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Protection conferred by live infectious bronchitis vaccine viruses against variant Middle East IS/885/00-like and IS/1494/06-like isolates in commercial broiler chicks

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ABSTRACT

The ability of the infectious bronchitis H120 (a Massachusetts strain) and CR88 (a 793B strain) live attenuated vaccine viruses to protect from two Middle East infectious bronchitis virus isolates, IS/885/00-like (IS/885) and IS/1494/06-like (IS/1494) in broiler chicks was investigated. Day-old chicks were separated into three groups, (I) vaccinated with H120 at day-old followed by CR88 at 14 days-old, (II) vaccinated with H120 and CR88 simultaneously at day-old and again with CR88 at 14 days-old, (III) control unvaccinated. At 30 days-old, each of the groups was challenged with virulent IS/885 or IS/1494. Protection was evaluated based on the clinical signs, tracheal and kidney gross lesions and tracheal ciliostasis. Results showed that administering combined live H120 and CR88 vaccines simultaneously at day-old followed by CR88 vaccine at 14 days-old gave more than 80 per cent tracheal ciliary protection from both of the Middle East isolates. In addition, this programme conferred 100 per cent protection from clinical signs and tracheal or kidney lesions. The other vaccination programme, H120 at day-old followed by CR88 at 14 days-old, the tracheal ciliary protection conferred were 60 per cent and 80 per cent from IS/885/00-like and IS/1494/06-like, respectively.

INTRODUCTION

Infectious bronchitis virus (IBV) is a ubiquitous virus with high mutation, and a large number of serotypes or genotypes of IBV strains have been reported worldwide (de Wit and others 2011). Variant strains emerge due to changes in the IBV genome through point mutations, deletions, insertions or RNA recombination and these variants are often responsible for IB outbreaks in vaccinated chicken flocks (Cavanagh and others 1988, Jia and others 1995, Liu and others 2007). Many countries have shown that multiple variant IBV strains are circulating in their poultry flocks (de Wit and others 2011).

IS/885/00 and IS/1494/06 or those with high similarities to these strains of IBVs have been reported throughout the Middle East and North Africa (Meir and others 2004), Iraq (Mahmood and others 2011), Egypt (Abdel-Moneim and others 2012), Turkey (Kahya and others 2013) and Libya (Awad and others 2014a). In most cases, severe respiratory distress and renal lesions with high mortality were observed in vaccinated flocks affected by these strains. It appears that the conventional H120 vaccines alone do not provide sufficient protection from these strains (Meir and others 2004, Kahya and others 2013). However, development of vaccines against each of these new variants is not generally an option due to the high cost and time required for product registration (Jackwood and others 2003, Bijlenga and others 2004). Furthermore, it could be a never-ending race as new variant IBVs are constantly emerging in major poultry producing countries. Instead, it has been recommended that protection conferred by available live IBV vaccines against new variants should be constantly evaluated (Alvarado and others 2003). Vaccination with one serotype does not ensure complete protection from heterologous strains (Cook and others 1999) but the use of different combinations of live IBV vaccines has been shown to be able to induce high and broad protection from challenges with several heterologous virulent IBV variants (Gelb and others 1991, Cook and others 1999, Cook and others 2001, Alvarado and others 2003, Martin and others 2007, Terregino and others 2008).

The objective of this study is to evaluate the protection conferred by available live IBV vaccines when used in a strategic manner against the two prominent Middle East variant IBVs that are related to IS/885/00 (Meir and others 2004) and IS/1494/06 (GenBank Accesion number: EU780077). In addition to the conventional vaccination programme (Mass at day-old followed by variant at 14 days-old), the authors also evaluated another vaccination programme where the Mass and variant live vaccines were given simultaneously at day-old followed by a variant vaccine at 14 days-old. Following challenge, protection was assessed based on a ciliostasis assay as recommended by European Pharmacopoeia (Europe 2007). In addition, clinical signs and gross lesions were evaluated.

MATERIALS AND METHODS
Chicks
Day-old commercial broiler chicks with IBV maternally derived antibodies (MDAs) were obtained from a commercial hatchery. The parent flock of these chicks had been vaccinated with live IBV H120+ D274 vaccines and with an inactivated IBV M41. Chicks were kept in an isolation unit at the University of Liverpool. The chicks were reared on deep litter with water and feed provided ad libitum.

IBV vaccines
Two different commercial live vaccines were used in this study; (i) H120, vaccine belonging to Massachusetts serotype (Merial S.A.S, Lyon, France) and (ii) CR88, vaccine belong to 793B serotype (Merial S.A.S, Lyon, France). The vaccines were kept at 4–8°C until used. Each vaccine was dissolved in sterile distilled water.

