In the Name of God

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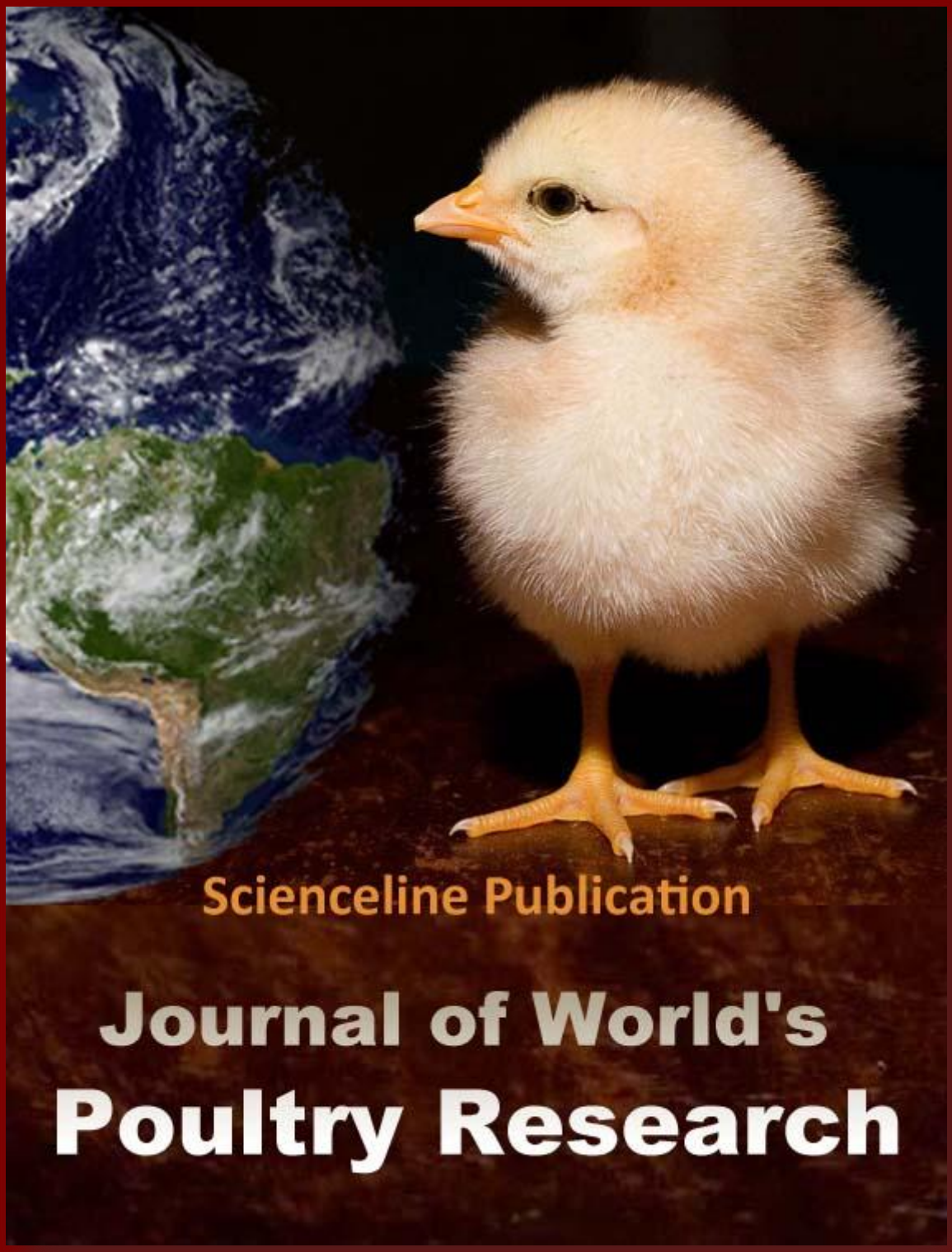

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ABSTRACT

Mycoplasma gallisepticum is an economically important pathogen of poultry worldwide, causing chronic respiratory disease in chickens and turkeys. Vaccination of poultry with *Mycoplasma gallisepticum* live vaccines is an approach to reduce susceptibility to infection and to prevent economic losses. The goal of this study was to develop an alternative method for evaluation of live and killed vaccine using quantitative differential real time PCR (rt-PCR) assay. Real time PCR assay was implemented for titration and identification of three types of *Mycoplasma gallisepticum* (F, ts-11 and field strain). Three groups of chicks were vaccinated by using F- strain, ts-11 and killed vaccine and the forth group was considered control. Challenge test was appliedby using *Mycoplasma gallisepticum* field strain (10^8 CFU) at three weeks post vaccination. Antibody ELISA titers against *Mycoplasma gallisepticum* were 319, 259 and 1009 for F, t-11 and killed vaccine respectively at 3 weeks post vaccination. The protection rates were 81.5%, 74%, and 66.6% for F- strain, ts-11 and killed vaccine respectively that was determined by air sac lesion scour. Using quantitative differential rt-PCR for necropsied birds at 5 days post challenge 7days post challenge and 14 days post challenge demonstrated that the F-strain vaccine had ability to prevent shedding of field strain at 14 days post challenge mean while the ts-11 and killed vaccine decreased shedding of field strain from $10^{8.1}$ and $10^{8.6}$ to $10^{5.1}$ and $10^{5.8}$ CFU respectively at 14 days post challenge. In this study, rt-PCR had ability to identify and quantify of two types of vaccines (F and ts-11) and field strain.

Keywords: *Mycoplasma*, rt-PCR, Vaccine, Poultry

ABSTRACT

Lithium concentration in surface and underground water, in some instances is higher than the standard level in places where lithium-rich brines and minerals occur, and in places where lithium batteries disposed of. This metal has numerous effects on human and other organisms, but there is no evidence about its effects on birds. For the first time we evaluated the effects of experimental lithium consumption in birds. The broiler chicks received daily 200 ppm lithium carbonate in their water, for 20 days and control group received water without lithium. At the end, blood samples collected for chemical analyses and the chickens were then euthanized and samples from brain, kidneys, gastrointestinal tract, heart and liver were collected for histopathological studies. Gross and microscopic lesions in organs were evaluated. Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT) and Uric acid also measured. The significant differences ($P<0.05$) between experimental group and control group were seen.

Keywords: Lithium, Toxicity, Bird, Histopathology, Clinical pathology

Research Paper

Effect of Cold Stress and Various Suitable Remedies on Performance of Broiler Chicken.

Qureshi S, Musadiq Khan H, Saleem Mir M, Ahmad Raja T, Alam Khan A, Ali H and Adil Sh.

J. World Poult. Res. 8(3): 66-73; pii: S2322455X1800010-8

ABSTRACT
A biological trial was conducted on commercial chicks during the winter months (December and January). Day old commercial meat type broiler chicks (273) were procured from a reputed source. Cold conditioning (20C to 80C) at third and fourth day of age for 3-4 hours was provided to 78 birds. These early cold conditioned birds were kept separate until distributed into respective treatment groups (fifth and sixth). At the end of second week, the chicks were individually weighed, distributed into 7 treatment groups of 3 replicates with 13 chicks in each replicate. Cold challenge @ 20C to 80C for 8 hours was provided from third week of age to sixth week of their age for all treatment groups except first and fifth treatment groups. The broiler birds in the treatment groups T1 and T5 were reared under normal temperature conditions (250C). Treatment group first (T1) was kept as control group. Antioxidant Vitamin E 250 mg per kg of feed was supplemented to the basal diet in the third treatment group. Chromium 0.1 gram per kg of feed was supplemented to the basal diet in the fourth treatment group. Chromium 0.2 gram per kg of feed was supplemented to the basal diet in the seventh treatment group. The data on individual body weight of the experimental birds and the cumulative feed consumption and feed conversion ratio on group basis were recorded at weekly intervals. Deaths were recorded daily and all dead birds were necropsied to identify ascites syndrome. There was no significant ($p< 0.05$) difference in the average body weight and body weight gain among various treatment groups throughout the experiment period. The cumulative feed consumption showed significant ($p< 0.05$) difference among various treatment groups throughout the experiment period. Highest feed consumption ($p< 0.05$) was observed in broiler chickens reared under cold conditions when compared with broiler birds reared under normal temperature conditions. Among the cold challenge treatment groups (T2, T3, T4, T6 and T7), there was significant ($p< 0.05$) improvement in feed conversion ratio (FCR) in the treatment groups T6 (early cold conditioning birds exposed to cold stress) and T7 (supplementation of chromium 0.2 g/kg of feed to birds exposed to cold stress). Among different treatment groups in general best FCR was observed in treatment group T5 (early cold conditioning group reared under normal conditions) followed by T1 (control group reared under normal conditions). At the end of the biological trial ascites linked mortalities showed significant ($p< 0.05$) difference among various treatment groups. There was no mortality reported in treatment groups kept under normal temperature conditions (T1 and T5). Highest ascites related mortality (23.07%) was observed in treatment group in which cold stress was provided and no measures were taken to alleviate the effect of cold stress on broiler birds (T2). The Vitamin E supplementation in the diet of broiler birds reared under cold stress (T3) showed significant ($p< 0.05$) reduction in ascites related mortality (10.25%).
Keywords: Ascites, Broiler chicken, Early cold conditioning, Chromium, Cold stress, Performance, Vitamin E

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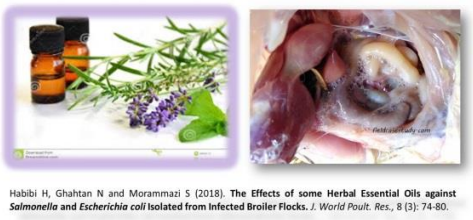
Research Paper

The Effects of some Herbal Essential Oils against Salmonella and Escherichia coli Isolated from Infected Broiler Flocks.



Habibi H, Ghahtan N and Morammazi S.

J. World Poult. Res. 8(3): 74-80; pii: S2322455X1800011-8

ABSTRACT
Escherichia coli and *Salmonella* spp are two bacterial infectious diseases responsible for heavy economic losses in the poultry industry. The emergence of antimicrobial resistance and its potential harmful threat to human health has led to a need to find safe alternatives for the control of these bacteria. To this end, the use of herbal remedies in poultry has been suggested. In this study, we have investigated the effect of essential oils extracted from five different herbal plants against *Salmonella spp* and *Escherichia coli* that have been isolated directly from infected broiler flocks. Standard Disk-diffusion method, Minimum Inhibition Concentration and minimum bactericidal concentration were used to determine the inhibitory effect of these essential oils. Also, tetracycline was used as a control group. Among the essential oils, *Carum copticum* had the highest antibacterial properties. The maximum inhibition zone in diameter against *Salmonella* and *Escherichia coli* were respectively 26.7 and 22.5 mm that concern about *Carum copticum* essential oils. According to the results of this study, it was found that some of the essential oils have a stronger antibacterial effect than tetracycline. So, after the complementary studies, some of these herbal plants can be suggested as alternatives to antibiotics for treating infections caused by these bacteria in poultry industry.
Keywords: Essential oil, Herbal plant, *Escherichia coli*, *Salmonella*

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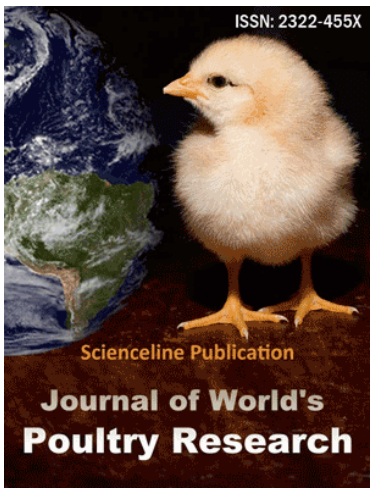

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Real Time PCR Quantification and Differentiation of both Challenge and Vaccinal *Mycoplasma gallisepticum*s trains Used in Vaccine Quality Control

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ABSTRACT

Mycoplasma gallisepticum is an economically important pathogen of poultry worldwide, causing chronic respiratory disease in chickens and turkeys. Vaccination of poultry with *Mycoplasma gallisepticum* live vaccines is an approach to reduce susceptibility to infection and to prevent economic losses. The goal of this study was to develop an alternative method for evaluation of live and killed vaccine using quantitative differential real time PCR (rt-PCR) assay. Real time PCR assay was implemented for titration and identification of three types of *Mycoplasma gallisepticum* (F, ts-11 and field strain). Three groups of chicks were vaccinated by using F- strain, ts-11 and killed vaccine and the forth group was considered control. Challenge test was appliedby using *Mycoplasma gallisepticum* field strain (10^8 CFU) at three weeks post vaccination. Antibody ELISA titers against *Mycoplasma gallisepticum* were 319, 259 and 1009 for F, t-11 and killed vaccine respectively at 3 weeks post vaccination. The protection rates were 81.5%, 74%, and 66.6% for F- strain, ts-11 and killed vaccine respectively that was determined by air sac lesion scour. Using quantitative differential rt-PCR for necropsied birds at 5 days post challenge 7days post challenge and 14 days post challenge demonstrated that the F-strain vaccine had ability to prevent shedding of field strain at 14 days post challenge mean while the ts-11 and killed vaccine decreased shedding of field strain from $10^{8.1}$ and $10^{8.6}$ to $10^{5.1}$ and $10^{5.8}$CFU respectively at 14 days post challenge. In this study, rt-PCR had ability to identify and quantify of two types of vaccines (F and ts-11) and field strain.

