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Research article

Congenital abnormalities in dead-in-shell chicks associated with mixed bacterial infections

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ABSTRACT

Determination of the chick embryonic developmental period at which embryonic mortalities occur could help in establishing the cause of such mortalities. The late stage of embryonic development has particular importance due to its dramatic effect on life after hatching. This study was conducted to investigate the occurrence, frequency and bacterial isolates from dead-in-shell chick embryos in Northern Jordan. A total of 1,000 unhatched eggs were collected at hatching day from 10 hatcheries located in Northern Jordan. Out of 1,000 eggs, 357 (35.7%) were fertile, of which 210 (58.8%) were dead-in-shell embryos. Approximately 50.5% of the dead embryos displayed abnormalities, including neck muscles with subcutaneous petechial haemorrhages (44.3%), beak abnormalities (3.8%), eye deformities (1.9%) and anencephaly (0.5%). Sixty-six bacterial isolates were identified from 82 samples from the dead-in-shell embryos. The isolates were 22 (33.3%) *Escherichia coli*, 18 (27.3%) *Klebsiella pneumoniae*, 14 (21.2%) *Staphylococcus aureus*, 5 (7.6%) *Pseudomonas aeruginosa*, 4 (6.1%) *Salmonella enteritidis*, 2 (3%) *Bacillus cereus* and 1 (1.5%) *Proteus vulgaris*. Mixed growth was also recorded in 16 (19.5%) samples. There was a significant ($P < 0.05$) association between *Escherichia coli* as a bacterial isolate and the occurrence of neck and beak abnormalities. In this study, infection of check embryos with several bacterial species, particularly *Escherichia coli*, was identified as an important cause of multiple congenital abnormalities involving the neck and beak of unhatched chicks.

1. Introduction

Chick embryo development consists of three distinctive periods: early, middle and late. More than 65% of total chick embryo mortality occurs during the early and late periods [1, 2]. Embryonic mortality can be caused by genetic or environmental factors [3]. Late phase mortality generally coincides with the period of highest demand for efficient respiratory gas exchange [4]. Other main causes related to high mortality are the conditions of incubation, nutritional deficiencies and malformation [5]. Previously, bacteriological examination of dead-in-shell embryos has demonstrated that different bacterial isolates could be implicated in such embryonic mortalities including *Salmonella* spp., *Escherichia coli (E. coli)*, *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *Proteus* spp., *Bacillus subtilis*, *Klebsiella* spp., *Micrococcus* spp. and *Mycoplasma* spp [6].

Northern Jordan contains 10 (23%) of the 43 hatcheries in the country that have a total capacity of 8 million chicks/year; they represent about 3.2% of the total annual chick production in Jordan. There are no studies to describe the rate and causes and conditions associated with dead-in-shell chick embryos in Jordan. Therefore, this study highlights the frequency and developmental abnormalities of dead-in-shell embryos, and the most common bacterial isolates associated with them, in hatcheries located in Northern Jordan.

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2. Materials and methods

2.1. Ethical approval

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Jordan University of Science and Technology (JUST).

2.2. Study area collection of eggs

A total of 1,000 unhatched eggs were randomly collected at the hatching day (21–21.5 days of incubation) from 10 commercial hatcheries in Northern Jordan. The total samples collected per hatchery ranged between 60 and 180 eggs. Eggs originated from Hubbard breeder flocks with an age from 30 to 64 weeks. Hatchery records were also reviewed to determine the hatchability percentage. Eggs were transported in an ice box and were examined within 24 h after collection.

2.3. Examination of unhatched eggs

Unhatched eggs were candled upon arrival to the laboratory to separate cracked from intact eggshells and to determine eggs with no embryonic development from eggs with developing embryos. The breakout procedure and fertility analysis were carried out according to defined criteria [7, 8]. Briefly, egg surfaces were swabbed using 70% alcohol and precautions were taken to prevent contamination of the embryos when eggs were opened from the top of the air cell. The eggs were carefully inspected for any abnormalities. Eggs with shell abnormalities and breaks were excluded. Dead embryos were classified according to their developmental age into three categories: 0–7 days (early dead): small in size, usually a decomposing embryo; 8–14 days (mid dead): more developed embryo and larger in size compared with early dead, feather tracts had appeared; and 15–21 days (late dead: embryo occupies most of the space within the egg). Dead-in-shell embryos were carefully examined to record any embryonic deformities. Egg contents were gently poured into sterile Petri dishes for further examination.