IBV challenge strains
Virulent strains of IBV IS/885/00-like (referred here as IS/885) and IS/1494/06-like (referred here as IS/1494) were used as challenge viruses. Both viruses had been isolated from a recent outbreak of high mortality and respiratory disease in broiler flocks in Egypt. The isolates were submitted to the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy as third passage allantoic fluid. At this laboratory, the allantoic fluid was screened against avian influenza virus, Newcastle disease virus and IBV. It was negative for avian influenza virus and Newcastle disease virus but positive for IBV. At the University of Liverpool, the IS/885 strain was identified by reverse transcriptase-PCR and part-S1 sequence analysis showed 90 per cent nucleotide level identity to the Israeli strain IS/885/00 (Meir and others 2004). Based on part-S1 analysis, the IS/1494 strain showed 99 per cent nucleotide level identity to the Israel strain IS/1494/06. A pairwise comparison of the S1 gene sequences of IS/885/00-like and IS/1494/06-like showed low level identity to the Mass and 793B types (Awad and others 2014a). The titre of the isolates were determined in specific pathogen-free embryonated chickens eggs and expressed as 50 per cent egg infectious doses (EID_{50}) following the method of Reed and Muench (Reed and Muench 1938).

Experimental design
Ninety one-day old commercial broiler chicks were divided into three groups of 30 chicks and housed in different isolation rooms. At one day of age, Group I was inoculated with H120 vaccine. Group II, was inoculated with combined H120 and CR88 vaccines. At 14 days of age, birds in Group I and II were revaccinated with CR88 vaccine. Group III was sham-inoculated at 1-day and 14 days old. Each chick was inoculated via oculo (50 µl)-nasal (50 µl) routes. Dosages were given as recommended by the manufacturer. Following the vaccinations, the birds were observed daily for clinical signs. Blood was collected prior to vaccination (at 1 day) and 30 days old from eight chicks in each group for antibody responses. On the same day, 10 chicks from each group (vaccinated and control groups) were transferred to another isolation room and challenged by the oculonasal route with 0.1 ml of virulent IS/885 to provide $10^{4.66}$ EID_{50}/chick. Another 10 chicks from each group were similarly transferred and challenged with 0.1 ml of virulent IS/1494 to provide $10^{5.00}$ EID_{50}/chick. The remaining 10 chicks (vaccinated or unvaccinated control) in each group were left as unchallenged controls. The birds were observed daily for clinical signs during the postchallenge period. Five days after challenge, all ten chicks in each unchallenged and challenged group were humanely euthanased by wing vein injection of 0.5–1 ml of Euthenal (pentobarbitone sodium, Rhone Merrieux, Ireland). Vaccine protection was evaluated by a ciliostasis test and examination of trachea and kidneys for gross lesions.

Detection of antibody responses
Sera collected prior to vaccination and 30 days old were tested using commercial ELISA (BioChek, Gouda, the Netherlands) following the protocols recommended by the manufacturer. The haemagglutination inhibition (HI) test was carried out as described before (Alexander and Chettle 1977). The IBV antigens used for the HI assay were M41 and 793B which were purchased from Animal Health Service, Deventer, The Netherlands. The haemagglutinin antigens of IS/885 and IS/1494 were prepared in the authors' laboratory based on the method described before (Alexander and Chettle 1977, King and Hopkins 1983).

Ciliostasis test
Assessment of protection from the challenge viruses were carried out as described by others (Cook and others 1999). Five days after challenge, birds were humanely killed, a section of trachea (immediately after larynx to thoracic inlet) was carefully dissected out and placed into warm (37°C) tracheal organ culture medium (Eagle's serum-free minimum essential medium with

glutamine and streptomycin (50 mg/ml) and penicillin (50 iu/ml)). The trachea was cut using a tissue chopper (The Mickle Laboratory Engineering) to give 6 μm thickness of rings, and rings of three from the top and bottom, and four from the middle trachea were examined for ciliary activity using a low-power microscope (×100 magnification). For each of the rings, the ciliary activity was scored; 0, all cilia beating; 1, 75 per cent beating; 2, 50 per cent beating; 3, 25 per cent beating; and 4, none beating (100 per cent ciliostasis). For each bird, out of the 10 rings examined, the maximum possible ciliary score is 40, which indicates a total lack of protection (no cilia beating in all 10 rings). The mean ciliary scores for each bird was calculated and percentage protection for each group using a formula described by others (Cook and others 1999); [1−(mean ciliostasis score for vaccinated/challenge group)/mean ciliostasis score for corresponding challenge controls)]×100.