Keywords: *Mycoplasma*, rt-PCR, Vaccine, Poultry

INTRODUCTION

Mycoplasma gallisepticum (MG) infects a wide variety of gallinaceous birds including chickens, turkeys and pheasants (Yoder,1990). MG is the most important notifiable disease. MG is a cause of chronic respiratory disease, especially in the presence of other respiratory microorganism or environmental stresses. The disease is characterized by coryza, conjunctivitis, sneezing, and sinusitis particularly in turkey and game birds. It results in loss of production, downgrading of meat-type birds and loss of egg production. MG strains vary in infectivity and virulence, and infections may sometimes be inapparent (OIE, 2013). Vaccination with bacterin has been shown to control, but not eliminate colonization, it is felt that bacterins are of minimal value in long-term prevention on commercial layer (Ley, 2008; Moraes et al., 2013). Live vaccines that have been used to control MG include F strain (Burnham et al., 2002), and more recently, ts-11 (Whithear et al., 1990). Culture methods, are often labor-intensive and require specially formulated media, so the improvement of diagnostic tools for direct detection of mycoplasma is necessary (Feberwee et al., 2005).

The quality control of live MG vaccine depends on identity, titration, safety, sterility and potency tests. The identity test was determined by conventional Polymerase Chain Reaction (PCR) that does not differentiate between the types of live MG vaccines (Mettifogo et al., 2015 and Thilagavathi et al., 2017). On the other hand, the bacterial titration by Colony Changing Unit (CCU) or by Colony Forming Unit (CFU) per dose takes long time (5-14days) (Stewke and Robertson, 1982).

Raviv et al. (2008) and Ehtisham et al. (2015) established a Real-time PCR assay that had an inherent quantitative nature using dual-labeled probe (Taqman) advantageous for microorganisms strain differentiation

owing to the superior sensitivity and improved specificity endowed by 3 hybridizing oligonucleotides (two primers and a probe)

The bacteriologicl examination and conventional PCR of MG live vaccine, that didn't have ability to differentiate between live vaccines, field strains) (Stewke and Robertson, 1982). This lack of ability to differentiate between the participating strains (F, ts11 and field strain) limits the level of control and the amount of information that could be gained from MG vaccine evaluation studies. Present study developed an alternative tool for the qualitative and quantitative differentiation between MG strains (F, Ts11 and field strain) in live and killed MG vaccine quality control especially challenge test and shedding determination that improve reliability and efficiency of the vaccine quality control studies in addition to strain differentiation.

MATERIALS AND METHODS

MG vaccine

Three types of commercial MG vaccinesused in this study, two live (vaccine F- strain and ts-11) and one killed vaccine.

MG field strain

It was obtained from the Reference Strain Bank at Central loboratory for Evaluation of Veterinary Biologics (CLEVB).

MG live vaccine and field strain titration

Live vaccines were reconstituted in the given manufacturer diluent. Vaccinesweрediluted ten–fold, different dilutions were streaked on pleuropneumonia like organism (PPLO) solid media. Starting dilution was 10^{10}CFU.Field strain was adjusted to10^{10}CFU and diluted ten-fold serial dilution.

rt-PCR for live MG vaccine (f strain and ts -11) and field strain :

Each ten dilution of two live MG vaccines and field strain ware centrifuged for 30 minutes, at 14,000 gandat 4°C. The supernatant was carefully removed and the pellet was suspended in 25 µl PCR grade water. The tube and the contents were boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 gfor 5 minutes. Half amount of DNA extract (12.5 µl) of each vaccine dilution was tested by rt-PCR.The primers and labeled probes (FAM , HEX and ROX) are summarized in table 1 and according to Raviv et al. (2008).

rt-PCRwas performed in stratagene MX 3005P. Thermoprofilewas 95 °C for 15 min with optics OFF, and 40 cycles of 94 °C for15s followed by reaction specific primers and probe (Table 1). Annealing/extension temperatures for 60 s with optics ON. Cycle threshold number (CT value) was determined asthe PCR cycle number at which the fluorescence of thereaction crosses the florescence threshold. Any reaction with CT value was considered positive and any reaction without CTvalue, was considered negative. Standard curves were established according to Ehtisham et al. (2015). The quantitation and detection limit of each of the study's rt-PCRs were determined by one run of each concentration for MG live vaccines and field strain. Final results were doubled as half amount of DNA extract was used.

Experimental design

Four groups contained of thrity four Specific Pathogen Free chickens two weeks ago. Three groups were vaccinated with F-strain, ts -11 and killed vaccine as recommended by vaccine manufacturer and the forth group was kept separately as control. Three weeks of post vaccination, all groups were challenged with 0.5 ml containing 1×10^{8}CFU of overnight culture of MG field strain.

Air sac lesion scoring

The air sac lesion scoring was carried out at7 and 14 days post challenge to determine the level of protection. Ten birds from each of the experimental groups were necropsied at each three time 5, 7 and 14 days post challenge (DPC). The level of protection was evaluated by gross air sac lesion scoring on a scale from 0 to 4 air sac lesions examination. Also the protection rate was determined according to Whithear (1996) Protection rate = (protective vaccinated birds-protected un vaccinated bird)/ unprotected unvaccinated birds (Kleven et al., 1972).

Serological evaluating

10 Serum sample were taken from all group preandpost vaccination for four times (0, 5, 7, 14 DPC test). The serum samples were used for determining the level of the immune response by using MG ELISA antibody test kit (Synbioytics , Pro FLOK, Zoitis USA) (Javedet al., 2005; Zulfekar et al., 2015).

Shedding determination

Ten birds per each group were necropsied and sampled three times (5,7and 14 DPC).rt- PCR was carried out on laryngeal wash samples. Briefly, the larynx was cut at the base and put in 10 ml sterile plastic tubes filled with 5 ml Phosphate Buffer Saline (PBS), and vortexed for 30s. A 0.5 ml laryngeal wash solution was submitted for DNA extraction. The final results were powered 20× as 10% amount of laryngeal wash and half amount of DNA extract were used

Ethical aporoval

This study was approved procedures from Centeral laboratory for evaluation of veterinary biologics, Cairo, Egypt for humane handling of experimental animals.

RESULTS

Each reaction standard curve was determined by independent runs of each reaction using 10fold serial dilutions (10^{10}–10^{1} copies per reaction) of the reaction's

standard DNA control. The mean CT values, the linear equation and the R-squared value of the obtained standard curves are summarized in table 2 and figure 2. The minimal concentration of F-strain and ts-11, MG live vaccines were 10 and 10^3 CFU per sample respectively,while for field strain it was 10^2 CFU per sample as shown in table 2.

The rt PCR was highly specificity and differentiating for the target strain and gave negative to opposite strain as demonstrated in figure 1.

The protection rate was analyzed by air sac lesion, incase of f-strain vaccination frist group the healthy necropsied birds (no air sac lesion) were8,8 and 9 birds at 5, 7 and 14 DPC respectively, and for the ts-11 strain vaccination second group the healthy necropsied birds were 8, 7 and 8 birds at 5, 7 and 14 DPC respectively while incase of the killed vaccination third group , the healthy necropsied birds were 7, 6 and 8 birds at 5 , 7 and 14 DPC respectively but the positive control group 4 (non vaccinated group), the healthy necropsied birds were 1, 1 and 1 bird at 5, 7 and 14 DPC (Table 3). So the protection rate for F-strain and ts-11 stain and killed vaccine were 81.5%, 74% and 66.6 % respectively (Table 3).

As shown in table 4 the antibody titer against MG, F-strain, ts-11 strain and killed in sera were increased from 122 pre-vaccination level to 319, 259 and1009 at 3 weeks post vaccination and to 954, 763 and 1643 at 3 weeks postchallenge.

The four groups were sampled three times at 5D , 7D and 14DPC and the quantitative rt- PCR for different MG strains (F , ts -11 and field strain) was carried out on laryngeal wash. Vaccinated birds with F- strain vaccine demonstrated sharp decrease of the field strain count ($10^{5.7}$ and $10^{4.8}$CFU CFU) at 5DPC and 7DPC respectivel, then shedding was stopped at14DPC, but for F-strain was continuously shedding even at 14DFC. Vaccinated birds with ts-11 strain vaccine demonstrated decrease of the field strain count ($10^{8.1}$, $10^{7.1}$ and $10^{5.1}$CFU) at 5D, 7D and 14 DPC, butts-11 strain was continuously shedding. vaccinated birds with killed vaccine demonstrated slight decrease of field strain count ($10^{8.6}$, $10^{7.9}$ and $10^{5.8}$CFU) at 5D, 7D and 14 D post challenge also the shedding wasn't stopped (Table 4).

DISCUSSION

The evaluation of avian mycoplasma vaccines and the study of their mechanism of action as serological, protection rate and shedding determination have lacked the ability to differentially identify and quantify the participating strains within the vaccine quality control. The conventional PCR is not suitable for multi strain infection situations (Muhammad et al., 2017). The lack of ability to differentially identify the participating strains imposed significant limitations to the level of control that could be achieved in MG vaccines evaluation studies (Thilagavathi et al., 2017).

In this study The rt-PCR had ability to identify and quantify the two types of vaccines (F, ts-11 and Field strain) strains at the same reaction by using different labeled probe (FAM, HEX and ROX) respectively. The sensitivity (minimal CFU that gave positive results) of the rt PCR for F-strain, ts-11 and field strain were 10 , 10^3and10^2CFU / sample respectively (table 2). Ehtisham et al. (2015) detected 10^2CFU MG / sample using rt-PCR taqman labeled probe while Raviv et al. (2008) detected 6.5×10^1 CFU MG / sample**.**

the antibody titer against MG, F-strain, ts-11 strain and killed in sera were increased from 122 pre-vaccination level to 319, 259 and1009 at 3 weeks of post vaccination and to 954, 763 and 1643 at 3 weeks of postchallenge. The birds taken killed vaccine apparently gave immune response than two live vaccine (F-strain and ts-11).The results were similar to the results of Avakian et al. (1988) and Pakpinyo et al. (2014).

The protection rate for birds vaccinated with F-strain vaccine was higher (81.5%) than ts-11 (74) and killed vaccine (66.6%) against field strain. This result was similar to the results of Jacob et al. (2014) and Jacob et al. (2015).

Regarding the F-strain live vaccine had ability to stop the shedding of the field strain at 14 DPC. On the contrary the ts-11 and killed vaccine didn't have ability to stop the shedding till at 14 DPC. Moreover, Raviv et al. (2008) recorded that birds vaccinated with 6/85 and K5831 strain live vaccine demonstrated a stopping the shedding of challenge strain.

Results of molecular assay showed that the ability to differentiate between a known array of Mycoplasma strains (F, ts-11 and field strain) in a mixed sample. The rt-PCR with dual-labeled probe technology endowed the method with its superior sensitivity, specificity and quantitative properties. The initial application of this quantitative strain differentiating tool was designed for live and killed mycoplasma vaccine quality control and indeed provided a significant upgrade to this area of research. The demonstrated concept of differential rt-PCR is general and could be considered for a variety of research applications in mycoplasmology and microbiology.

