Standardisation of a new model of H9N2/ *Escherichia coli* challenge in broilers in the Lebanon

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Summary

Primary infection by low pathogenic avian influenza (LPAI) predisposes for secondary infection by *Escherichia coli* in poultry, leading to significant economic losses. Future research in control of this ailment requires the establishment of a successful controlled challenge by avian influenza virus (AIV)/*E. coli*. Six groups of broilers (6 birds/group) were included for the standardisation of the controlled challenge by AIV/*E. coli*. Birds in groups 1, 2, 3, 4 and 5 received an intra-tracheal challenge of 0.5 ml of two haemagglutinating units of H9N2 virus at 20 days of age. At the age of 23 days, birds in group 1 received an intra-thoracic (right air sac)-*E. coli* challenge equivalent to 1.6×10^9 colony-forming units (cfu)/0.5 ml/bird, while birds in groups 2, 3, 4 and 5 received *E. coli* by the same route and in the following respective decreasing order of viable cells: 1.6×10^6, 1.6×10^5, 1.6×10^4 and 1.6×10^3 cfu. Birds in control group 6 were deprived of H9N2 and *E. coli* challenge. Results showed significant early mortality in group 1 that was challenged with the highest number of *E. coli*, in comparison to groups 2-6 ($p<0.05$); however, the average weight at 28 days of age was similar in surviving birds of groups 2-6 ($p>0.05$). The frequencies of four signs at 2 days and at 5 days post *E. coli* challenge (conjunctivitis, diarrhoea, ocular exudates and rales) in the surviving birds of groups 2-5 were most often higher than those observed in control group 6 ($p<0.05$). These four signs and five gross lesions (abdominal airsacculitis, left thoracic airsacculitis, pericarditis, right thoracic airsacculitis and tracheitis) had a decreasing pattern of frequency related to a decrease in the *E. coli* count used in the challenge.

Keywords

Avian influenza, Broiler, Challenge, *Escherichia coli*, Lebanon, Standardisation, Virus.

Standardizzazione di un nuovo modello di challenge H9N2/ *Escherichia coli* nei broiler in Libano

Riassunto

L'infezione primaria per influenza aviaria a bassa patogenicità (LPAI) predispone i polli ad un'infezione secondaria per Escherichia coli, *causando significative perdite. La ricerca futura sul*

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controllo di questo alimento necessita di un efficace sistema di challenge per il virus dell'influenza aviaria (AIV)/E. coli. *Per la standardizzazione del challenge di controllo per AIV*/E. coli *sono stati esaminati sei gruppi di broiler (6 volatili/gruppo). I volatili dei gruppi 1, 2, 3, 4 e 5 hanno ricevuto un challenge intratracheale di 0,5 ml di due unità emoagglutinanti di virus H9N2 al 20° giorno dalla nascita. A 23 giorni gli esemplari del gruppo 1 hanno ricevuto un challenge intratoracico (sacco aereo destro) di* E. coli *equivalente a* $1,6 \times 10^9$ *unità formanti le colonie (cfu)/0,5 ml/uccello, mentre gli esemplari dei gruppi 2, 3, 4 e 5 hanno ricevuto* E. coli *attraverso la stessa modalità e rispettivamente nel seguente ordine decrescente di cellule vive:* $1,6 \times 10^6$, $1,6 \times 10^5$, $1,6 \times 10^4$ *e* $1,6 \times 10^3$ *cfu. Agli esemplari del gruppo di controllo 6 non è stato somministrato il challenge H9N2*/E. coli. *I risultati evidenziano una significativa precoce mortalità nel gruppo 1, che è stata sottoposto al challenge con il più elevato numero di* E. coli, *rispetto ai gruppi 2-6* ($p<0,05$); *tuttavia, il peso medio al 28° giorno di vita è risultato simile negli esemplari in vita dei gruppi 2-6* ($p>0,05$). *La frequenza di quattro segni al 2° e al 5° giorno successivi al challenge* E. coli *(congiuntivite, diarrea, essudato oculare e rantoli) negli esemplari rimasti in vita dei gruppi 2-5 è risultata molto spesso superiore a quella osservata nel gruppo di controllo 6* ($p<0,05$). *Questi quattro segni e cinque importanti lesioni (aerosacculite addominale, aerosacculite toracica sinistra, pericardite, aerosacculite toracica destra e tracheite) hanno mostrato un modello di frequenza decrescente correlato alla diminuzione della conta di* E. coli *utilizzata nel challenge.*

Parole chiave
Broiler, Challenge, *Escherichia coli*, Influenza aviaria, Libano, Standardizzazione, Virus.

