. Assiut Veterinary Medical Journal 66, 24–33. 10.21608/avmj.2020.168452.

Adams, S.C., Xing, Z., Li, J., Cardona, C.J., 2009. Immune-related gene expression in response to H11N9 low pathogenic avian influenza virus infection in chicken and Pekin duck peripheral blood mononuclear cells. Molecular Immunology 46, 1744–1749. 10.1016/j.molimm.2009.01.025.

Ahmed, H.A., El-Hofy, F.I., Shafik, S.M., Abdelrahman, M.A., El-said, G.A., 2016. Characterization of virulence-associated genes, antimicrobial resistance genes, and class 1 integrons in *Salmonella* enterica serovar Typhimurium isolates from chicken meat and humans in Egypt. Foodborne Pathogens and Disease 13, 281–288. 10.1089/fpd.2015.2097.

Ahmed, K.A., Saxena, V.K., Ara, A., Singh, K.B., Sundaresan, N.R., Saxena, M., Rasool, T.J., 2007. Immune response to Newcastle disease virus in chicken lines divergently selected for cutaneous hypersensitivity. International Journal of Immunogenetics 34, 445–455. 10.1111/j.1744-313X.2007.00722.x.

Akbarmehr, J., Salehi, T., Brujeni, G., 2010. Identification of salmonella isolated from poultry by MPCR technique and evaluation of their hsp groEL gene diversity based on the PCR-RFLP analysis. African Journal of Microbiology Research 4, 1594–1598.

Akeila, M.A., Ellakany, H.F., Sedik, M.E., El-Bahr, H.M., 2013. Characterization and plasmid profiling of *Salmonella* Enteritidis isolated from broiler chickens. Alexandria Journal of Veterinary Sciences URL: https://www.alexjvs.com/?mno=38655.

Attia, Y., Ellakany, H., Abd El-Hamid, H., Bovera, F., Ghazaly, S., 2012. Control of *Salmonella* Enteritidis infection in male layer chickens by acetic acid and/or prebiotics, probiotics and antibiotics. European Poultry Science 76, 239– 245.

Awad, A., Gwida, M., Khalifa, E., Sadat, A., 2020. Phenotypes, antibacterial-resistant profile, and virulence-associated genes of *Salmonella* serovars isolated from retail chicken meat in Egypt. Veterinary World 13, 440–445. 10.14202/vetworld.2020.440-445.

Balan, K.V., Babu, U.S., 2017. Comparative responses of chicken macrophages to infection with *Salmonella* enterica serovars. Poultry Science 96, 1849–1854. 10.3382/ps/pew477.

Berndt, A., Wilhelm, A., Jugert, C., Pieper, J., Sachse, K., Methner, U., 2007. Chicken cecum immune response to *Salmonella* enterica serovars of different levels of invasiveness. Infection and Immunity 75, 5993–6007. 10.1128/IAI.00695-07.

Borrmann, E., Berndt, A., Hänel, I., Köhler, H., 2007. *Campylobacter*-induced interleukin-8 responses in human intestinal epithelial cells and primary intestinal chick cells. Veterinary Microbiology 124, 115–124. 10.1016/j.vetmic.2007.04.041.

Caffrey, N., Agunos, A., Gow, S., Liljebjelke, K., Mainali, C., Checkley, S.L., 2021. *Salmonella* spp. prevalence and antimicrobial resistance in broiler chicken and turkey flocks in Canada from 2013 to 2018. Zoonoses and Public Health 68, 719–736. 10.1111/zph.12769.

Capua, I., Alexander, D.J. (Eds.), 2009. Avian influenza and Newcastle disease. Springer Milan, Milano. 10.1007/978-88-470-0826-7.

Castro-Vargas, R.E., Herrera-Sánchez, M.P., Rodríguez-Hernández, R., Rondón-Barragán, I.S., 2020. Antibiotic resistance in *Salmonella* spp. isolated from poultry: A global overview. Veterinary World 13, 2070–2084. 10.14202/vetworld.2020.2070-2084.

Coble, D.J., Redmond, S.B., Hale, B., Lamont, S.J., 2011. Distinct lines of chickens express different splenic cytokine profiles in response to *Salmonella* Enteritidis challenge. Poultry Science 90, 1659–1663. 10.3382/ps.2010-01279.