Table 1. The primer and labeled probe (FAM and HEX) rt- PCR specifications of *mycoplasma*live vaccine and field strain

Types of mycoplasma Strains	Gene and GenBank sequence accession #	Forward (F) primer sequence (5-3)	Reverse (R) primer sequence (5- 3)	Probe (P) Sequence (5-3)	Type of fluorescence	Oligos location on GenBank sequence	PCR product size (bps)	Anneling/eextentiontemputure
F strain	mgc2, AY556230	gttcaagaaccaactcaacca	Gattaagaccgaattgtggattg	caaccaggatttaatcaacctcag	FAM	F: 217–237 R: 328–306 P: 280–303	112	61 ℃
ts-11 strain	mgc2 AY556232	ctcaagaaccaactcaacca	Ggggattaggaataaattgcggat		HEX	F: 218–237 R: 331–308 P: 280–303		
Field strain	pvpA, AY556306	ttctcaaccacgcccaatg	ggttagatccaccaactccca	Caatgggtgctccaaatcctcaac	ROX	F: 246–264 R: 364–344 P: 290–313	119	61 ℃

FAM, HEXandRox were fluorescence dye

Table 2. Summary of the mean CT values, the linear equations and the R-squared values of the rt- PCRs for F-strain ,ts-11 and field strain

Dilution (CFU/ sample)	F-strain	ts-11	Field strain
10^{10}	11.21	13.41	13.1
10^{9}	14.59	16.9	16.4
10^{8}	17.25	20.17	19.17
10^{7}	20.91	23.46	22.77
10^{6}	24.69	26.79	24.98
10^{5}	27.55	30.36	29.01
10^{4}	31.71	33.98	32.18
10^{3}	32.68	39.09	37.26
10^{2}	33.98	Negative	38.77
10	39.09	Negative	Negative
Linear equation	**Y=** -0.3219X+13.691	**Y=** -0.305X+14.167	Y=-0.3136X+14.0833
R-squared	**0.9877**	**0.9811**	**0.9911**

Table 3. The summary of the airsac lesion and the protection rate for F-strain, ts-11 and killed *Mycoplasma gallisepticum* vaccine

Group	Group 1 (F- strain)			Group 2 (ts-11)			Group 3(killed vaccine)			Group 4 (Positive control)		
Day post challenge	5	7	14	5	7	14	5	7	14	5	7	14
Protected bird /total number	8/10	8/10	9/10	8/10	7/10	8/10	7/10	6/10	8/10	1/10	1/10	1/10
	25/30			23/30			21/30			3/30		
Protection rate*	**81.5%**			**74%**			**66.6%**			0		

*Protection rate = (protective vaccinated birds-protected un vaccinated bird)/ unprotected unvaccinated birds

Table 4. Serological evaluation (ELISA antibodies mean titer) for F-strain, ts-11 and *Mycoplasma gallisepticum* killed vaccine

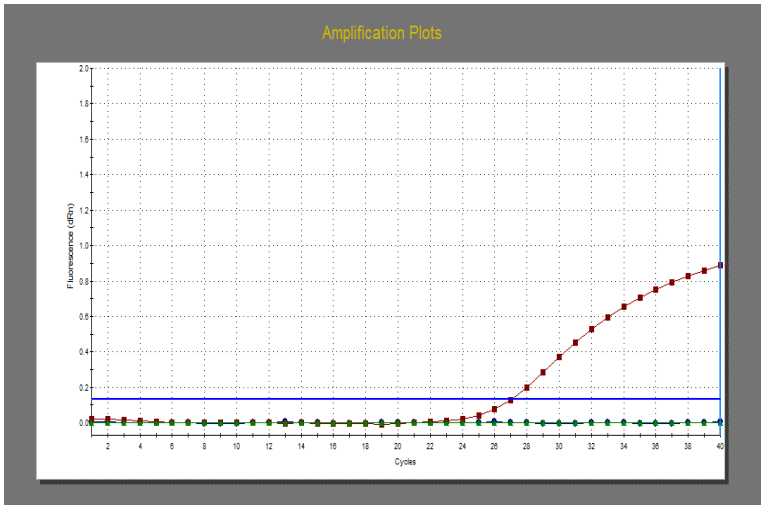
Items	Group 1 (F- strain)	Group 2 (ts-11)	Group3 (killed vaccine)	Group 4 (Positive control)
0 day of vaccination	122	122	122	122
3 weeks of post vaccination	319	259	1009	113
first week of post challenge	706	543	1203	116
second week of post challenge	811	546	1508	234
third week of post challenge	954	763	1643	467

Table 5. Determination of the amount of microbial shedding for the F-strain , ts-11, and killed *Mycoplasma gallisepticum*vaccineafter 5, 7 and 14 days post challenge

		Group 1 (F- strain)			Group 2 (ts-11)			Group3 (killed vaccine)			Group 4 (Positive control)		
Type of Probe fluorescence		FAM (F-strain)	HEX (Ts-11)	ROS (Challenge strain)	FAM (F-strain	HEX (Ts-11	ROS (Challenge strain)	FAM (F-strain)	HEX (Ts-11)	ROS (Challenge strain)	FAM (F-strain)	HEX (Ts-11)	ROS (Challenge strain)
Linear equation		**Y=** -0.3219X +13.691	**Y=** -0.305X +14.167	Y=-0.3136X +14.083	**Y=** -0.3219X +13.691	**Y=** -0.305X +14.167	Y=-0.3136X +14.083	**Y=** -0.3219X +13.691	**Y=**-0.305X +14.167	Y=-0.3136X +14.083	**Y=** -0.3219X +13.691	**Y=** -0.305X +14.167	Y=-0.3136X +14.083
Mean Ct for 10birds	5 DPC	22.2	No Ct	30.3	No Ct	26.4	23.1	No Ct	No Ct	21	No Ct	No Ct	22.1
	7 DPC	23.6	No Ct	33.2	No Ct	33.9	25.9	No Ct	No Ct	23	No Ct	No Ct	20.7
	14 DPC	27.9	No Ct	No Ct	No Ct	37.8	32.2	No Ct	No Ct	30	No Ct	No Ct	22.9
Mean titer for 10 birds*	5 DPC	$10^{7.7}$	**-ve**	$10^{5.7}$	**-ve**	$10^{7.3}$	$10^{8.1}$	**-ve**	**-ve**	$10^{8.6}$	**-ve**	**-ve**	$10^{8.2}$
	7 DPC	$10^{6.2}$	**-ve**	$10^{4.8}$	**-ve**	$10^{4.9}$	$10^{7.1}$	**-ve**	**-ve**	$10^{7.9}$	**-ve**	**-ve**	$10^{8.6}$
	14 DPC	$10^{5.8}$	**-ve**	**-ve**	**-ve**	$10^{3.7}$	$10^{5.1}$	**-ve**	**-ve**	$10^{5.8}$	**-ve**	**-ve**	$10^{7.9}$

*The titer was powered 20 × as 10% amount of laryngeal wash and half amount of DNA extract were used; Ct: cycle threshold value. DPC = days post challenge. FAM, HEXandRox were fluorescence dye

Figure 1. Specificity test for differentiation between different F- strain ,ts -11 and field *Mycoplasma gallisepticum* strain



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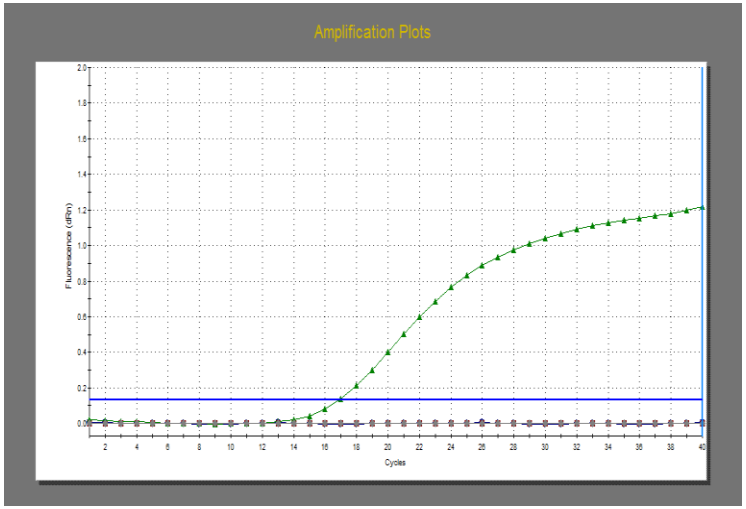

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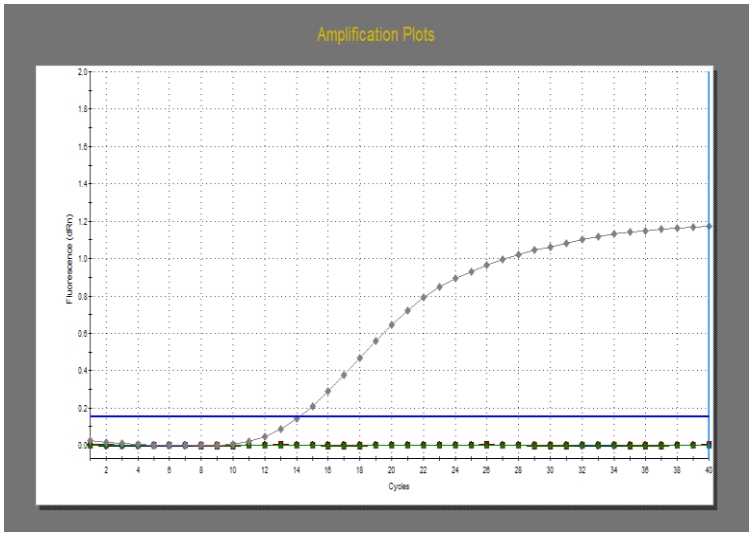

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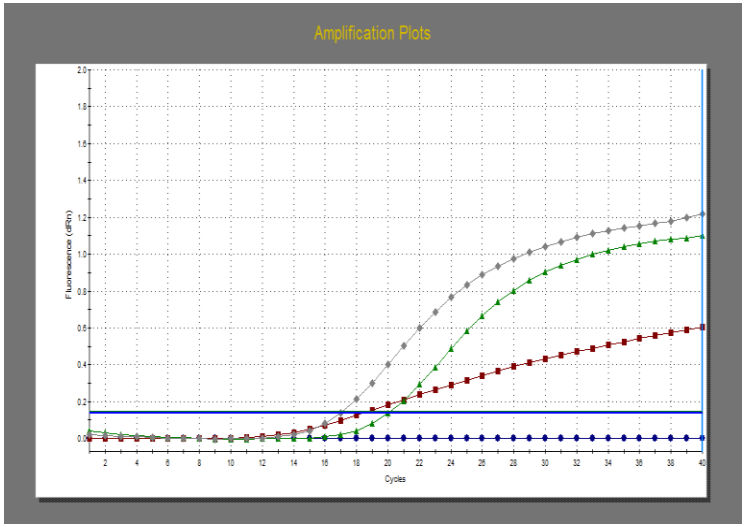

D

- **Run A:** the sample contain DNA extract from Fstrain was carried out using primers and probs for F-strain (red) , ts-11 (green) and field strain (gray) and gave signal for F-strain only.
- **Run B:** the sample contain DNA extract from ts-11 was carried out using primers and probs for F-strain (red) , ts-11 (green) and field strain (red) and gave signal for ts -11 strain only.
- **Run C:** the sample contain DNA extract from challenge strain was carried out using primers and probs for F-strain (red) , ts-11 (green) and field strain (red) and gave signal for field strain only.
- **Run D (two samples):** the sample no. 1 contain mix DNA extract from F-strain (red) , ts-11 (green) and field strain (red) was carried out using all primers and probs and gave signals for all strains. On the anther hand the sample no 2 contain normal saline thatno gave signal (blue).

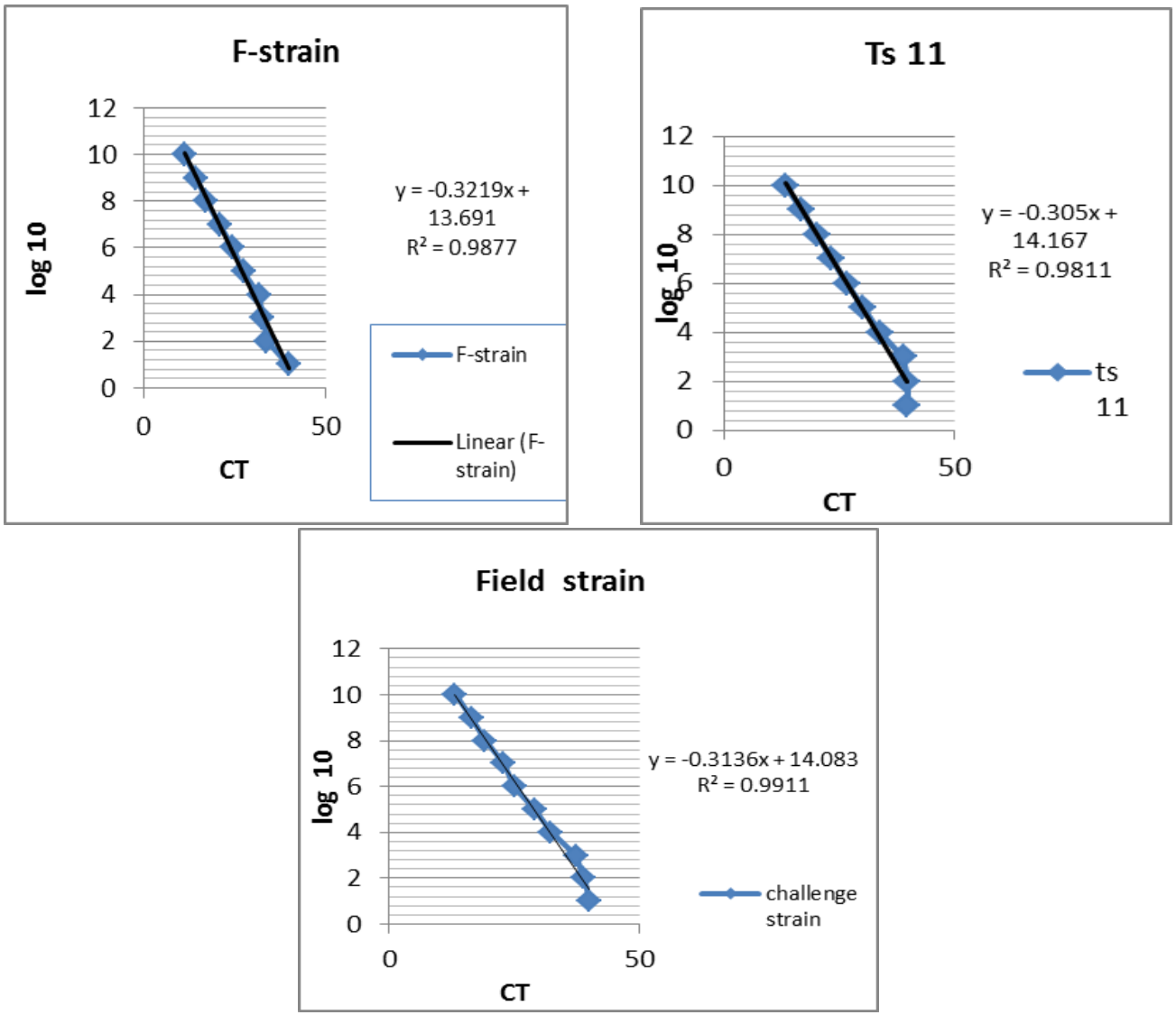

Figure 2. Standard curve and the calculation equation for F-strain , ts-11, and field *Mycoplasma gallisepticum* strain**s** Log10 = 10^2, 10^3, 10^4etc. CT = Cycle threshold

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors participated in making the design,performing the experiment, analyses of the data, and writing the paper.