2.4. Bacteriological isolation and identification

Cracked eggs were excluded from bacterial examination. A total of 82 out of 210 dead-in-shell chick embryos were randomly selected and subjected to bacterial isolation. Swab samples from unabsorbed yolk sac, liver, heart, lungs and apparent lesions found in the dead-in-shell embryos were aseptically obtained for bacterial culture [9]. A 0.1% buffered peptone solution was used as a diluent to prepare direct inoculum on nutrient agar, blood agar and MacConkey agar plates (Oxoid, Hampshire, UK). The plates were then incubated at 37 °C for 18–24 h. Bacterial identification was carried out based on colony morphology, Gram staining characteristics and biochemical tests using commercially available kits and reagents. *Pseudomonas aeruginosa* was identified and confirmed using API 20 NE strips typing system (BioMerieux, France) [10]. *E. coli* and *Klebsiella pneumonia* were identified using the API 20E kit (BioMerieux) [11, 12]. *Staphylococcus aureus* was identified according to Rosario et al. [13] For salmonella isolation, swabs were immersed in tetrathionate broth as an enrichment medium followed by separation on selective XLD medium and slide agglutination polyvalent O and H kit (Oxoid) and were further confirmed using polymerase chain reaction (PCR) as described by Woodward and Kirwan [14]. PCR was conducted using two primer pairs, namely ST11 and ST15, which are specific for *Salmonella* spp., [15], and Sef167 and Sef478, which specifically detects the *sefA* gene that encode the major fimbrial protein sefA in *Salmonella enteritidis.* [14].

2.5. Histological examination

Skeletal muscle tissue samples were collected from the neck region of normal embryos as well as embryos with neck deformities and fixed in 10% buffered formalin for 24 h. The tissues were processed routinely for histopathological examination [16].

2.6. Statistical analysis

The chi-square and Fisher's exact tests (SPSS software, version 23; IBM, Armonk, NY, USA) were used to determine the significance regarding proportions and associations between the recorded abnormalities and bacterial isolates.

3. Results

The hatchability rate of the sampled hatcheries ranged from 59% to 76% (mean = 69.4%, median = 72%). The total percentage of hatchability losses because of early, mid and late dead-in-shell embryos ranged was 17%–22%, with a mean of 19.5% and median of 21.5%.

Out of the 1,000 unhatched eggs, 357 (35.7%) were fertile and contained dead embryos of various stages of development. Of the 357 dead-in-shell embryos, 100 (28%) eggs were early (first week), 47 (13.2%) were mid (second week) and 210 (58.8%) were late dead-in-shell embryos. Of the late dead-in-shell embryos, 106/210 (50.5%) were found to have developmental anomalies. The most encountered anomaly was neck muscle with subcutaneous petechial haemorrhages in 93 out of 210 (44.3%) dead-in-shell chicks. There were also beak abnormalities (brachygnathia) in 8/210 (3.8%), eye deformities in 4/210 (1.9%) and skull deformities (anencephaly) in 1/210 (0.5%). Thickened outer and inner shell membrane, which appeared as a white thick tissue, were observed only in 3/210 (1.4%).

3.1. Bacteriological culture

Sixty-six bacterial isolates (80%) were recovered from a total of 82 randomly cultured samples of the dead-in-shell embryos. The isolates were 22 (33.3%) *E. coli*, 18 (27.3%) *K. pneumoniae*, 14 (21.2%) *S. aureus*, 5 (7.6%) *P. aeruginosa*, 4 (6.1%) *S. enteritidis*, 2 (3%) *Bacillus cereus* and one (1.5%) *Proteus vulgaris*. Mixed growth was also recorded in 16 samples (19.5%). *S. aureus* and *E. coli* were present in most of the mixed growth cultures, representing 42% and 29% of the total isolates, respectively.

There was a significant ($P < 0.05$) association between the occurrence of neck and beak abnormalities and isolation of *E. coli* from dead-in-shell embryos (Table 1). *E. coli* was isolated from neck lesions in 70% of examined embryos. No other bacterial isolates were recovered from neck or head lesions nor from the livers, heart or lungs of dead-in-shell embryos.