Collee, J., Miles, R.S., Watt, B., 1996. Tests for identification of bacteria, in: Collee, J., Mackie, T., McCartney, J. (Eds.), Mackie and Mccartney Practical Medical Microbiology. 14th ed.. Elsevier, New York, USA.

Colom, K., Pérez, J., Alonso, R., Fernández-Aranguiz, A., Lariño, E., Cisterna, R., 2003. Simple and reliable multiplex PCR assay for detection of bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes in *Enterobacteriaceae*. FEMS Microbiology Letters 223, 147–151. 10.1016/S0378-1097(03)00306-9.

Ding, K., Shang, K., Yu, Z.H., Yu, C., Jia, Y.Y., He, L., Liao, C.S., Li, J., Zhang, C.J., Li, Y.J., Wu, T.C., Cheng, X.C., 2018. Recombinant-attenuated *Salmonella* pullorum strain expressing the hemagglutinin-neuraminidase protein of Newcastle disease virus (NDV) protects chickens against NDV and *Salmonella* pullorum challenge. Journal of Veterinary Science 19, 232–241. 10.4142/jvs.2018.19.2.232.

Duc, V.M., Nakamoto, Y., Fujiwara, A., Toyofuku, H., Obi, T., Chuma, T., 2019. Prevalence of *Salmonella* in broiler chickens in Kagoshima, Japan in 2009 to 2012 and the relationship between serovars changing and antimicrobial resistance. BMC Veterinary Research 15, 108. 10.1186/s12917-019-1836-6.

Fernández, A., Lara, C., Loste, A., Calvo, S., Marca, M.C., 2001. Control of *Salmonella* Enteritidis phage type 4 experimental infection by fosfomycin in newly hatched chicks. Comparative Immunology, Microbiology and Infectious Diseases 24, 207–216. 10.1016/s0147-9571(00)00028-x.

Flynn, J.L., Chan, J., 2001. Immunology of tuberculosis. Annual Review of Immunology 19, 93–129. 10.1146/annurev.immunol.19.1.93.

Grimont, P.A., Weill, F., 2007. Antigenic formulas of the *Salmonella* serovars. 9th ed., WHO, Collaborating Centre for Reference and Research on *Salmonella*, Paris, France. URL: https://www.pasteur.fr/sites/default/files/veng_0.pdf.

Harrington, L.E., Janowski, K.M., Oliver, J.R., Zajac, A.J., Weaver, C.T., 2008. Memory CD4 T cells emerge from effector T-cell progenitors. Nature 452, 356–360. 10.1038/nature06672.

He, H., Genovese, K.J., Swaggerty, C.L., Nisbet, D.J., Kogut, M.H., 2013. Nitric oxide as a biomarker of intracellular *Salmonella* viability and identification of the bacteriostatic activity of protein kinase A inhibitor H-89. Plos One 8, e58873. 10.1371/journal.pone.0058873.

Hojati, Z., Zamanzad, B., Hashemzadeh, M., Molaie, R., Gholipour, A., 2015. The *fimh* gene in uropathogenic *Escherichia coli* strains isolated from patients with urinary tract infection. Jundishapur Journal of Microbiology 8, e17520. 10.5812/jjm.17520.

Hong, Y.H., Lillehoj, H.S., Lillehoj, E.P., Lee, S.H., 2006. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Veterinary Immunology and Immunopathology 114, 259–272. 10.1016/j.vetimm.2006.08.006.

Ibekwe, A.M., Murinda, S.E., Graves, A.K., 2011. Genetic diversity and antimicrobial resistance of *Escherichia coli* from human and animal sources uncovers multiple resistances from human sources. Plos One 6, e20819. 10.1371/journal.pone.0020819.

Ijaz, A., Veldhuizen, E.J.A., Broere, F., Rutten, V.P.M.G., Jansen, C.A., 2021. The interplay between *Salmonella* and intestinal innate immune cells in chickens. Pathogens 10. 10.3390/pathogens10111512.

Ishola, O.O., 2010. Effects of challenge dose on crop colonization of *Salmonella* Enteritidis in experimentally infected chickens. Veterinarski Arhiv 80, 71–80. URL: https://hrcak.srce.hr/55678.

Jiang, H., Yang, H., Kapczynski, D.R., 2011. Chicken interferon alpha pretreatment reduces virus replication of pandemic H1N1 and H5N9 avian influenza viruses in lung cell cultures from different avian species. Virology Journal 8, 447. 10.1186/1743-422X-8-447.