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Effect of Lithium Toxicity in Broiler

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ABSTRACT

Lithium concentration in surface and underground water, in some instances is higher than the standard level in places where lithium-rich brines and minerals occur, and in places where lithium batteries disposed of. This metal has numerous effects on human and other organisms, but there is no evidence about its effects on birds. For the first time we evaluated the effects of experimental lithium consumption in birds. The broiler chicks received daily 200 ppm lithium carbonate in their water, for 20 days and control group received water without lithium. At the end, blood samples collected for chemical analyses and the chickens were then euthanized and samples from brain, kidneys, gastrointestinal tract, heart and liver were collected for histopathological studies. Gross and microscopic lesions in organs were evaluated. Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT) and Uric acid also measured. The significant differences ($P<0.05$) between experimental group and control group were seen.
Keywords: Lithium, Toxicity, Bird, Histopathology, Clinical pathology

INTRODUCTION

Lithium is the lightest metal that has an atomic weight of seven and closely resembles sodium chemically. Compared with other alkali metals, it is less reactive than sodium and much less reactive than potassium. Lithium is generally found naturally in the aquatic and terrestrial environment but in small concentrations (Aral and Vecchio-Sadus, 2008) According to the Australian Capital Territory Environment Protection Regulation (EPA), lithium is listed as a pollutant that causes environmental harm in irrigation water supplies. Since lithium is found in natural brines and lakes, therefore it can be considered as a main source of lithium poisoning for birds, especially for wild birds. Another source of lithium poisoning impact to the environment, especially for pet birds, is spent because the consumers routinely dispose of batteries along with other garbage in the municipal solid waste. Because lithium has many industrial applications (Meshram et al., 2014) therefore, professional intoxications from the industrial applications are possible and environment can be considered as a source of pollution with lithium in such circumstances. This metal has numerous effects on human and other creatures (Haussmann et al., 2015; Moore and Committee, 1995; Phiel and Klein, 2001). Denoted that prolong administration of lithium resulted in significant inflammatory and hyperemic changes in kidneys, liver and brain in rats (Dimitrova et al., 2013) (Socaciu and Leucuta, 1999). There are limited evidences concerning the effects of this metal on different organs of birds. It has been reported that lithium can be accumulated in the organs of birds, and the rate of lithium accumulation is higher in the terrestrial birds than in the aquatic ones (Horai et al., 2007).

Since lithium is poorly absorbed across the skin, so dermal contact and inhalation are not likely to be significant routes of exposure to this metal and ingestion appears to be the most popular route of exposure. When ingested in excessive amounts, lithium primarily affects the gastrointestinal tract, central nervous system, liver and kidneys. Although it can be life threatening, but unfortunately, no studies have quantified the risk of lithium toxicity in birds. Most of the toxicity information has been obtained from the ingested lithium salts by humans (Hardman and Lant, 1996). Therefore, the present study investigated the pathological and clinic pathological effects of experimental lithium poisoning in broilers.

MATERIALS AND METHODS

Ethical approval

All experiments in this study were performed in accordance with the guidelines for animal research from the School of Veterinary Medicine, Shiraz University, Shiraz, Iran. Also, we used the recommendations of European Council Directive (2010/63/EU).

Experimental design

Twenty eight day old broiler from both sexes were purchased from a local hatchery and were kept at 32±1^{o}C, 40-50% humidity, controlled electrical heating batteries and at12/12h light-dark cycle. They were maintained as a flock and were provided with commercial diet and water ad-libitum. After 20 days they were randomly divided into two control (A) and experimental (B) groups, each having 14 chickens. Each group was then divided into two subgroups A1, A2 and B1, B2, each having 7 chickens. The chickens of the experimental group (B) received daily 200 ppm lithium carbonate in their water, for 20 days, which was calculated on the base of the producer recommendation and our previous experiences.

The animals of the control group received water without lithium. At the end of the experiment, blood samples were collected for chemical analyses of the Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyrovic Transaminase (SGPT) enzymes (Reitman and Frankel, 1957) and uric acid. The animals were then euthanized and all organs were carefully examined and samples from brain, kidneys, gastrointestinal tract, heart and liver were collected for histopathological examination. After fixation in 10% neutral buffered formalin, the tissue samples were washed, dehydrated by graded ethanol, cleared, embedded in paraffin wax, sectioned at 4-5 µm, stained with haematoxylin and eosin and examined by a light microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analysis of the blood SGOT, SGPT and uric acid between the lithium treated and control groups was done by the independent sample *t-test*. Data were analyzed by SPSS 21 using one-way ANOVA.

RESULTS

The chickens of the control group remained healthy and active throughout the experiment, but those in the experimental group were lethargic and drowsy and some of them started to perish about 10-12 days after receiving lithium and after necropsy, the hydropericard lesions were seen. No pathological changes were noticed on gross and microscopic examinations of the organs of chickens of the control group; however, the cell lining in the tubules of the kidneys of the chickens in the experimental group showed degenerative and necrotic changes. The epithelium of proximal convoluted tubules was vacuolated and, in some sections, showed necrotic and sloughing changes (Figure. a). Proteinaceous casts and cellular debris were present in some of the degenerated or necrotic tubules (Figure b and c). The basement membrane, epithelial cells and lumen of some of the necrotic tubules showed mineralization (Figure a).

The glomeruli showed hyperemia, glomerular atrophy and dilatation of the urinary spaces. The blood vessels were hyperemic and foci of hemorrhages were evident in the interstitial tissue of these organs (Figure. b). The hepatocytes of the experimental animals showed different extents of fatty infiltration and in some instances focal necrosis (Figure d). The portal area showed chronic portal hepatitis and were infiltrated with lymphocytes, plasma cells and macrophages (Figure e and f). The blood vessels were hyperemic and multifocal or diffused hemorrhages were seen in the parenchyma of the liver of the experimental animals. The white matter of cerebrum showed cellular vacuolation and spongiosis (Figure g and h). The submucosa and lamina propria of different parts of the gastrointestinal tract were infiltrated by lymphocytes, plasma cells and macrophages. Hyperemia and hemorrhages were other consistent changes in the submucosa and lamina propria of intestine.

The Serum Glutamate Pyruvate Transaminase (SGPT) and Uric acid levels between the two groups showed significant differences ($P<0.05$), but there was no significant difference ($P>0.05$) between the Serum Glutamate Oxaloacetate Transaminase (SGOT) values among the groups (Table 1).

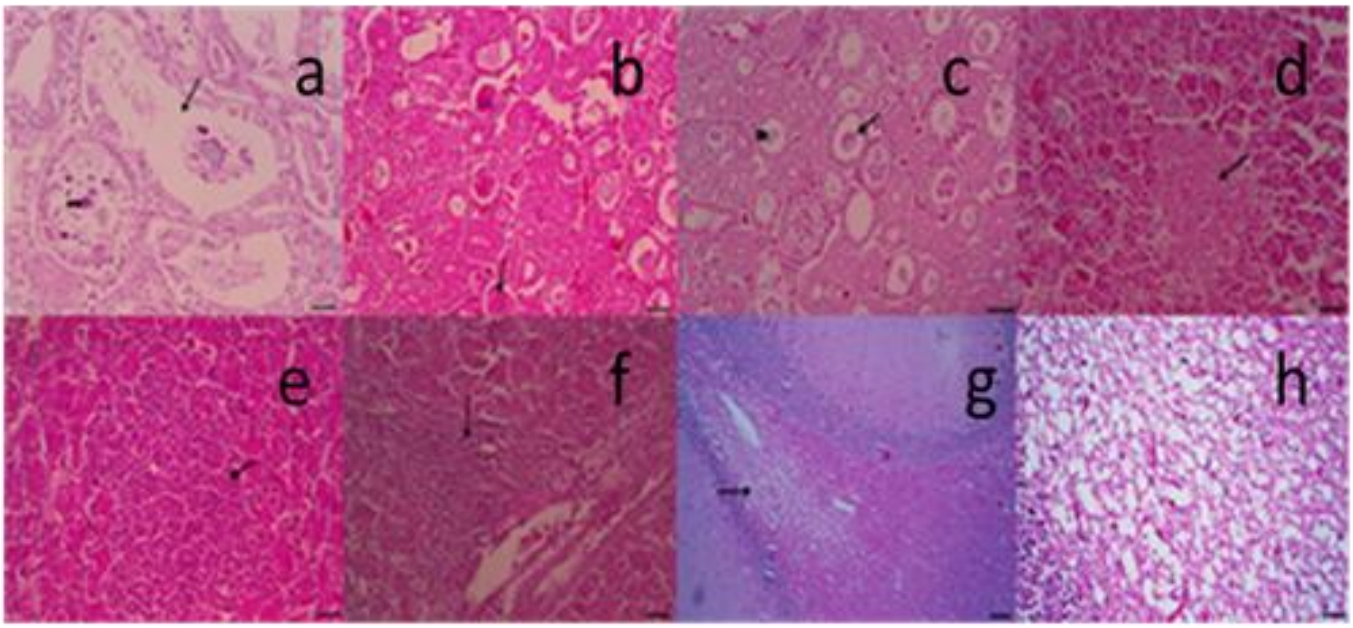

Figure 1. Microscopic lesions of kidney, liver and brain in broilers under lithium toxicity

Mineralization and vacuolation of proximal convoluted tubules (**Figure a**; H & E, scale bar=200 μm), hyperemia, glomerular atrophy and dilatation of the urinary spaces (**Figure b**; H & E, scale bar=250 μm), Proteinaceous casts and cellular debris in some of the degenerated or necrotic tubules (**Figure b** and **c**; H & E, scale bar=250 μm), fatty infiltration and focal necrosis (**Figure d**; H & E, scale bar=250 μm), infiltration of lymphocytes, plasma cells and macrophages in portal area (**Figure e** and **f**; H & E, scale bar=250 μm) and cellular vaccuolation and spongiosis of cerebrum (**Figure g**; H & E, scale bar=370 μm and **Figure h**; H & E, ×40 scale bar=105 μm).

Table 1. Means and standard deviations of serum activities of Serum Glutamate Pyruvate Transaminase (SGPT), Glutamate Oxaloacetate Transaminase (SGOT) and uric acid of broilers at the end of experiment

Group	SGPT (U/L)	SGOT (U/mL)	Uric acid (mg/dL)
Control	5.849±1.727a*	274.09±52.48b	5.2±1.18a
Lithium	14.103±383b	298.95±49.11b	9.5±2.19c

*Different small letters in the same row indicate significant difference among groups (P<0.05).