3.2. Pathological examination

The gross appearance of the neck abnormalities in dead-in-shell chicks is presented in Figure 1. The affected necks exhibited diffuse and extensive haemorrhages that involved most of the dorsal parts of the neck and extended cranially towards the skull and both sides of the face. Histologically, the examined skeletal muscles from the non-affected necks showed normal morphology with no prominent histopathological lesions (Figure 2). The skeletal muscles of the affected necks showed distinctive histopathological changes (Figures 3 and 4). The perimysium and endomysium were diffusely expanded by a large amount of serofibrinous material admixed with a variable number of mixed inflammatory cells and haemorrhage. The myocytes were shrunken with hypereosinophilic cytoplasm and fragmented or lost myofibers (necrotic). In multiple areas, moderate numbers of heterophils and lymphocytes replaced and infiltrated the myofibers and extended into the interstitial

Table 1. Association between *Escherichia coli* positivity and late dead-in-shell chick embryonic abnormalities.

Level	No.	Lesion type	Positivity (%)	X^2	d.f.	P	OR	95% CI
+	22	Beak	30	10.6	1	0.01	13.5	2.2, 64.9
-	60		3					
+	22	Neck	75	7.5	1	0.01	5.1	1.5, 18.4
-	60		40					
+	22	Eye	15	5.1	1	0.09	9.3	0.8, 132
-	60		2					
+	22	Skull	5	0.03	1	0.95	2.8	0.1, 2.1
-	60		2					

Note. CI, confidence interval; OR, odds ratio.

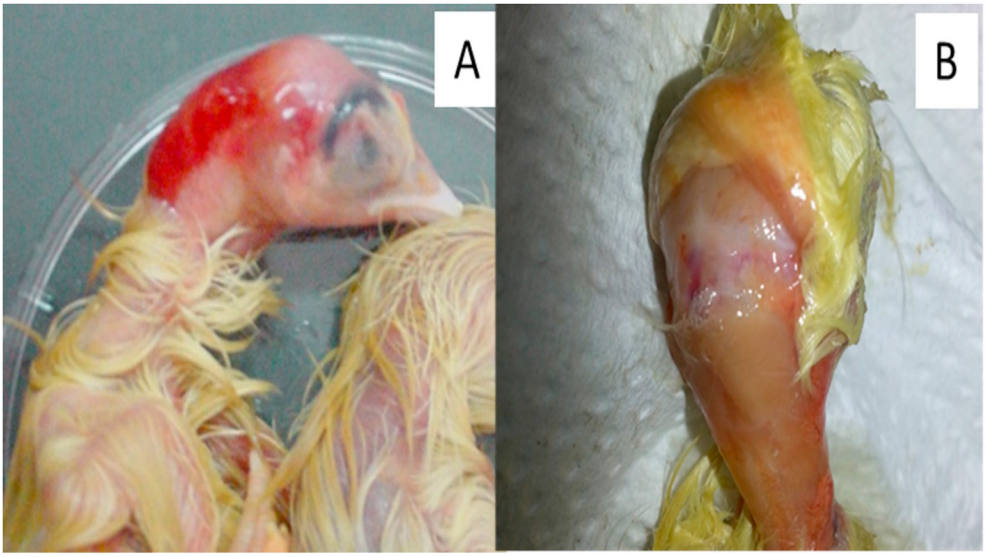

Figure 1. A late dead-in-shell chick embryo with a haemorrhagic neck lesion (A) and a normal neck in a non-affected normal chick (B).

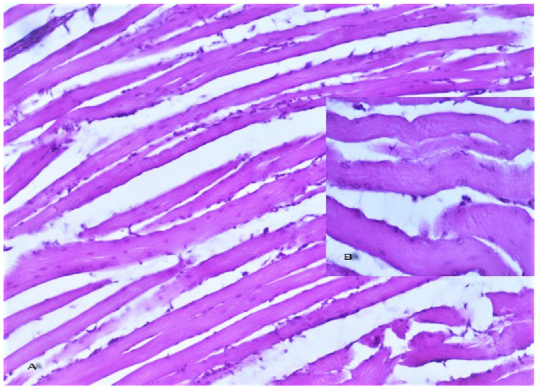

Figure 2. Haematoxylin and eosin–stained section of skeletal muscles of the neck from a normal late dead-in-shell chick embryo. Notice the normal skeletal muscle morphology with no significant histopathological lesions (A; 10×magnification). Normal skeletal muscle histology of late dead-in-shell chick embryo (B; 40×magnification).

tissues (Figure 3). In some sections, moderate fibrosis with collagen deposition that replaced the necrotic myofibers was evident (Figure 4). Adjacent to the fibrotic areas, myocytes exhibited variable degrees of degeneration, myofibers loss and necrosis.