Kaiser, P., Rothwell, L., Galyov, E.E., Barrow, P.A., Burnside, J., Wigley, P., 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Gallinarum. Microbiology 146 Pt 12, 3217–3226. 10.1099/00221287-146-12-3217.

Kaiser, P., Stäheli, P., 2014. Avian cytokines and chemokines, in: Avian Immunology. Elsevier, pp. 189–204. 10.1016/B978-0-12-396965-1.00010-8.

Kano, R., Konnai, S., Onuma, M., Ohashi, K., 2009. Cytokine profiles in chickens infected with virulent and avirulent Marek's disease viruses: Interferon-gamma is a key factor in the protection of Marek's disease by vaccination. Microbiology and Immunology 53, 224–232. 10.1111/j.1348-0421.2009.00109.x.

Kauffmann, F., 1974. Kauffmann-White Scheme. Annales de Microbiologie 125, 427–432. URL: https://www.ncbi.nlm.nih.gov/pubmed/4467767.

Kogut, M.H., McGruder, E.D., Hargis, B.M., Corrier, D.E., DeLoach, J.R., 1994. Dynamics of avian inflammatory response to *Salmonella*-immune lymphokines. changes in avian blood leukocyte populations. Inflammation 18, 373–388. 10.1007/{BF01534435}.

Kogut, M.H., McGruder, E.D., Hargis, B.M., Corrier, D.E., DeLoach, J.R., 1995. *In-vivo* activation of heterophil function in chickens following injection with *Salmonella* Enteritidis-immune lymphokines. Journal of Leukocyte Biology 57, 56–62. 10.1002/jlb.57.1.56.

Kogut, M.H., Rothwell, L., Kaiser, P., 2005. IFN-gamma priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella* enterica serovar Enteritidis. Journal of Interferon and Cytokine Research 25, 73–81. 10.1089/jir.2005.25.73.

Koneman, E.W., 1992. Color Atlas and Textbook of Diagnostic Microbiology. 4th ed., J.P. Lippincott, Philadelphia.

Kumar, S., Nayak, B., Collins, P.L., Samal, S.K., 2011. Evaluation of the Newcastle disease virus F and HN proteins in protective immunity by using a recombinant avian paramyxovirus type 3 vector in chickens. Journal of Virology 85, 6521–6534. 10.1128/JVI.00367-11.

Liu, X., Jones, G.W., Choy, E.H., Jones, S.A., 2016. The biology behind interleukin-6 targeted interventions. Current Opinion in Rheumatology 28, 152–160. 10.1097/BOR.0000000000000255.

Lowenthal, J., 2000. Avian cytokines: The natural approach to therapeutics. Developmental & Comparative Immunology 24, 355–365. 10.1016/S0145-305X(99)00083-X.

Malorny, B., Hoorfar, J., Hugas, M., Heuvelink, A., Fach, P., Ellerbroek, L., Bunge, C., Dorn, C., Helmuth, R., 2003. Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. International Journal of Food Microbiology 89, 241–249. 10.1016/s0168-1605(03)00154-5.

Martins, M., McCusker, M., Amaral, L., Fanning, S., 2011. Mechanisms of antibiotic resistance in *Salmonella*: Efflux pumps, genetics, quorum sensing and biofilm formation. Letters in Drug Design & Discovery 8, 114–123. 10.2174/157018011794183770.

Matulova, M., Varmuzova, K., Sisak, F., Havlickova, H., Babak, V., Stejskal, K., Zdrahal, Z., Rychlik, I., 2013. Chicken innate immune response to oral infection with *Salmonella* enterica serovar Enteritidis. Veterinary Research 44, 37. 10.1186/1297-9716-44-37.

Murugkar, H.V., Rahman, H., Dutta, P.K., 2003. Distribution of virulence genes in *Salmonella* serovars isolated from man & animals. The Indian Journal of Medical Research 117, 66–70. URL: https://www.ncbi.nlm.nih.gov/pubmed/12931840.

Oliveira, S.D., Rodenbusch, C.R., Cé, M.C., Rocha, S.L.S., Canal, C.W., 2003. Evaluation of selective and non-selective enrichment PCR procedures for *Salmonella* detection. Letters in Applied Microbiology 36, 217–221. 10.1046/j.1472-765x.2003.01294.x.