DISCUSSION

Normally lithium is not present in significant amounts in body fluids and doesn't appear to be an essential element for life (Shahzad et al., 2017; Léonard et al., 1995). Lithium can substitute for sodium or potassium, thus providing a pathway for lithium entry into cell cytoplasm (Timmer and Sands, 1999). The pathways for transporting lithium out of cells are more limited, resulting in lithium accumulating intracellularly (Timmer and Sands, 1999). When lithium is ingested in excessive amounts, it primarily affects the gastrointestinal (GI) tract, central nervous system and kidneys. Lithium is well absorbed from the GI tract (Casarett and Doull, 1975; Ellenhorn and Barceloux, 1997; Schrauzer, 2002). It primarily affects the GI tract. Hyperemia and hemorrhages with infiltration of mononuclear cells in the sub-mucosa and lamina propria of the GI organs in the experimental chickens of the present study indicate that lithium is harmful and results in consistent lesions in GI organs. Lithium is excreted almost entirely by the kidneys (McCartney et al., 2014). Lithium is freely filtered by the glomeruli since it is not bound to serum proteins and in the proximal tubules it is handled similar to sodium (McCartney et al., 2014). Approximately 80% of the lithium that is filtered by the glomeruli is reabsorbed; the remainder is excreted in the urine. Of the filtered lithium 60% is reabsorbed in the proximal tubules and 20% between the loop of Henle and the collecting ducts (Okusa and Crystal, 1994). The nephrotoxic effects of lithium have been divided into three main categories: nephrogenic diabetes insipidus, acute intoxication, and chronic renal disease (Markowitz et al., 2000). Nephrogenic diabetes insipidus (NDI) is the most

common renal side effect of lithium therapy. Patients present with polyuria and polydipsia due to a urinary concentrating defect that can lead to significant volume depletion. Acute lithium intoxication due to lithium overdose includes acute renal failure and volume depletion and is mostly seen in long-term lithium therapy (Ott et al., 2016). The predominant form of chronic renal disease associated with lithium therapy is a chronic tubulo-interstitial nephropathy (CTIN) that is heralded by the insidious development of renal insufficiency, often in the setting of chronic NDI (Hasegawa et al., 2017). Biopsy findings in patients with lithium-induced CTIN include tubular atrophy and interstitial fibrosis, typically out of proportion to the degree of glomerulo-sclerosis or vascular disease (Aurell et al., 1981; Hestbech et al., 1977; Hetmar et al., 1987; Jørgensen et al., 1984). Majority of studies have shown infrequent and relatively, mild renal insufficiency attributable to lithium therapy (Gitlin, 2016). Much less has been reported about the potential glomerular toxicity of lithium and this particular aspect has been underappreciated. Walker et al. reported occurrence of mild nephrotoxicity in association with lithium therapy and the New Zealand White rabbits treated with lithium developed a pattern of CTIN with tubular cysts that was virtually identical to the human disease, with progressive renal insufficiency (Walker et al., 1986) . In another experiment the male wistar rats treated with lithium developed nephrogenic diabetes insipidus and a distal tubulopathy marked by tubular dilatation (Zardawi et al., 2013). Lithium salts induce renal toxicological symptoms such as sclerotic glomeruli and tubular damage (Chmielnicka and Nasiadek, 2003). In their experiment the inability of the nephrons to concentrate urine during lithium treatment was correlated to the occurrence of histological changes. Chmielnicke and Nasiadeks (2003) showed that oral administration of lithium carbonate induced renal toxicity as well as injurious symptoms which were found to be directly related to the dose effect and to the concentration of this metal in serum and urine in rat (Chmielnicka and Nasiadek, 2003). Walker et al. also reported morphological changes corresponding with Li-NDI induce tubulo-interstitial fibrosis, vacuolation and swelling of the cytoplasm, as well as accumulation of glycogen-like PAS positive material in tubular epithelial cells (Walker et al., 1986). In some instances tubular atrophy and glomerular sclerosis have been observed. The above results were almost comparable to our findings that showed vacuolation and swelling of the cytoplasm resulting in tubular degeneration and necrosis. In addition, in the present study, there was a significant increase ($P<0.05$) in the serum level of uric acid as a marker for renal function. The principal site of uric acid secretion appears to be in the proximal tubules of the cortical nephrons (Thrall et al., 2012) where most portion of the lithium is reabsorbed. Uric acid is the major end product of nitrogen metabolism in birds and evaluation of the serum or plasma uric acid concentration has been widely used in the detection of kidney diseases in birds (Thrall et al., 2012). Liver is the main site of metabolization of xenobiotics. Histopathologic examination of the liver in the lithium group revealed multifocal necrosis and infiltration of inflammatory cells, mainly lymphocytes, plasma cell and macrophages in the portal tracts and hepatic parenchyma. Hepatocellular fatty degeneration, hemorrhages and hyperemia were evident in some of the experimental animals. These findings were comparable to those of Sharif et al. who showed focal infiltration of mononuclear cell infiltration with hemorrhagic areas in the paranchyma and portal tracts in the animals which were naturally exposed to lithium poisoning (Sharif et al., 2011). Measurement of the serum levels of SGOT/AST and SGPT/ALT showed a significant ($P<0.05$) increase in SGPT level and slight but not significant changes in SGOT, from which the hepatic dysfunction can be demonstrated. However, the aminotransferase level has also been recently found in skeletal muscle, heart muscle, brain and kidneys, which makes interpretation of increased plasma AST activity challenging (Thrall et al., 2012). But enhanced serum concentration of the SGPT and SGOT in the lithium group is probably suggestive of liver and intestinal damage as suggested by Sinclair et.al. (1984). Another important organ that can be affected in lithium poisoning is the central nervous system and the toxic effects of lithium salts on the central nervous system have been described for nearly a century (Kjølholt et al., 2003). Persistent neurological deficits have been reported in cases of lithium carbonate intoxication (Nagaraja et al., 1987; Niethammer and Ford, 2007). Previous findings showed that lithium absorption by the central nervous system is not uniform; it may be remain in the plasma and high level in the brain at the therapeutic level (Won and Kim, 2017). The symptoms of lithium neurotoxicity at therapeutic levels are mostly neurological and differ only in the extent of injury from those described in cases of toxicity with high levels. The clinical signs and lesions can occur in both acute and chronic therapy; the most common presentation is one of an encephalopathy. Rarely focal neurological disturbances, motor disturbances, psychotic episodes and specific cognitive deficits may occur (Bell et al., 1993; Sheean, 1990; Verdoux and Bourgeois, 1990). These findings suggest that presence of serum lithium concentrations above the therapeutic levels is not mandatory and that lithium neurotoxicity mechanism seems to be a multifactorial entity. Comparable to the

previous studies, we observed some degenerative lesions in the brain that mainly contained white matter spongiosis and cellular vacuolation. Association between lithium toxicity and cerebral degeneration has similarly been suggested by neuropathological studies which have demonstrated spongiform changes of the white matter and changes in the dentate nucleus. The heart didn't show any lesions. There is no evidence about pathological changes of heart subsequent to lithium toxicity, but some clinical signs have previously been reported. It has been indicated that intravenous lithium salts depressed the heart's action and caused a fall in blood pressure in animals (Good, 1903). The dose of lithium salts necessary to stop the heart has been found to be much larger than the dose of potassium salts necessary to produce the same effect. Leonard et al. indicated that no information on the possible carcinogenic effects of lithium compounds was available. However, this seems unlikely in view of the known biological mechanisms of action of lithium (Mohandas and Rajmohan, 2007). In general, we can say the type and severity of lesions depends on the level of accumulation of this chemical pollutant, that is dependent on the diet, the intensity of exposure, the time spent in a habitat, and various kinds of physiological dysfunctions (Bos et al., 2012; Esselink et al., 1995), and each one can permute the severity of toxicity and consequent lesions.

DECLARATIONS

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Author`s contributions

The authors declare that there is no conflict of interest. R.R., I.S. and M.A. contributed to the conception, design and interpretation of data. A.O, M.S, H.R. was also involved in the collection of data, and drafting of the manuscript. All authors check and approved the final manuscript.

Competing interests

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Effect of Cold Stress and Various Suitable Remedies on Performance of Broiler Chicken

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ABSTRACT

A biological trial was conducted on commercial chicks during the winter months (December and January). Day old commercial meat type broiler chicks (273) were procured from a reputed source. Cold conditioning (2^0C to 8^0C) at third and fourth day of age for 3-4 hours was provided to 78 birds. These early cold conditioned birds were kept separate until distributed into respective treatment groups (fifth and sixth). At the end of second week, the chicks were individually weighed, distributed into 7 treatment groups of 3 replicates with 13 chicks in each replicate. Cold challenge @ 2^0C to 8^0C for 8 hours was provided from third week of age to sixth week of their age for all treatment groups except first and fifth treatment groups. The broiler birds in the treatment groups T_1 and T_5 were reared under normal temperature conditions (25^0C). Treatment group first (T_1) was kept as control group. Antioxidant Vitamin E 250 mg per kg of feed was supplemented to the basal diet in the third treatment group. Chromium 0.1 gram per kg of feed was supplemented to the basal diet in the fourth treatment group. Chromium 0.2 gram per kg of feed was supplemented to the basal diet in the seventh treatment group. The data on individual body weight of the experimental birds and the cumulative feed consumption and feed conversion ratio on group basis were recorded at weekly intervals. Deaths were recorded daily and all dead birds were necropsied to identify ascites syndrome. There was no significant ($p<0.05$) difference in the average body weight and body weight gain among various treatment groups throughout the experiment period. The cumulative feed consumption showed significant ($p<0.05$) difference among various treatment groups throughout the experiment period. Highest feed consumption ($p<0.05$) was observed in broiler chickens reared under cold conditions when compared with broiler birds reared under normal temperature conditions. Among the cold challenge treatment groups (T_2, T_3, T_4, T_6 and T_7), there was significant ($p<0.05$) improvement in feed conversion ratio (FCR) in the treatment groups T_6 (early cold conditioning birds exposed to cold stress) and T_7 (supplementation of chromium 0.2 g/kg of feed to birds exposed to cold stress). Among different treatment groups in general best FCR was observed in treatment group T_5 (early cold conditioning group reared under normal conditions) followed by T_1 (control group reared under normal conditions). At the end of the biological trial ascites linked mortalities showed significant ($p<0.05$) difference among various treatment groups. There was no mortality reported in treatment groups kept under normal temperature conditions (T_1 and T_5). Highest ascites related mortality (23.07%) was observed in treatment group in which cold stress was provided and no measures were taken to alleviate the effect of cold stress on broiler birds (T_2). The Vitamin E supplementation in the diet of broiler birds reared under cold stress (T_3) showed significant ($p<0.05$) reduction in ascites related mortality (10.25%).

Keywords: Ascites, Broiler chicken, Early cold conditioning, Chromium, Cold stress, Performance, Vitamin E

INTRODUCTION

The State of Jammu and Kashmir falls in the north-western region of the great Himalayas in India. The average altitude of the valley of Kashmir is 1850 metres above the mean sea level. It is surrounded by mountains, which are always snow-clad. The climate in the valley of Kashmir has its own peculiarities. The season of winter is quite cold which lasts from November to March. These months are characterised by the onset of snow and rain as a consequence of Mediterranean depressions (Raina, 2002). The temperature from December 24 to March 8 is often below zero (Raina, 2002). According to the nineteenth livestock Census report, the total poultry population in the country is 729.2 million and there has been growth in the poultry production by 12.39%. It is estimated that Indian poultry industry contributes about 422 million United States dollars to the GDP (gross domestic production) of the country. But the state of Jammu and Kashmir with only 8 million poultry population, ranks seventh in the Poultry population in the country (Anonymous, 2014).

Thus, the situation in Kashmir regarding the poultry sector is different from the rest of the country. The economy of Kashmir Valley is badly affected due to outflow of the money to outside states owing to the poultry imports into the valley of Kashmir (Gilani, 2009). Our state is also a worse hit when it comes to unemployment and it is assuming enormous proportions with every passing day. The poultry industry is one of the activities which could generate employment. To counter the problem of unemployment we can turn to the self-employment schemes (Banday et al., 2013). This provides us with plenty of avenues to absorb the educated unemployed youth. But there are certain problems related to poultry sector in Kashmir (Gilani, 2009).

Physiological tolerance of organisms is a strong determinant of the environmental conditions in which they inhabit. At certain range of environmental temperature the organisms maintain a normal body temperature with least involvement of thermoregulatory mechanism. This range of ambient temperature is called a zone of thermo-neutrality (Kampen et al. 1979). The environmental temperature beyond the upper and lower limit of the thermoneutral zone is supposed to produce heat or cold stress in animals (Meltzer, 1983). The adverse climatic condition produces physiological stress which has profound economic influence on the productive efficiency including health and disease resistant capacity (Phuong et al., 2016). Exposure of poultry birds to extreme temperature stressor modulates the immune responsiveness and haemato-biochemical parameters of birds (Hangalapura et al., 2004). Among all the environmental stressors, cold stress induces physiological responses which are of high priority and energy demanding for homeotherms. Cold temperature can increase ascites susceptibility by increasing both metabolic oxygen requirements and pulmonary hypertension (Stolz et al., 1992). The biggest obstacle in raising broilers at high altitudes and cold conditions is the ascites syndrome. This condition can be characterized by an accumulation of fluid in the abdominal cavity and elevated mortality that tends to peak between 4-6 weeks of age (James, 2005).

In addition to this, the winter rearing of broiler chickens is associated with excess moisture content of the litter material, which in turn results in elevated levels of air contaminants, such as ammonia (Campbell et al., 2008). Chickens can be imbued with better thermal stress tolerance during pre-natal and early post-natal period by epigenetic adaptation mechanisms, characterized as genomic imprinting, which occur to pre-adapt the organism for the expected post-natal environmental conditions (Nichelmann et al., 2001; Nichelmann, 2002; Tzschentke and Basta, 2002). It is based on the influence that environmental conditions may have on the set point of the physiological control systems (D.orner, 1974). It can also be achieved during early post-natal period by thermal conditioning (Arjona et al., 1988 and 1990; Yahav and Hurwitz, 1996; Yahav et al., 1997), or during life span, by acclimation to extreme environmental temperatures (Hurwitz et al., 1980; Yahav et al., 1995). Shinder et al. (2002) reported that short-term cold conditioning of chickens at an early age could induce an improvement either in thermotolerance during cold challenge or in performance of chickens exposed to an optimal environmental temperature.