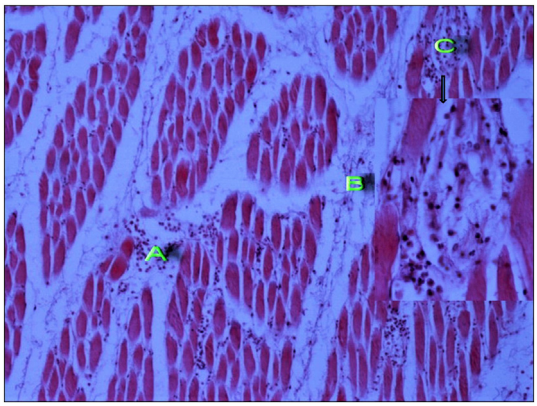

Figure 3. Haematoxylin and eosin–stained section of the skeletal muscles from late dead-in-shell chick embryo with a neck abnormality. Notice the perimysium and endomysium are markedly expanded by serofibrinous material admixed with variable numbers of mixed inflammatory cells (B; 10× magnification). There are moderate numbers of heterophils and lymphocytes that have infiltrated and replaced the myofibres (C; 10× magnification) that extend into the interstitial tissue (A; 10× magnification). The myocytes are characterised by shrunken myocytes, hypereosinophilic cytoplasm and fragmented or lost myofibres. The inset (C) shows a higher magnification of A (40×).

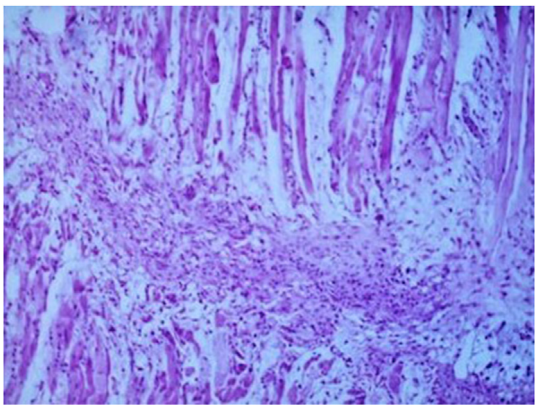

Figure 4. Haematoxylin and eosin–stained section of the skeletal muscles of late dead-in-shell chick embryo with a neck abnormality. Notice the moderate area of fibrosis with associated collagen that replaces the necrotic myofibres. The myofibres adjacent to the fibrosis exhibit variable degrees of degeneration and necrosis (10× magnification).

4. Discussion

In recent years, the poultry industry in Jordan has experienced a rapid development due to the widespread appearance of specialised poultry companies that import and distribute the necessary poultry production equipment and material to the farmers [17]. Moreover, these companies also represent a substantial part of the poultry industry production chain. Good hatchery practices play an important role in the poultry industry chain because hatcheries could be a source of microbial contamination and dissemination to poultry farms [18]. Moreover, failure of the egg hatching process reduces reproductive efficacy and is therefore of economic interest to the players in the poultry industry [19].

From the 357 fertile unhatched eggs, only 210 (58.8%) were dead-in-shell embryos. This mortality rate is relatively lower than the 65% mortality rate for early and late embryonic mortality reported by Jassim et al. [2], but in agreement with the 60% rate for late mortality mentioned by Jordan and Pattison [20]. Such differences can be attributed to several factors related either to the egg itself or the environment or management conditions during egg storage, incubation, breeder nutrition, diseases or genetic causes [20, 21, 22, 23, 24].

In this study, the total percentage of hatchability losses because of early, mid and late dead-in-shell embryos ranged from 17% to 22%, with a mean of 19.5% and median of 21.5%; these values exceed the reported acceptable levels for un-hatched embryos [1]. The most commonly reported abnormality involved the neck and represented 93/140 (66.4%) of all late dead-in-shell deformities. Factors that are known to cause embryonic malformation and abnormal deformities are lethal genes [5] or improper egg handling and storage [25]. Nutritional deficiencies may also cause abnormalities [26]. In this study, there was a significant association between different types of neck and beak deformities and *E. coli*. These findings were similar to previously reported data [27].