Introduction

It is documented in literature that *Escherichia coli* infection in poultry is the major secondary infection that is responsible for economic losses worldwide (3). This infection by *E. coli* comes secondary to many primary factors including microbial and environmental elements. Among the primary microbial factors are bacterial (*Mycoplasma* spp., *Pasteurella multocida*), viral (low pathogenic avian influenza, Newcastle disease virus, infectious bronchitis virus, infectious bursal disease virus) and protozoal (*Eimeria* spp., *Histomonas* spp.) (3).

At present, the H9N2 virus is infecting poultry in many countries, including the Lebanon (2), Iran, Saudi Arabia, Kuwait, Iraq, Germany and Italy (1), causing serious economic losses (2, 5, 6, 7), especially due to secondary infection by *E. coli* (9).

Research related to reproducibility of *E. coli* pathological effects in poultry has been faced with difficulties which has led to inadequate development of vaccines and therapeutics against this economic secondary infection. This standardisation of *E. coli* secondary infection to other primary agents has been the subject of research for the past decade (7, 8).

The purpose of this paper is to attempt to find a new successful model for the standardisation of a controlled challenge of primary infection by H9N2-avian influenza virus, followed by a secondary infection by *E. coli* in broilers to contribute to future research, targeting the control and/or treatment against this economic impediment.

Materials and methods

Birds

Thirty-six day-old broilers were divided into six groups (six birds per group), with no significant differences in the mean weight among the groups ($p>0.05$); all birds were kept on the floor and grouped into separate isolation rooms. The birds were of Ross 308 breed which is the predominant breed of broilers in Lebanon. These day-old broilers were healthy; there was no presence of omphalitis or any respiratory or enteric signs.

H9N2 challenge

The primary low pathogenic avian influenza (LPAI) virus (H9N2) was administered intratracheally to each bird in groups 1-5 at 20 days of age, in a density of two haemagglutinating units (HA)/0.5 ml. This challenge dose of H9N2 was previously standardised in our

facility resulting in apparent histopathological effects on the trachea and air sacs of broilers.

Escherichia coli challenge

The secondary *E. coli* challenge was administered in the right thoracic air sac using sterile needles and syringes. The *E. coli* strain was recovered from a severe colibacillosis outbreak caused by a primary Newcastle disease virus, on a major broiler farm in the Bekaa Valley of the Lebanon (BVL-strain). This *E. coli* strain when administered alone in 1.7×10^9 colony-forming units (cfu)/0.5 ml/bird in broilers will not result in colibacillosis due to its negligible pathogenicity in the absence of a primary viral infection (4). Birds in groups 1-5 at 23 days of age, received the following respective viable *E. coli* counts, in decreasing order, namely, 1.7×10^9, 1.7×10^6, 1.7×10^5, 1.7×10^4 and 1.7×10^3 cfu/0.5 ml/bird. Group 6 was left as a control, without challenge by H9N2 and *E. coli*. No other control group, challenged only with H9N2, was included in this design, since the main objective was to standardise the level of *E. coli* challenge as a secondary infection to the H9N2 virus.

Clinical signs, mortality and weights

The frequency of clinical signs in each of the six groups was recorded at an age of 20, 25 and 28 days, including: ocular exudates, conjunctivitis, rales, diarrhoea, huddling, nasal discharge, and thick oral saliva. In the period between the H9N2 and the *E. coli* challenges, most birds showed one sign, namely: sneezing. The cumulative mortality percentage up to the age of 28 days, and the live weight at this age were recorded.

Gross lesions

The frequency of each of nine gross lesions in each of the six groups was recorded when the birds were sacrificed (28 days of age). The gross lesions included the following:

- tracheitis
- right thoracic airsacculitis
- left thoracic airsacculitis
- abdominal airsacculitis
- splenomegaly
- pericarditis
- perihepatitis
- enteritis
- pancreatitis.

Statistical methods

One-way analysis of variance (ANOVA) followed by the Tukey test were used for the mean weight comparison. Chi-square test was used for the comparison of frequencies of signs, lesions and mortality percentage among the six groups. Statistical differences among means and among frequencies were reported at $p<0.05$. Both tests were performed using statistical computing software (SPSS 15.0, SPSS Inc., Chicago).

Results

Results related to the new model for standardisation of the secondary infection of *E. coli*, by the intra-thoracic air sac route, following the primary H9N2 challenge, are shown in Tables I, II, III and IV. There was a significant mortality of 83.3% in birds of group 1 that each received the highest viable count of *E. coli*, equivalent to 1.7×10^9 cfu/0.5 ml) (Table I).

Four of the seven signs observed were present at 2 days after the intra-thoracic air sac challenges in groups 2, 3, 4 and 5, namely: ocular exudates, rales, diarrhoea and huddling (Table II). Data for group 1 is missing from Table II due to the high mortality (83.3%) that occurred in this group within a period of four days following the administration of the high *E. coli* dose.