Antioxidant plays an important role in both nutrition and production performance in poultry. Dietary supplementation of vitamin E at levels of higher than the National Research council (NRC, 1994) recommendations for poultry enhanced the immune response (Lin et al., 2004) and general performance (Guo et al., 2001). The higher doses of vitamin E had positive influence on the productive performance than lower doses in quails (Biswas et al., 2008). It is also suggested that high vitamin E supply can alleviate oxidative stress in Pulmonary Hypertension Syndrome (Iqbal et al., 2002; Niu et al., 2018) and can be beneficial in reducing ascites mortality

in broilers (Bottje et al., 1995). Chromium is an essential micromineral, which is required for nutrient metabolism (Anderson, 1987). Moreover, Chromium content of poultry feed is very low, therefore its requirement increases during stress (Zulfiqar et al., 2016; Mayada et al., 2017). Such circumstances demand for supplementation of this essential trace element to optimize productive performance in poultry (Khan et al., 2014). Based on aforementioned facts, a research study was conducted to evaluate the effect of cold on performance in broiler chicken along with examining effect of early cold conditioning and use of anti-oxidants (Vitamin E and Chromium) on the ability to cope with cold exposure during their life span.

MATERIALS AND METHODS

Methodology

Day-old commercial meat type broiler chicks (273) were procured from a reputed source. Chicks were reared in battery cages until 14 days of age. During the first seven days period all the birds were provided with a pre-starter mash (23% crude protein). They were provided starter (crude protein 22%) and finisher (crude protein 19%) diets from periods first week to third week and fourth week to sixth week of their age respectively. The diets were iso-nitrogenous, isocaloric and formulated to meet the recommendations of the bureau of Indian standards (BIS, 1992). Birds had free access to feed and water throughout and were maintained on a constant 24-hour light schedule. All chicks were vaccinated against Ranikhet disease on 5th day with F1 strain vaccine and IBV-95 vaccine against infectious bursal disease on 16th day. Chicks were checked twice daily for mortality, if any.

Experiment design

A biological trial was conducted on commercial chicks during the winter months (December and January) in the farm of division of Livestock Production and Management, Faculty of Veterinary Sciences at Shuhama, SKUAST-K. Cold conditioning (2^0C to 8^0C) at third and fourth day of age for 3-4 hours was provided to 78 birds. These early cold conditioned birds were kept separate until distributed into respective treatment groups (fifth and sixth). On fourteenth day (end of second week), the chicks were individually weighed, distributed into seven treatment groups of three replicates with 13 chicks in each in a completely randomized design so that the treatment means differ as little as possible. Cold challenge 2^0C to 8^0C for 8 hours was provided from third week of age to sixth week of their age for all treatment groups except first and fifth treatment groups. The broiler birds in the treatment groups T_1 and T_5 were reared under normal temperature conditions (25^0C). Treatment group first (T_1) was kept as control group. Antioxidant vitamin E 250 mg per kg of feed was supplemented to the basal diet in the third treatment group. Chromium 0.1 gram per kg of feed was supplemented to the basal diet in the fourth treatment group. Chromium 0.2 gram per kg of feed was supplemented to the basal diet in the seventh treatment group. E-Care (Vitamin E) from Gujarat Liqui Pharmacaps India was source of Vitamin E. Chromisac from Zeus Biotech Limited India was source of chromium. The birds were reared on deep litter system throughout the experimental period. The treatment group second was subjected to cold challenge and no antioxidant supplementation of any kind was added to the basal diet.

Parameter recorded

The data on individual body weight of the experimental birds and the cumulative feed consumption and feed conversion ratio on group basis were recorded at weekly intervals. Deaths were recorded daily and all dead birds were necropsied to identify ascites syndrome.

Ethical approval

The study was conducted after approval of research committee and institutional ethical committee (registration no: 1809/GO/ReBi/S/15/CPCSEA).

Statistical Analysis

The data obtained were statistically assessed by the analysis of variance (ANOVA) through General Linear Model procedure of SPSS (10.0) software considering replicates as experimental units and the values were expressed as means±standard error. Duncan's multiple range test (Duncan 1955) was used to test the significance of difference between means by considering the differences significant at $p<0.05$.

RESULTS AND DISCUSSION

There was no significant ($p>0.05$) difference in the average body weight and body weight gain among various treatment groups throughout the experiment period (Table 1 and 2). It shows that cold stress did not adversely affect body weight in broiler chicken. The results are in agreement with Blahova et al. (2007). He reported that cold stress did not significantly ($p>0.05$) effect body weight in broiler chicken. However, Aksit et al. (2008) reported lower body weight gains when broiler birds were subjected to cold stress. But, Leenstra and Cahaner (1991),

in a study investigated genotype and environmental temperature interactions and reported that the low temperature caused the highest growth rate in all genotypes. Actually, body weight of broiler birds reared under cold stress conditions is closely related to their feed consumption.

The cumulative feed consumption showed significant ($p<0.05$) difference among various treatment groups throughout the experiment period (Table 3). The difference was discernible clearly after third week of their age. At the end of third week lowest feed consumption was recorded in treatment groups reared under normal temperature conditions (T_1 and T_5). The broiler birds reared under cold stress showed significantly ($p<0.05$) higher feed consumption at the end of third week when compared with the control group. The cumulative feed consumption at the end of sixth week showed similar significant ($p<0.05$) impact of cold stress on feed consumption and metabolism pattern of broiler chicken (Table 3). Highest cumulative feed consumption ($p<0.05$) was observed in broiler birds reared under cold conditions when compared with broiler birds reared under normal conditions (Table 3). The results are in concordance with Blahova et al. (2007) and Aksit et al. (2008). They independently reported the increase in feed consumption in broiler chicken reared under cold stress conditions. Poultry are homeotherm animals that can live comfortably only in a relatively narrow zone of thermoneutrality (Blahova et al. 2007). It is in order to balance their body temperatures, birds are forced to increase feed consumption under low temperatures (Aksit et al. 2008). This explains the finding regarding less feed consumption in the treatment groups (T_1 and T_5) as they were not subjected to cold challenge.

Table 1. Average weekly body weight (kg) of broiler chicken reared under cold conditions (2^0C to 8^0C for 8 hours) at the farm of faculty of veterinary sciences SKUAST-K in Kashmir region, India

Week	Treatment Groups						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
2	368.15±0.27	371.99±1.57	368.73±4.22	370.24±3.11	373.68±0.66	367.86±3.64	369.57±2.27
3	624.83±2.47	621.22±5.51	625.76±3.68	619.88±6.58	622.92±7.35	627.38±10.32	627.98±5.92
4	981.74±5.38	978.48±9.26	983.11±6.71	980.28±2.16	984.74±5.38	979.53±6.42	986.36±7.35
5	1324.91±0.58	1329.53±6.18	1321.74±3.81	1329.67±2.16	1319.67±2.16	1332.88±2.56	1335.18±3.61
6	1708.29±7.65	1713.39±1.96	1706.87±3.45	1714.46±3.98	1719.10±3.95	1721.62±4.22	1718.15±7.24

Table 2. Average body weight gain (kg) of broiler chicken reared under cold conditions (2^0C to 8^0C for 8 hours) at the farm of faculty of veterinary sciences SKUAST-K in Kashmir region, India

Age in Weeks	Treatment Groups						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
2-3	256.68±0.79	249.23±3.30	257.03±2.10	249.68±3.66	249.24±2.59	259.24±1.89	258.41±4.98
2-4	613.59±3.63	606.49±4.40	614.38±8.31	611.55±4.45	611.06±4.60	611.67±8.28	616.79±4.11
2-5	956.76±9.46	957.54±3.64	953.01±3.17	959.43±4.95	945.67±1.67	965.02±2.70	965.61±4.49
2-6	1340.14±8.56	1341.4±3.89	1338.14±4.43	1344.22±6.48	1345.42±3.27	1353.76±5.46	1348.58±9.75

Table 3. Average weekly feed consumption (grams) of broiler chicken reared under cold conditions (2^0C to 8^0C for 8 hours) at the farm of faculty of veterinary sciences SKUAST-K in Kashmir region, India

Age in Weeks	Treatment Groups						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
2-3	$410.68±1.27^b$	$418.70±3.95^c$	$431.81±1.51^d$	$416.96±2.85^c$	$398.78±0.85^a$	$433.39±1.96^d$	$431.54±102^d$
2-4	$1135.14±2.95^a$	$1225.11±1.97^b$	$1241.04±1.85^b$	$1229.21±3.35^b$	$1124.35±3.05^a$	$1211.10±1.23^b$	$1215.07±0.85^b$
2-5	$1923.08±2.26^a$	$2135.31±2.50^b$	$2115.68±1.63^b$	$2129.93±2.03^b$	$1881.88±2.25^a$	$2074.79±2.35^b$	$2085.71±1.29^b$
2-6	$3082.02±0.85^a$	$3407.15±3.67^c$	$3385.49±2.26^{bc}$	$3400.87±1.25^c$	$3054.10±3.5^a$	$3357.32±0.95^b$	$3344.47±1.38^b$

Means within the same row with different superscripts are significantly different ($p\leq0.05$)

Table 4. Average weekly feed conversion ratio of broiler chicken reared under cold conditions (2^0C to 8^0C for 8 hours) at the farm of faculty of veterinary sciences SKUAST-K in Kashmir region, India

Age in Weeks	Treatment Groups						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
2-3	1.6±0.03^a	1.68±0.01^b	1.68±0.03^b	1.67±0.01^b	1.60±0.02^a	1.67±0.01^b	1.67±0.5^b
2-4	1.85±0.01^a	2.02±0.05^c	2.02±0.08^c	2.01±0.02^{bc}	1.84±0.08^a	1.98±0.01^b	1.97±0.05^b
2-5	2.01±0.05^a	2.23±0.08^c	2.22±0.03^c	2.22±0.01^c	1.99±0.05^a	2.15±0.04^b	2.16±0.05^b
2-6	2.3±0.08^b	2.54±0.06^d	2.53±0.04^d	2.53±0.03^d	2.27±0.05^a	2.48±0.05^c	2.48±0.03^c

Means within the same row with different superscripts are significantly different ($p \leq 0.05$)

Table 5. Related mortality percentage of ascites in broiler chicken reared under cold conditions (2^0C to 8^0C for 8 hours) at the farm of faculty of veterinary sciences SKUAST-K in Kashmir region, India

Treatment group	Mortality percentage
T_1	0± 0.0^a
T_2	23.07± 4.43^d
T_3	10.25± 2.51^b
T_4	20.51± 256^d
T_5	0±0.0^a
T_6	15.38± 0.0^c
T_7	15.38± 4.43^c

Means within the same row with different superscripts are significantly different ($p \leq 0.05$)

The significant ($p<0.05$) difference in FCR was observed among various treatment groups (Table 4). Among the cold challenge treatment groups (T_2, T_3, T_4, T_6 and T_7), there was significant ($p<0.05$) improvement in FCR in the treatment groups T_6 (early cold conditioning birds exposed to cold stress) and T_7 (supplementation of chromium 0.2 g/kg of feed to birds exposed to cold stress). Among different treatment groups in general best FCR was observed in treatment group T_5 (early cold conditioning group reared under normal conditions) followed by T_1 (control group reared under normal conditions). The results related to effect of cold stress on FCR are in agreement with Blahova et al. (2007) and Aksit et al. (2008). They reported that FCR was negatively affected by cold stress. The negative effect is attributed to the adverse effect of cold on immune response, physiological responses, haemato-biochemical parameters and oxygen availability to tissues (Balog et al., 2003; Yardimici et al., 2006; Blahova et al., 2007; Aksit et al., 2008; Phuong et al., 2016). In the present study it was found that Vitamin E 250 mg/ kg of feed did not significantly ($p>0.05$) improved FCR in the broiler chicken reared under cold conditions. The result is in harmony with the finding of Aksit et al. (2008). The results achieved regarding effect of early cold conditioning on the performance of broiler chicken reared under cold stress or normal conditions are in harmony with other workers (Shinder et al., 2002 and Yardimci et al., 2006) who reported that early cold conditioning improved performance of broiler chicken both under normal and cold stress conditions. Short-term cold conditioning of chickens at an early age can induce an improvement either in thermotolerance during cold challenge, or in performance of chickens that are exposed to an optimal environmental temperature (Shinder et al., 2002). Chickens can be imbued with better thermal stress tolerance during pre-natal and early post-natal period by epigenetic adaptation mechanisms, characterized as genomic imprinting, which occur to pre-adapt the organism for the expected post-natal environmental conditions (Nichelmann et al., 2001; Nichelmann, 2002; Tzschentke and Basta, 2002).

The beneficial effect of chromium in alleviating the effect of cold stress in poultry was also reported by Sahin and Sahin (2001). They suggested that a diet containing chromium can be considered as a protective practise in poultry to lessen the depressive effects of cold stress to certain extend if not completely. The beneficial impacts of chromium have been linked with improvement in the metabolism and immune system in the poultry (Mayada et al., 2017). Dietary supplementation of chromium stimulate the secretion of digestive enzymes by improving the functions of liver and pancreas (Sahin et al., 2005; Onderci et al., 2005; Toghyani et al., 2010; Noori et al.,

2012; Ebrahimzadeh et al., 2013; Hesham et al., 2014; Zulfiqar et al., 2017).