The bacteriological examination of dead-in-shell eggs showed the presence of *E. coli*, *K. pneumoniae*, *S. aureus*, *P. aeruginosa*, *S. enteritidis*, *B. cereus* and *P. vulgaris*. These findings are similar to previously reported data regarding bacterial isolates from dead-in-shell chick embryos [6, 11, 12, 13]. Other bacterial isolates from dead-in-shell embryos such as *Micrococcus* spp., *Streptococcus* spp. and *Mycoplasma* spp. have been reported at various percentages in previously published articles, but without great concern about specification the developmental stage of dead embryos [6, 11, 22].

Based on the bacteriological results in this study, *E. coli* was the most prevalent bacteria isolated from the third-week embryonic mortalities. *E. coli*, *Klebsiella* spp. and *Staphylococcus* spp. accounted for approximately 82% of the total isolates in this study. *E. coli* is a common avian pathogen that is mainly associated with yolk sac infections [11, 13]. In addition, *E. coli* infection of fertile eggs in hatcheries has been associated with poor hygienic egg selection and unhygienic hatchery management. Furthermore, *E. coli* problems in chickens are becoming increasingly critical because of the number of isolates resistant to multiple antibiotics and the lack of effective vaccines [23]. In Jordan, it has recently been found that the antimicrobial resistance of avian pathogenic *E. coli* (APEC) is widespread in poultry farms and the environment [24].

The histopathological findings of myocyte degeneration and necrosis with variable degrees of inflammatory cell infiltrates are consistent with the infectious process among the grossly affected neck muscles. This, along with the presence of a significant association between the isolation of *E. coli* and neck deformities, supported our findings that the aetiology of the neck deformities was due to infectious process.

In this study, the frequency of dead-in-shell chick embryos with different developmental deformities during the late incubation period was reported. Although several bacterial species were isolated from dead embryos, there were only statistically significant associations between neck and beak deformities and the isolation of *E. coli*. Therefore, an infectious cause of dead-in-shell embryos with various developmental deformities must be suspected in hatcheries with high hatching percentage losses.

Declarations

Author contribution statement

Wael M. Hananeh: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammad Q. Al-Natour: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Akram R. Alaboudi: Performed the experiments; Wrote the paper.

Mahmoud N. Abo-Shehada: Analyzed and interpreted the data; Wrote the paper.

Zuhair A. Bani Ismail: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

The authors do not have permission to share data.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

[1] S. Tullett, Investigating hatchery practice. Ross Technology: Scotland, Available at, http://www.aviagen.com. (Accessed 2 August 2019).

[2] E. Jassim, M. Grossman, W. Koops, et al., Multiphasic analysis of embryonic mortality in chickens, Poultry Sci. 75 (4) (1996) 464–471.

