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-thoracic 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 × 10⁹ 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 × 10⁸, 1.6 × 10⁷, 1.6 × 10⁶ and 1.6 × 10⁵ 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.

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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...
controlo di questo alimento necessita di un efficace sistema di controllo 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 × 10^8 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 × 10^7, 1,6 × 10^6, 1,6 × 10^5 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 sottoposta 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 intra-tracheal 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 × 10⁸ 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 × 10⁷, 1.7 × 10⁶, 1.7 × 10⁵, 1.7 × 10⁴ and 1.7 × 10³ 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 × 10⁷ 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
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 $1.7 \times 10^9$ 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 ($1.6 \times 10^8$-$1.6 \times 10^9$ 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 1085.8 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.

### Table I

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

<table>
<thead>
<tr>
<th>Group(a)</th>
<th>Challenge</th>
<th>E. coli dose per bird</th>
<th>Mortality(b) (%)</th>
<th>Average weight aged 28 days (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>$1.7 \times 10^9$</td>
<td>83.3(c)</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>$1.7 \times 10^6$</td>
<td>0.0(d)</td>
<td>936.7(d)</td>
</tr>
<tr>
<td>3</td>
<td>++</td>
<td>$1.7 \times 10^5$</td>
<td>0.0(d)</td>
<td>1085.8(d)</td>
</tr>
<tr>
<td>4</td>
<td>++</td>
<td>$1.7 \times 10^4$</td>
<td>16.7(d)</td>
<td>979.2(d)</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>$1.7 \times 10^3$</td>
<td>0.0(d)</td>
<td>1010.0(d)</td>
</tr>
<tr>
<td>6</td>
<td>– –</td>
<td>N/A</td>
<td>0.0(d)</td>
<td>920.8(d)</td>
</tr>
</tbody>
</table>

(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.

(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.

(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)**

<table>
<thead>
<tr>
<th>Group(a)</th>
<th>Frequency of birds with specific signs/Number tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ocular exudates</td>
</tr>
<tr>
<td>2</td>
<td>6/6(b)</td>
</tr>
<tr>
<td>3</td>
<td>6/6(b)</td>
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<tr>
<td>4</td>
<td>4/6(b)</td>
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<tr>
<td>5</td>
<td>6/6(b)</td>
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<tr>
<td>6</td>
<td>0/6(b)</td>
</tr>
</tbody>
</table>

(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.

(b, c, d) Percentages and averages in a column followed by (b), (c) and (d) superscripts are significantly different ($p<0.05$).
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

<table>
<thead>
<tr>
<th>Group</th>
<th>Ocular exudates</th>
<th>Conjunctivitis</th>
<th>Rales</th>
<th>Diarrhoea</th>
<th>Huddling</th>
<th>Nasal discharge</th>
<th>Thick oral saliva</th>
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<tbody>
<tr>
<td>2</td>
<td>6/6&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>6/6&lt;sup&gt;(b)&lt;/sup&gt;</td>
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</table>

(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.

(b) Frequencies followed by (b) superscripts are significantly different p<0.05

### Table IV

<table>
<thead>
<tr>
<th>Group</th>
<th>Tracheitis</th>
<th>Right airsacculitis</th>
<th>Left airsacculitis</th>
<th>Abdominal airsacculitis</th>
<th>Splenomegaly</th>
<th>Pericarditis</th>
<th>Perihepatitis</th>
<th>Enteritis</th>
<th>Pancreatitis</th>
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**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 result 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.

**References**