The ascites related mortality rate during the experiment are given per treatment group in table 5. At the end of the biological trial ascites linked mortalities showed significant ($p<0.05$) difference among various treatment groups. There was no mortality reported in treatment groups kept under normal temperature conditions (T_1 and T_5). Highest ascites related mortality percentage (23.07%) was observed in treatment group in which cold stress was provided and no measures were taken to alleviate the effect of cold stress on broiler birds (T_2). Cold temperature increase ascites susceptibility by increasing both metabolic oxygen requirements and pulmonary hypertension (Stolz et al., 1992). Lowest ascites related mortality was reported in treatment group (T_3) in which broiler birds kept under cold stress were supplemented with vitamin E 250 mg/ kg of feed. In these birds, dietary vitamin E supplementation could not entirely prevent ascites mortality induced by cold stress but caused significant ($p<0.05$) decrease to 10.25%. The result is in agreement with Aksit et al. (2008) who reported vitamin E significantly ($p<0.05$) decreased ascites related mortalities in broiler birds exposed to cold stress. Bottje et al. (1995) have shown that vitamin E reduced ascites-induced mortality probably by providing an increase in antioxidant defence against free radicals (Niu et al., 2018).

The mortality percentage in the treatment group in which early cold conditioning before broiler birds were subjected to cold stress was done (T_6) and the treatment group (T_7) in which supplementation of chromium 0.2 g per kg of feed was given to broiler birds kept under cold stress was equal (15.38%). The mortality percentage was significantly ($p<0.05$) lower when compared with the treatment group T_2. The early cold conditioning significantly ($p<0.05$) decreased ascites related mortalities has been reported by other workers also (Shinder et al., 2002; Bahadoran and Hassanzadeh 2009). Schinder et al. (2007) reported that early cold conditioning increased the ability of broiler birds to maintain body temperature and thermotolerance during second cold challenge in later part of their life which in turn decreased incidence of ascites related mortality. This could be related to the change in the endogenous functions of chickens, such as the levels of plasma corticosterone and thyroid hormones. The change of these important parameters is important to epigenetic adaptions that might be beneficial to the metabolic rate or the structural size of the cardiopulmonary systems in broiler chicken (Bahadoran and Hassanzadeh, 2009).

The stress increased production of free radicals which damages the body cells and result in increased poultry mortality and chromium is able to reduce stress due to its antioxidant property which in turn reduces the mortality (Mayada et al., 2017). As cold stress exacerbate a marginal chromium deficiency or increase in requirement, thus implying chromium should be supplemented in the diets of broiler chicken reared under cold stress (Sahin and Sahin, 2002).

CONCLUSION

It can be very well put forward that the temperature of environment is one of the most significant abiotic factors that can influence metabolism and subsequently the production of broiler chickens to the great extent. But various remedies such as providing early cold conditioning to chicks, supplementing vitamin E 250 mg per kg of feed and Chromium 0.2 g per kg of feed can help reduce cold stress.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Author's contributions

All the authors have made substantive contribution to the study.

Consent to publish

All the authors gave their informed consent prior to their inclusion in the study.

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The Effects of some Herbal Essential Oils against *Salmonella* and *Escherichia coli* Isolated from Infected Broiler Flocks

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ABSTRACT

Escherichia coli and *Salmonella* spp are two bacterial infectious diseases responsible for heavy economic losses in the poultry industry. The emergence of antimicrobial resistance and its potential harmful threat to human health has led to a need to find safe alternatives for the control of these bacteria. To this end, the use of herbal remedies in poultry has been suggested. In this study, we have investigated the effect of essential oils extracted from five different herbal plants against *Salmonella spp* and *Escherichia coli* that have been isolated directly from infected broiler flocks. Standard Disk-diffusion method, Minimum Inhibition Concentration and minimum bactericidal concentration were used to determine the inhibitory effect of these essential oils. Also, tetracycline was used as a control group. Among the essential oils, *Carum copticum* had the highest antibacterial properties. The maximum inhibition zone in diameter against *Salmonella* and *Escherichia coli* were respectively 26.7 and 22.5 mm that concern about *Carum copticum* essential oils. According to the results of this study, it was found that some of the essential oils have a stronger antibacterial effect than tetracycline. So, after the complementary studies, some of these herbal plants can be suggested as alternatives to antibiotics for treating infections caused by these bacteria in poultry industry.

Keywords: Essential oil, Herbal plant, *Escherichia coli*, *Salmonella*

INTRODUCTION

The use of antibiotics has improved poultry performance effectively and economically however there has been a developing controversy and much criticism surrounding the use of antibiotics as growth promoters in the poultry industry (Abd El-Galil and Mahmoud, 2015). The high incidence and rising frequency of antibiotic resistance among the bacteria populating poultry presents many public health issues. Based on the theories suggesting that pathogenic bacteria have the ability to become resistant to specific antibiotics, it is difficult to develop drugs and treatments with the abilities to kill them (Hoffman-Pennesi and Wu, 2010). These antibiotic resistant bacteria can be transmitted from poultry to humans through the food chain with serious consequences on public health (Abiala et al., 2016). It is generally accepted that enteric infection and its associated gastrointestinal dysfunction is the major stress that impairs intestinal function and compromises the immunity of food-producing poultry, therefore leading to growth retardation and increased morbidity and mortality of poultry. Strategies including the use of enzymes, organic acids, probiotics, prebiotics, and of the implication of herbal plants for controlling enteric infection, improving poultry immunity, and ameliorating intestinal function are common approaches in order to reduce and maximize poultry production (Gong et al., 2013). Nowadays, herbal plants are used on a large-scale in medicines against drug-resistant bacteria, which are considered as one of the most important reasons for the lack of success of treatment in infectious diseases. Herbal plants are a major source of new medicines and may be considered as an alternative to the usual drugs (Al-Mariri

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et al., 2014). Traditionally, many plant extracts and oils are used as medicinal plants in Iran for many purposes, particularly for respiratory and gastrointestinal disorders (Feiz Haddad et al., 2017). The essential oils of 5 herbal plants used in this work include *Pulicaria gnaphalodes* L. *Ducrosia anethifolia* L, *Carum copticum Benth* L, *Foeniculum vulgare* Mill and *Majorana hortensis Minch* L (Habibi et al, 2017). Essential oils can be beneficial as a feed additive to promote the gut health of chickens and help to reduce the risk of bacterial infections. As consumers are trending toward more health-conscious eating and natural alternatives instead of artificial products, essential oils obtained from herbal plants can be used as natural feed additive for poultry (Hoffman-Pennesi and Wu, 2010). The aim of this study was to screen the in vitro antibacterial activity of 5 plants essential oils against two gram-negative bacteria including *Escherichia coli* (*E. coli*) and *Salmonella* spp that were isolated from broiler flocks, were then compared to common antibiotics used in the poultry industry.

MATERIALS AND METHODS

Collection of herbal samples

The various medicinal plants were obtained from the mountainous area in south of Iran between December 2016 and May 2017. The plants include: *Pulicaria gnaphalodes* L, *Ducrosia anethifolia* L, *Carum copticum Benth* L, *Foeniculum vulgare* Mill and *Majorana hortensis Minch*.

Plants essential oil

In order to provide essential oils, 100 g of each plant (*P. gnaphalodes* L, *D. anethifolia* L, *C. copticum*, *F. vulgare Mill* and *M. hortensis*) was introduced in the distillation flask (1 L), which was connected to a steam generator via a glass tube and to a condenser to retrieve the oil. Aromatic molecules of the essential oils were released from the plant material and evaporated into the hot steam. The hot steam forced the plant material to release the essential oils without burning the plant material itself. Then, steam containing the essential oils was passed through a cooling system in order to condense the steam. The steam was applied for 3 hours. Afterward, the essential oils were collected in tightened vials and stored in a refrigerator. For the carried out of antimicrobial activity test, use of suitable chemical solution, therefore the essential oils were diluted to 100 mg mL^{-1} in dimethyl sulfoxide (DMSO) (Habibi et al, 2017).

Isolation of bacteria

Samples were collected over a period of three weeks from four poultry farms in the south of Iran. Two of these samples showed signs of colibacillosis such as mucous nasal discharge, sneezing, conjunctivitis, facial swelling, Perihepatitis, Peritonitis and Cellulitis over the abdomen. Two other samples showed salmonellosis symptom including depression, ruffled feathers, closed eyes, white diarrhea, vent pasting, loss of appetite, intestinal inflammation and unabsorbed yolk sacs. The sampling in colibacillosis was obtained from the liver and air sack swaps. But the samples in salmonellosis were from cloacal swaps.

Identification of bacteria

Cotton swabs were moistened with autoclaved and placed in sterile bags prior to use in the processing plant. Swabs sampling has done from ventral cloaca, cecum, air sace and trachea that approximately 30 s using a vigorous back and forth motion. The swabs were placed in a tube containing a medium suitable for bacterial transport (Transwab; Medical Wire and Equipment Co. Ltd., Corsham, England) and were sent to the laboratory by ordinary mail. On arrival, the 10 swabs were pooled in a tube containing 3 ml of sterile water. The swabs were whirl-mixed in the tube and were left for approximately 5 min sat room temperature to release the bacteria. For the isolation of *E. coli*, 200 µl of above solution was cultured onto MAC plates and incubated at 35°C for 24 h. Following incubation, lactose-positive colonies (3-5 coloni) were streaked onto eosin-methylene blue agar plates. Typical *E. coli* colonies on eosin-methylene blue agar (green and shiny or with dark or purple centers) were subcultured in 10 ml of Trypticase soy broth and were incubated for 24 h at 37°C. The broth cultures were tested for indole production, and indole-positive cultures were confirmed to be *E. coli* by using API 20E (Biomerieux Vitek, Inc., Hazelwood, Mo). To isolate *Salmonella*, 200 µl of *salmonella* suspicious solution was mixed with the same volume of double-concentrated lactose broth. After incubation at 35°C for 24 h, 1.0 ml of the enrichment broth was transferred into 9.0 ml of tetrathionate broth and incubated at 42°C for 24 h. Following 24 h of incubation, the broth culture was streaked onto xylose-lysine-tergitol 4 agar plates and incubated for 24 h at 37°C. Presumptive *Salmonella*

colonies (3-5 coloni) on xylose-lysine-tergitol 4 plate were selected and used to inoculate triple sugar iron slants, which were then incubated for 24 hrs at 37°C. The identities of *Salmonella* isolates were confirmed by the use of the oxidase test and biochemical strips (API20E, BioMerieux) (Drobniewski, 1993).

Antimicrobial assay

Agar gel disk diffusion test (qualitative method) and Minimum Inhibitory Concentration (MIC) as well as Minimum Bactericidal Concentration (MBC) were used in this study.

Disc diffusion susceptibility

Antibacterial susceptibility assay Muller-Hinton Broth (MHB, Merck) medium was used to grow the test isolates for 22 h at 37°C. Final bacterial numbers were standardized to 1×10^6 cfu/ml. A total of 0.1 ml of bacterial suspension was poured into each plate, containing MHA. The surface culture was prepared by sterile L shape pipet pastor and allowed to remain in contact for 1 min. Thereafter, a 5% concentration of each plant extract and the essential oils was prepared. The sterile filter paper discs (6-mm diameter) were placed on the cultures, and 24 h after incubation at 37°C, the inhibition zone was measured in mm. Tetracycline was used as positive control standard, to determine the sensitivity of each bacterial species tested. All the tests were performed in triplicate (Karami et al, 2017).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

For each extract and essential oil a set of 9 sterile test tubes were used. The stock solutions (500 mg/ml) were further diluted in a 2-fold serial dilution to obtain the following concentrations: 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.91, 1.95, and 0.98 mg/ml. One test tube as a negative control and tetracycline as positive control were used. An aliquot of 1 ml of the bacterial suspension was inoculated into each tube. The negative control tubes were inoculated with the same quantity of extracts. All tubes were incubated at 37 C for 24 hours. The lowest concentration that did not permit any visible growth when compared with the control was considered as the MIC. The contents of all the tubes that showed no visible growth were cultured on MHA, incubated at 37 C for 24 hrs. The MIC was considered as the lowest concentration that could not produce a single bacterial colony and the MBC was defined as the lowest concentration of the extract at which 99.9% of the inoculated microorganisms were killed (Aboaba et al., 2006).

Statistical analysis

The data was analyzed with Statistical Package for the Social Sciences (SPSS), version 16.0 software. All bacterial counts were converted to log10 cfu/ml (g) for analysis and ANOVA was performed. Statistical significance was set at a P-value of $P \leq 0.05$.