Gross lesions
All chicks that were euthanased at five day post challenge (dpc) were also examined individually for gross tracheal and kidney lesions and scored accordingly using a four-point scale. For the trachea, 0=no lesions, 1=congestion, 2=mucoid exudate in the trachea and bronchi, 3=caseous exudate plug in the trachea and bronchi, air sacculitis, perihepatitis and pericarditis, (ii). For kidneys, 0=no lesions, 1=swollen and pale, 2=swollen with visible urates, 3=large swelling, pale with tubules and ureters distended with urates. Total gross lesions scores for each group were calculated based on the mean of scores observed per total number of chicks.

Statistical analysis
The ELISA, HI antibody titres and gross lesions between groups were analysed statistically using analysis of variance followed by Tukey's test for comparison of means. Differences were considered to be significant when $P \leq 0.05$. All analyses were conducted using the GraphPad Prism software, V.6.0.1.

RESULTS
Clinical signs post vaccination
There were no clinical signs found in the unvaccinated control chicks. In both vaccinated groups, mild clinical respiratory signs began to appear at six days post vaccination (dpv). The signs included head shaking, tracheal râles and sneezes. These signs subsided by 12 dpv.

Serology
The mean ELISA antibody titre prior to vaccination (at day-old, MDA) was 4174±636. The means of IBV HI titres against IBV M41, 793B, IS/885 and IS/1494 prior to vaccination (at day-old, MDA) were $\log_2 6.3$, $\log_2 5.6$, $\log_2 3.5$ and $\log_2 3.1$, respectively.

At 30 days of age (on the day of challenge), vaccinated groups showed significantly higher levels of IBV ELISA antibody titre than the unvaccinated control group. Chicks of group II (d0:H120+CR88, d14:CR88) exhibited significantly higher antibody titre (P<0.05) than group I (d0:H120, d14:CR88) (Fig 1). The HI response to M41 and 793B were higher than the heterologous antigens (IS/885 and IS/1494) (Fig 2). Using the M41 as antigen, the vaccinated chickens showed high level of HI antibody titre compared with the control group (Group III). The antibody titre against 793B antigen in group II was significantly higher than group I (Fig 2).

Clinical signs post challenge
The unvaccinated and vaccinated-unchallenged groups remained free of clinical signs. All the 10 chicks of unvaccinated-challenged chicks showed signs of typical IBV infection at 1 dpc which including depression with ruffled feathers, listlessness and huddling, head shaking, tracheal râles, sneezing and coughing. These signs were continued up to 5 dpc. In contrast, no clinical signs were observed in both of the vaccinated-challenged groups.

Tracheal ciliary assessment
The percentage ciliary protection was calculated for each group. The unvaccinated and vaccinated-unchallenged groups had greater than 98 per cent protection. The unvaccinated (group III) birds challenged with either IS/885 or IS/1494 viruses had 0 per cent protection (Table 1). Group II (d0:H120+CR88, d14:CR88) and group I (d0:H120, d14:CR88) showed 83 per cent and 60 per cent ciliary protection from IS/885 challenge, respectively (Table 1). Following challenge with IS/1494, group I and group II gave 80 per cent and 94 per cent protection, respectively (Table 1).

Gross lesions
The unvaccinated and vaccinated-unchallenged control groups remained free of gross lesions. Five days after the IS/885 challenge, congestion of the trachea and pale swollen kidneys were observed in all chicks in group III (unvaccinated-challenged), which were significantly different from vaccinated groups (P=0.02). These lesions were also found in group I (Table 1). However, no significant difference was observed between group I and group III regarding kidney lesions (P=0.06). Birds in group II were free of these gross lesions. Following the IS/1494 challenge, congestion of trachea and pale swollen kidney was observed in one bird in group I, while it appeared normal in the rest of the birds (Table 1). Chicks in group II showed no tracheal or kidney lesions. However, no significant difference was observed between the two vaccinated groups.