Two signs had a dose effect at two days post the *E. coli* challenge, namely: conjunctivitis and huddling, decreasing in frequency as the *E. coli* challenge decreased in its viable cell count. Four signs were present at five days post the *E. coli* challenge (Table III), with a clearer decreasing trend in frequency of three signs, in relation to a decrease in *E. coli* dose, namely: the signs of ocular exudates, conjunctivitis and rales. It is worth noting that the control birds in group 6 did not show any signs and there was no case of mortality during the experiment.

This new model of H9N2/*E. coli* challenge was able to induce the presence of nine of the nine

Table I
Mortality percentage and average weight in each broiler group at 28 days of age

Group[a]	Challenge		*E. coli* dose per bird	Mortality[b] (%)	Average weight aged 28 days (g)
	H9N2	*E. coli*			
1	+	+	1.7×10^9	83.3[c]	N/A
2	+	+	1.7×10^6	0.0[d]	936.7[d]
3	+	+	1.7×10^5	0.0[d]	1 085.8[d]
4	+	+	1.7×10^4	16.7[d]	979.2[d]
5	+	+	1.7×10^3	0.0[d]	1 010.0[d]
6	–	–	N/A	0.0[d]	920.8[d]

(a) Each bird in groups 1-5 was challenged intra-thoracically with 0.5 ml of an *Escherichia coli* suspension in the right air sac three days after an intra-tracheal challenge with 2 HA units/0.5 ml of H9N2 avian influenza virus administered at 20 days of age
The *E. coli* suspension given to group 1 had a transmittance rate of 3%, corresponding to a viable cell count of 1.7×10^9 cfu/0.5 ml
Groups 2, 3, 4, and 5 received the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution of the suspension given to group 1
Group 6 was the control, which remained unchallenged
Mortality of birds in group 1 occurred within the period of 4 days after *E. coli* challenge
(b) The mortality reported is cumulative, from 23 to 28 days of age; the H9N2 and *E. coli* challenges were administered at 20 and 23 days of age, respectively
N/A Not applicable
(c, d) Percentages and averages in a column followed by (c) and (d) superscripts are significantly different ($p<0.05$)

Table II
Morbidity signs at 25 days of age (2 days post *Escherichia coli* challenge)

Group[a]	Frequency of birds with specific signs/Number tested						
	Ocular exudates	Conjunctivitis	Rales	Diarrhoea	Huddling	Nasal discharge	Thick oral saliva
2	6/6[b]	5/6[c]	6/6[c]	6/6[d]	5/6[d]	0/6[b]	0/6[b]
3	6/6[b]	4/6[c]	6/6[c]	5/6[c, d]	4/6[c, d]	0/6[b]	0/6[b]
4	4/6[b]	3/6[c]	6/6[c]	3/6[c]	2/6[b, c]	0/6[b]	0/6[b]
5	6/6[b]	0/6[b]	5/6[c]	4/6[c, d]	2/6[b, c]	0/6[b]	0/6[b]
6	0/6[c]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]

(a) Each bird in groups 1-5 was challenged intra-thoracically with an *Escherichia coli* suspension in the right air sac three days after an intra-tracheal challenge with 2 HA units/0.5 ml of H9N2 avian influenza virus administered at 20 days of age
The *E. coli* suspension given to birds in group 1 had a transmittance rate of 3%, corresponding to a dose of 1.7×10^9 cfu/0.5 ml, resulting in high and early mortality, leading to the absence from this Table of signs of morbidity in group 1
Groups 2, 3, 4, and 5 received the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution of the suspension given to group 1
Group 6 was the control which remained unchallenged
(b, c, d) Percentages and averages in a column followed by (b), (c) and (d) superscripts are significantly different ($p<0.05$)

observed lesions at the age of 28 days (Table IV). Three of the nine observed lesions had a decreasing trend in frequency with a decrease in *E. coli* dose used in the challenge, namely: tracheitis and right and left thoracic airsacculitis. The control birds in group 6 did not show any of the nine lesions, except for a 50% frequency of pancreatitis.

Discussion

The mortality of 83.3% in birds of group 1 occurred within a short period (4 days), following the *E. coli* challenge, reflecting the acute nature of the 1.7×10^9 cfu/0.5 ml of viable *E. coli* per bird, predisposed by H9N2-LPAI challenge. The other lower *E. coli* challenges in groups 2, 3, 4 and 5 (1.6×10^6-1.6×10^3 cfu/0.5 ml/bird) resulted in low mortality ranging from 0% to 16.7% ($p>0.05$). The average live weight did not differ significantly among the six groups, including the control-unchallenged group ($p>0.05$), resulting in mean body weights in groups 2-6 ranging between 920.8 g to 1 085.8 g. The short period of 5 days after the *E. coli* challenge could be the reason for not finding differences in live weight. Future investigations will focus on the chronic phase

that follows a field challenge by *E. coli* up to the market age of 43-45 days.