RESULTS

In the present study, antibacterial activity of *Pulicaria gnaphalodes* L, *Ducrosia anethifolia* L, *Carum copticum Benth* L, *Foeniculum vulgre Mill* L and *Majorana hortensis Minch* L essential oils were recorded against *salmonella* spp and *E. coli* isolated from broiler flocks. For the evaluation of bacterial susceptibility to herbal agents, we carried out three standard tests including disc diffusion assay, MIC and MBC. Based on the results summarized in Table 1, essential oils from leaves of different herbal plants showed potential activity against *E. coli* that were isolated from broiler flocks with the mean zone of inhibition ranging between 10-26.7 mm. The results, as seen in Table 1, shows that the *E. coli* was the most susceptible to essential oil obtained from *Carum copticum* Benth with an inhibition zone range of 26.7 mm in diameter that was more than tetracycline with inhibition zone of 22 mm in diameter. The lowest effect of essential oil against *E. coli* was related to *Pulicaria gnaphalodes*, with 10 mm of inhibition zone. The activities of the 5 essential oils of herbal plants showed that *Carum copticum* Benth have the highest inhibition zone diameter against *salmonella* spp, and the second susceptibility of *salmonella* spp was related to *Majorana hortensis* Minch essential oil but the other essential oil had no effect on *salmonella spp* (Table 1).

The inhibition zone in diameter of *Carum copticum Benth* (22.5 mm) was more than the control positive agent (tetracycline). The MIC and MBC of essential oils at the concentrations range from 250 mg/ml to 0.98 mg/ml compared with the activity of tetracycline are shown in Table 2. In general, *E. coli* was more susceptible than *salmonella* spp to herbal agents. The results of the MBC method indicated that three herbal plants had antibacterial activity against *Salmonella* spp but *E. coli* was susceptible toward each five herbal plant agents (Table 2).

Table 1. Evaluation of inhibitory effects of plant essential oils using disc diffusion method (mm)

Bacteria	*P.gnaphalodes*	*D.anethifolia*	*F.Vulgare*	*M.hortensis*	*C.copticum*	Tetracycline	Control negative
Salmonella spp	0	0	0	18±2.8^{cd}	22.5±4*^{e}	18.5±2^{cd}	0
E. coli	10^{a}	11±2.3^{a}	14.7±3^{b}	16.5±3.8^{bc}	26.7±3.8^{f}	20±4de	0

P.gnaphalodes: Pulicaria gnaphalodes, D.anethifolia: Ducrosia anethifolia, F.Vulgare: Foeniculum vulgare, C.copticum: Carum copticum. The different superscripts are significantly different (P < 0.05), *Mean ± standard deviation

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (mg/ml) values of essential oils of the selected plants against isolated bacteria

Herbal Essential Oil	***Salmonella* spp**		***E. coli* spp**	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
P.gnaphalodes	125	NO	125	250
D.anethifolia	62.5	NO	7.8125	15.625
F.Vulgre Mill	62.5	250	3.91	15.625
M.hortensis	3.91	7.8125	3.91	7.8125
C.copticum	1.95	3.91	0.98	1.95
Tetracycline	1.95	3.91	0.98	3.91
Control negative	NO	NO	NO	NO

NO; No effect. *P.gnaphalodes: Pulicaria gnaphalodes, D.anethifolia: Ducrosia anethifolia, F.Vulgare: Foeniculum vulgare, C.copticum: Carum copticum.*

DISCUSSION

In populations, the alarming prevalence of antimicrobial resistance is a result of antibiotic consumption and because of the pressure exerted by these antibiotics, the spread of these resistant bacteria has increased (Dayaram et al., 2017). This is an indication that the indiscriminate use of conventional antimicrobials in the livestock and poultry industries has led to a steady increase in the antibiotic resistance. One of the most important reasons for the unusual use of antibiotics in the poultry industry is the presence of bacterial infections such as salmonellosis and colibacillosis which are caused by different isolates of *salmonella* and *E. coli*, respectively. Avian colibacillosis and salmonellosis are considered to be the major bacterial diseases in the poultry industry worldwide that are communicable to humans (Lutful Kabir, 2010). Both of these diseases are considered as the most important causes of severe financial loss by its association with high mortality and performance losses in the broiler industry. Therefore, researchers are trying to find out alternatives for antibiotics in order to control and treat these bacterial diseases. Herbal Plants have been documented as one of the sources that possess antimicrobial traits which are chiefly synthesized during secondary metabolism. Plant based antimicrobial compounds have great therapeutic potentials as they can serve the purpose without any side effects associated with synthetic drugs (El-Mahmood and Doughari, 2008). In the current study, essential oils obtained from five herbal plants were used against two pathogenic bacteria. *Carum copticum* Benth essential oil was found to have the most effective antimicrobial property on *E. coli* among all the tested essential oils. Gas chromatography analysis of the essential oils from Iranian *Carum copticum* shows that the three most important constituents of these oils include Thymol, terpinolene and o-cymene (Mohagheghzadeh et al., 2007). Thymol and cymene are two potential antimicrobial agents that exist in *Carum copticum* Benth essential oil. The result of the disc's antibacterial susceptibility, MIC and MBC testing showed that both of the pathogen bacteria are highly susceptible to *Carum copticum* Benth essential oil. Previous studies have reported that the strong antimicrobial potential of the *Carum copticum* Benth can be attributed to thymol and its precursors, cymene and terpinene, have strong antimicrobial activities (Marino et al., 1999; Hassan et al., 2016). Based on current evidence, Ajowan EO can inhibit food-borne pathogenic microorganisms such as

Staphylococcus aureus (Vazirzadeh et al, 2013). The antimicrobial activity of thymol may be induced via modification of the cell membrane permeability and leakage of intracellular material. P-cymene, a major compound detected in *Carum copticum* oil, is a hydrophobic molecule and causes swelling of the cytoplasmic membrane (Burt, 2004). The antimicrobial potential of thymol, p-cymene, Carvacrol, and γ-terpinene against *E. coli* and *Staphylococcus aureus* has been reported in literature reviews (Cristani et al., 2007; Hassan et al., 2016). The results of our study are in consistent with the results of other researchers that have indicated the antimicrobial potential of *Carum copticum* oil against, *Escherichia coli*, *Corynebacterium diphtheriae*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Klebsiella* spp, *Proteus vulgaris* and *Salmonella typhimurium* (Singh et al., 2002; Goudarzi et al., 2011; Hassan et al., 2016). *Majorana hortensis* has been used since times immemorial to treat a wide range of infections. It has been subjected to quite extensive phytochemical, experimental and clinical investigations. The results of this study show that *salmonella* spp *and Escherichia coli* are susceptible to the action of *Majorana hortensis Minch* essential oil. The inhibition zone of *Majorana hortensis* Minch essential oil against both of these bacteria were less than tetracycline, but *salmonella* was more sensitive to marijuana than *Escherichia coli*. In the previous studies, the antibacterial effect of this plant on Proteus vulgaris, Pseudomonas aeruginosa, and Shigella boydii was determined (Habibi et al, 2018). The main component of marjoram essential oils was carvacrol which represented more than 80% in all of the components (Dorman and Deans, 2004). It was explained that the antimicrobial mode of action of the marjoram essential oil is considered to rise mainly from their hydrophobic potential to introduce into the bacterial cell membrane (Mathlouthi et al., 2012). Moreover, marjoram essential oil components can penetrate into the interior of the cell and interact with intracellular sites critical for bacterial activities. More precisely, they are able to inhibit glucosyltransferase enzyme activity, which is responsible in bacteria adhesion to its sites (Cristani et al., 2007; Omara et al., 2014). New research reveals that the marjoram essential oil has antimicrobial activity against *Salmonella* and *E. coli* species that is consistent with the results of this study (Leeja and Thoppil 2007; Omara et al. 2014). The results of antibacterial activity of *Foeniculum vulgare* Mill essential oil showed medium inhibition against *E. coli* and no inhibition against *Salmonella* spp in disc diffusion method but have the less inhibitory effect on *salmonella* in MIC and MBC method. Similar to these results, Aprotosoaie et al. (2008); Tarek et al. (2014); Abdurahim et al. (2017) reported that *E. coli* are susceptible to *Foeniculum vulgare* Mill. Our results were opposed to those obtained by Bisht et al. (2014) who found that the *Foeniculum vulgare* Mill essential oil showed high antimicrobial activity against *salmonella typhimurium* that this might be difference in bacterial species. According to our study, *Ducrosia anethifolia* essential oil has a moderate inhibitory effect on *E. coli* but has no inhibitory effect on *Salmonella* spp. The main components of the essential oil of leaves and stem of *Ducrosia anethifolia* (α-pinene, myrcene, limonene, terpinolene, and E-β-ocimene) were active against gram positive bacteria that among these components, limonene has the most efficient antimicrobial activity against some gram positive bacteria (Ibrahim, 2001). The previous studies also showed the antibacterial effect of this medicinal plant on various species of Mycobacterium, Bacillus cereus, Bacillus sphericus, Bacillus antheracoid, Bacillus coagulance, Bacillus subtlis and Listeria monocytogenes ATCC 1297 (Habibi et al, 2017; Stavri et al, 2003). So far, no research has been done on the antimicrobial effects of *Ducrosia anethifolia* essential oil on *Salmonella* spp and *E. coli* up to this date. Many studies have reported the antibacterial effects of different *Pulicaria* genus but no study has yet to report about the antibacterial activities of the *Pulicar gnaphalodes* species against *salmonella* spp and *E. coli* spp. However, it has been reported that the oils and extracts of the different Pulicaria species had antibacterial activity against *Proteus vulgaris, Pseudomonas aeruginosa,* and *Shigella boydii* (Habibi et al, 2018). It has further been reported that the MIC values of *P. gnaphalodes* against *Salmonella typhimurium* and *Staphylococcus aureus* were 0.2 and 0.1 v/v, respectively (Gandomi et al, 2015). The results of this study has shown that *Pulicaria gnaphalodes* essential oil has a moderate inhibitory effect on *E. coli* but has no inhibitory effect on *Salmonella* spp. Khani and Asghari (2012) reported that the most common components of *Pulicaria gnaphalodes* collected from central mountain of Iran include 65% monoterpenes, with *α-pinene* (34%) and 1,8-cineole (12%) as main compounds, and β-pinen (0.6%), *alloaromadendrene* (0.4%) and *trans-verbenol* (0.2%) as minor compounds were identified in the oil of this plant. Among of these components, the most antibacterial effect has been related to phenolic compound (Nabil Qaid et al., 2014). Nickavar et al. (2002) reported that the gram

positive bacterial strains were more sensitive than the gram negative ones. Nabil Qaid et al. (2014) reported that *E. coli* are more susceptible than *salmonella* spp with the effect of *Pulicaria Inuloides* species that is similar to the results of this study (Nabil Qaid et al. 2014).

CONCLUSION

Pulicaria gnaphalodes L, *Ducrosia anethifolia* L, *Carum copticum Benth* L, *Foeniculum vulgre* Mill and *Majorana hortensis* Minch essential oils have the most inhibitory effect against *E. coli* but there has been less inhibitory effect on *Salmonella* Spp. Avian colibacillosis and salmonellosis are considered to be the avian colibacillosis and salmonellosis are considered to be the major bacterial disease problems in the poultry industry world-wide. In conclusion, *Carum copticum* Benth essential oil contains potential antimicrobial components that may help to prevent and treat some of the poultry diseases associated with *E. coli* and *salmonella* spp.

DECLARATIONS

Competing interests

The authors have no competing interests to declare.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

Author`s contributions

Habibi and Ghahtan were involved in the collection of data, statistical analysis and drafting of the manuscript. Ghahtan and Moramezi read and approved the final manuscript.

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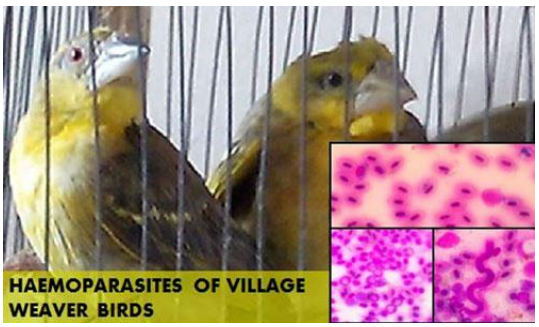

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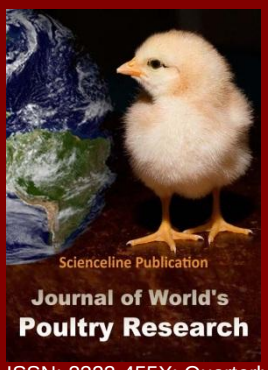

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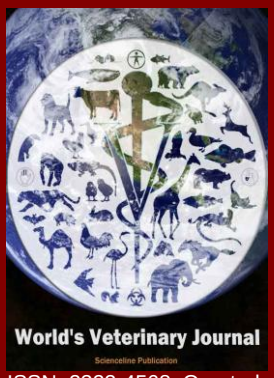

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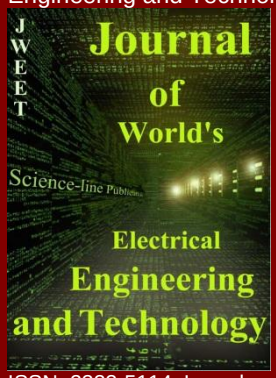

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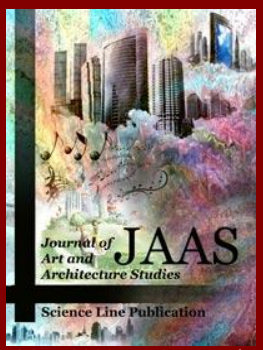

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