Penha Filho, R.A.C., Moura, B.S., de Almeida, A.M., Montassier, H.J., Barrow, P.A., Berchieri Junior, A., 2012. Humoral and cellular immune response generated by different vaccine programs before and after *Salmonella* Enteritidis challenge in chickens. Vaccine 30, 7637–7643. 10.1016/j.vaccine.2012.10.020.

Pineda, M., Kogut, M., Genovese, K., Farnell, Y.Z., Zhao, D., Wang, X., Milby, A., Farnell, M., 2021. Competitive exclusion of intra-genus *Salmonella* in neonatal broilers. Microorganisms 9. 10.3390/microorganisms9020446.

Quinn, P.J., Markey, B.K., Leonard, F.C., Hartigan, P., Fanning, S., Fitzpatrick, E.S., 2011. Manual of Veterinary Microbiology. 2nd ed., University of Toronto Libraries.

Radwan, I.A., Moustafa, M.M.M., Abdel-Wahab, S.H., Ali, A., Abed, A.H., 2022. Effect of essential oils on biological criteria of Gram-negative bacterial pathogens isolated from diseased broiler chickens. International Journal of Veterinary Science 11, 59–67. 10.47278/journal.ijvs/2021.078.

Sedeik, M.E., El-Shall, N.A., Awad, A.M., Elfeky, S.M., Abd El-Hack, M.E., Hussein, E.O.S., Alowaimer, A.N., Swelum, A.A., 2019. Isolation, conventional and molecular characterization of *Salmonella* spp. from newly hatched broiler chicks. AMB Express 9, 136. 10.1186/s13568-019-0821-6.

Sekelova, Z., Stepanova, H., Polansky, O., Varmuzova, K., Faldynova, M., Fedr, R., Rychlik, I., Vlasatikova, L., 2017. Differential protein expression in chicken macrophages and heterophils *in-vivo* following infection with *Salmonella* Enteritidis. Veterinary Research 48, 35. 10.1186/s13567-017-0439-0.

Sheela, R.R., Babu, U., Mu, J., Elankumaran, S., Bautista, D.A., Raybourne, R.B., Heckert, R.A., Song, W., 2003. Immune responses against *Salmonella* enterica serovar Enteritidis infection in virally immunosuppressed chickens. Clinical and Diagnostic Laboratory Immunology 10, 670–679. 10.1128/cdli.10.4.670-679.2003.

Tulu, D., 2020. Newcastle disease and its different applicable control options in poultry in Ethiopia. International Journal of Agricultural Extension 8, 43–56. 10.33687/ijae.008.01.3010.

Wigley, P., Kaiser, P., 2003. Avian cytokines in health and disease. Revista Brasileira de Ciência Avícola 5, 1–14. 10.1590/S1516-635X2003000100001.

Wise, M.G., Suarez, D.L., Seal, B.S., Pedersen, J.C., Senne, D.A., King, D.J., Kapczynski, D.R., Spackman, E., 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42, 329–338. 10.1128/JCM.42.1.329-338.2004.

WOAH, 2021. Chapter 2.3.14. Newcastle disease (infection with Newcastle disease virus), in: World Organization of Animal Health (WOAH) Terrestrial Manual. URL: https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.03.14_Newcastle_DIS.pdf.

Yang, H.M., Zhao, J., Xue, J., Yang, Y.L., Zhang, G.Z., 2017. Antigenic variation of LaSota and genotype VII Newcastle disease virus (NDV) and their efficacy against challenge with velogenic NDV. Vaccine 35, 27–32. 10.1016/j.vaccine.2016.11.048.

Yu, Z., Ono, C., Aiba, S., Kikuchi, Y., Sora, I., Matsuoka, H., Tomita, H., 2015. Therapeutic concentration of lithium stimulates complement C3 production in dendritic cells and microglia via GSK-3 inhibition. Glia 63, 257–270. 10.1002/glia.22749.

Yuan, J.S., Reed, A., Chen, F., Stewart, C.N., 2006. Statistical analysis of real-time PCR data. BMC Bioinformatics 7, 85. 10.1186/1471-2105-7-85.

Zhu, Y., Lai, H., Zou, L., Yin, S., Wang, C., Han, X., Xia, X., Hu, K., He, L., Zhou, K., Chen, S., Ao, X., Liu, S., 2017. Antimicrobial resistance and resistance genes in *Salmonella* strains isolated from broiler chickens along the slaughtering process in China. International Journal of Food Microbiology 259, 43–51. 10.1016/j.ijfoodmicro.2017.07.023.