The absence of morbidity signs at 20 days of age, the time of administration of H9N2 to birds in groups 1, 2, 3, 4 and 5, was clearly apparent, revealing the healthy status of the birds. As time after the *E. coli* challenge elapsed from 2 to 5 days, the dose effect of the viable cell count of *E. coli* was more prominent, showing a decrease in frequency of three signs and three lesions with a decrease in *E. coli* count used in a challenge (Tables III and IV). This positive relationship between the frequency of specific signs and lesions and the viable cell count of secondary *E. coli* challenge is indicative of the success of this challenge model.

Table III
Morbidity signs at 28 days of age (5 days post *Escherichia coli* challenge)

Group[a]	Frequency of birds with a specific sign/Number tested						
	Ocular exudates	Conjunctivitis	Rales	Diarrhoea	Huddling	Nasal discharge	Thick oral saliva
2	6/6[c]	6/6[c]	6/6[c]	5/6[c]	2/6[b]	0/6[b]	0/6[b]
3	6/6[c]	6/6[c]	4/6[c]	3/6[c]	0/6[b]	0/6[b]	0/6[b]
4	5/5[c]	5/5[c]	4/5[c]	2/5[b, c]	0/5[b]	0/5[b]	0/5[b]
5	2/6[b]	1/6[b]	1/6[b]	4/6[c]	0/6[b]	0/6[b]	0/6[b]
6	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]

(a) Each bird in groups 1-5 was challenged intra-thoracically with 0.5 ml of an *Escherichia coli* suspension in the right air sac three days after an intra-tracheal challenge with 2 HA units/0.5 ml of H9N2 avian influenza virus administered at 20 days of age
The *E. coli* suspension given to birds in group 1 had a transmittance rate of 3%, corresponding to a dose of 1.7×10^9 cfu/0.5 ml, resulting in high and early mortality, leading to the absence from this Table of signs of morbidity in group 1
Groups 2, 3, 4, and 5 were given the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution of the suspension given to group 1
Group 6 was the control which remained unchallenged
(b, c) Frequencies followed by (b) and (c) superscripts are significantly different $p<0.05$)

Table IV
Frequency of birds showing lesions at 28 days of age (5 days post *Escherichia coli* challenge)

Group[a]	Frequency of birds with specific lesion/Number tested								
	Tracheitis	Airsacculitis		Abdominal airsacculitis	Spleno-megaly	Peri-carditis	Peri-hepatitis	Enteritis	Pancre-atitis
		Right thoracic	Left thoracic						
2	6/6[b]	3/6[c]	2/6[b]	1/6[b, c]	0/6[b]	4/6[c, d]	0/6[b]	4/6[b]	6/6[c]
3	5/6[b,c]	3/6[c]	2/6[b]	1/6[b, c]	1/6[b]	1/6[b]	0/6[b]	3/6[c, d]	6/6[c]
4	5/6[b,c]	2/6[b, c]	1/6[b]	3/6[b,c]	0/6[b]	2/6[b, c]	2/6[b, c]	2/6[b, c, d]	6/6[c]
5	3/6[b]	0/6[b]	0/6[b]	1/6[b, c]	0/6[b]	0/6[b]	0/6[b]	1/6[b, c]	5/6[b, c]
6	0/6[a]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	0/6[b]	3/6[b]

(a) Each bird in groups 1-5 was challenged intra-thoracically with 0.5 ml of an *Escherichia coli* suspension in the right thoracic air sac three days after an intra-tracheal challenge with 2 HA units/0.5 ml of H9N2 avian influenza virus administered at 20 days of age
The *E. coli* suspension given to birds in group 1 had a transmittance rate of 3%, corresponding to a dose of 1.7×10^9 cfu/ml, resulting in high and early mortality, leading to the absence from this Table of signs of morbidity in group 1
Birds in groups 2, 3, 4 and 5 received the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution of the suspension given to group 1
Group 6 was the control which remained unchallenged
(b, c, d) Frequencies followed by (b), (c) and (d) superscripts are significantly different $p<0.05$

Conclusion

In conclusion, this model for standardisation of a secondary *E. coli* challenge shows that the intra-thoracic air sac route of challenge helped to establish the *E. coli* dose that produces a high and acute mortality (83.3%) and the doses that results in low mortality and clear specific signs and lesions. It is recommended in future evaluations of new control and/or treatment of secondary *E. coli* to a predisposing H9N2 challenge in broilers, to adopt this successful challenge model in attempts to alleviate the pathological injuries of this widespread ailment, and consequently reduce the current significant economic losses in the poultry sector.

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