[3] B. Yoo, E. Wientjes, Rate of decline in hatchability with preincubation storage of chicken eggs depends on genetic strain, Br. Poultry Sci. 32 (4) (1991) 733–740.
[4] H. Rahn, A. Ar, C.V. Paganelli, How bird eggs breathe, Sci. Am. 240 (2) (1979) 46–55.
[5] C. Beaumont, N. Millet, E. Le Bihan-Duval, et al., Genetic parameters of survival to the different stages of embryonic death in laying hens, Poultry Sci. 76 (9) (1997) 1193–1196.
[6] N. Cox, M. Berrang, J. Cason, Salmonella penetration of egg shells and proliferation in broiler hatching eggs–a review, Poultry Sci. 79 (11) (2000) 1571–1574.
[7] J.A. Ranson, Troubleshooting flock fertility problems. Hubbard Technical Bulletin, Available at: https://www.hubbardbreeders.com/media/troubleshooting_flock_fertility_problems_jan_2005_082057200_1448_06012015.pdf. (Accessed 2 August 2019).
[8] Anonymous. Hubbard, Incubation guide, Available at: https://www.hubbardbreeders.com/media/incubation_guideen_053407700_1525_26062017.pdf. (Accessed 22 July 2019).
[9] G. Carter, M. Chengappa, A. Robert, Microbial nutrition, metabolism and growth. Essentials of Veterinary Microbiology, fifth ed., Williams & Wilkins, 1995, p. 394pp.
[10] S. Walker, J. Sander, J. Cline, et al., Characterization of Pseudomonas aeruginosa isolates associated with mortality in broiler chicks, Avian Dis. 46 (4) (2002) 1045–1050.
[11] R.D. Montgomery, L.S. Jones, C.R. Boyle, et al., The embryo lethality of Escherichia coli isolates and its relationship to various in vitro attributes, Avian Dis. 49 (1) (2005) 63–69.
[12] I. Orskov, F. Orskov, B. Jann, et al., Serology, chemistry, and genetics of O and K antigens of Escherichia coli, Bacteriol. Rev. 41 (3) (1977) 667–710.
[13] C. Rosario, C. López, I. Tellez, et al., Serotyping and virulence genes detection in Escherichia coli isolated from fertile and infertile eggs, dead-in-shell embryos, and chickens with yolk sac infection, Avian Dis. 48 (4) (2004) 791–802.
[14] M.J. Woodward, S. Kirwan, Detection of Salmonella enteritidis in eggs by the polymerase chain reaction, Vet. Rec. 138 (17) (1996) 411–413.
[15] S. Aabo, O. Rasmussen, L. Roseen, et al., Salmonella identification by the polymerase chain reaction, Mol. Cell. Probes 7 (3) (1993) 171–178.
[16] K.S. Suvarna, C. Layton, J.D. Bancroft, Bancroft's Theory and Practice of Histological Techniques E. Book, Elsevier Health Sciences, 2018.
[17] M.I. Abu-Iteleh, M.A. Rahahleh, M.S. Alrkaibat, M.N. Daood, Poultry sector country review, Jordan, FAO (2007). http://search.ebscohost.com/login.aspx?directtrue&dbcat02127a&ANfao.837033&siteeds-live. (Accessed 8 February 2021).
[18] T.A. Scott, C. Swetnam, Screening sanitizing agents and methods of application for hatching eggs II. Effectiveness against microorganisms on the egg shell, J. Appl. Poultry Res. 2 (1) (1993) 7–11.
[19] H. Ishaq, M. Akram, M. Baber, et al., Embryonic mortality in cobb broiler breeder strain with three egg weight and storage periods at four production phases, J. Anim. Plant Sci. 24 (6) (2014) 1623–1628.
[20] T.W. Jordan, M. Pattison, Poultry Diseases, W.B. Saunders, Philadelphia, PA, 1996.
[21] J. Thompson, E. Hipwell, H.V. Loo, et al., Effects of cadmium on cell death and cell proliferation in chick embryos, Reprod. Toxicol. 20 (4) (2005) 539–548.
[22] M.A. Khan, M. Ashfaque, I. Hussain, Bacteriology of dead-in-shell broiler embryos and antibiotic sensitivity of the isolates, Pakistan J. Biol. Sci. 2 (2) (1999) 442–444.
[23] L.H. Kahn, G. Bergeron, M.W. Bourassa, et al., From farm management to bacteriophage therapy: strategies to reduce antibiotic use in animal agriculture, Ann. N. Y. Acad. Sci. 1441 (1) (2019) 31–39.
[24] R.A. Ibrahim, T.L. Cryer, S.Q. Lafi, et al., Identification of Escherichia coli from broiler chickens in Jordan, their antimicrobial resistance, gene characterization and the associated risk factors, BMC Vet. Res. 15 (1) (2019) 159.
[25] R.A. Othman, M.R. Amin, S. Rahman, Effect of egg size, age of hen and storage period on fertility, hatchability, embryo mortality and chick malformations in eggs of Japanese quail (Coturnix coturnix japonica), IOSR-JAVS. 7 (1) (2014) 101–106.
[26] A. Monroe, K. Latimer, G. Pesti, et al., Pathology and histology of dietary tryptophan deficiency in broiler chicks, Avian Dis. 47 (4) (2003) 1393–1398.
[27] C.R. Cortés, G.T. Isaías, C.L. Cuello, et al., Bacterial isolation rate from fertile eggs, hatching eggs, and neonatal broilers with yolk sac infection, Rev. Latinoam. Microbiol. 46 (1-2) (2004) 12–16.