DISCUSSION
The evaluation of protection conferred by live vaccines against the virulent IS/885 and IS/1494 isolates was assessed based on ciliary activity in the tracheal explants
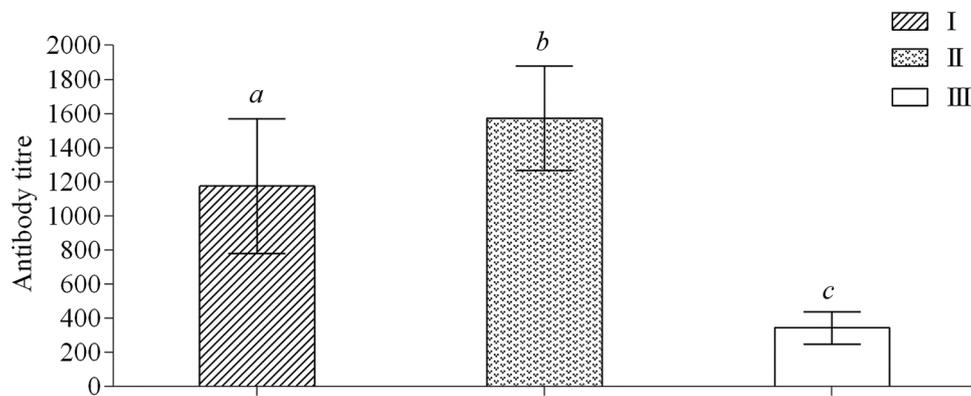
FIG 1: Mean ELISA antibody titres at 30 days of age. Group I=d0:H120, d14:CR88; group II=d0:H120+CR88, d14:CR88; group; III=control. Bars represent the mean±SD for eight birds in each group. Different superscript lowercase letters in the bar indicate significant difference (P<0.05), while data with same letters indicate that there were no significant differences (P>0.05). The cut-off titre=834

prepared from vaccinated-challenged chicks (Darbyshire 1980, Andrade and others 1982, Marquardt and others 1982, Snyder and others 1983, Cook and others 1999) and gross lesions in the trachea and kidneys following the challenge with the respective viruses. In this study, similar to other experimental work (Martin and others 2007, Terregino and others 2008) and field practices, the live vaccines were reconstituted and applied to chicks at dosages recommended by the vaccine manufacturers. The combined vaccination programme where both live H120 and CR88 vaccines were simultaneously given at one day-old followed by CR88 vaccine two weeks later (Group II) provided an excellent protection from both isolates. Following the challenges, there were no clinical signs or tracheal/kidney lesions and the ciliary protection was high (83–94 per cent).

Group 1, which had live H120 vaccine alone at day-old followed by CR88 vaccine two weeks later showed 60 per cent and 80 per cent protection from IS/885 and IS/1494/06-like, respectively. It appears that the protection conferred was much improved when the CR88 was given

together (Group II) with the H120 at day-old in contrast to H120 given alone (Group 1). It must be noted that the vaccine programme given to group II also offered 100 per cent kidney protection from both isolates. In a previous study performed in specific pathogen-free chicks, live H120 vaccination afforded protection of the trachea (92 per cent) and the kidney (25 per cent) on the basis of virus isolation when the birds were challenged with IS/885/00 (Meir and others 2004). Moreover, immunisation with live H120, H52 and D274 given singly was reported to produce little cross-protection from challenge with other nephropathogenic IBV strains (Albassam and others 1986, Lambrechts and others 1993, Pensaert and Lambrechts 1994). The present study shows that the vaccination programme of group II has further boosted the ciliary and tracheal/kidney protection from IS/885 compared with that of group I.

Both vaccination programmes used in this experiment provided excellent protection from the virulent IS/1494 challenge. A better protection observed in group II (d0:
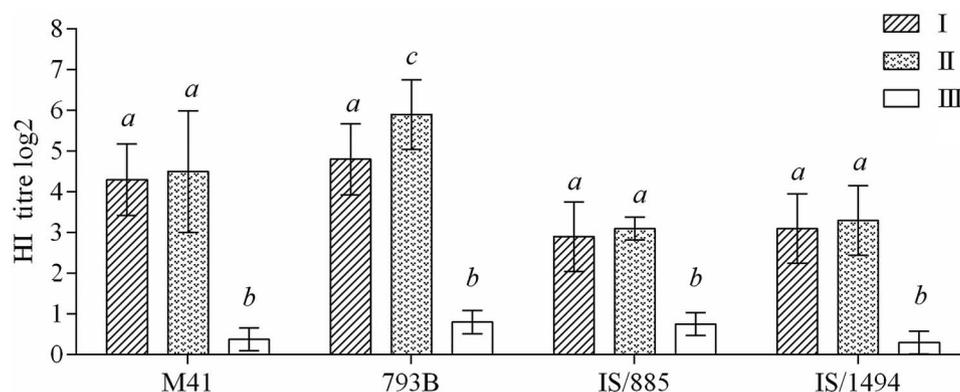

FIG 2: Haemagglutination inhibition (HI) antibody titres $\log_2$ against infectious bronchitis virus (IBV) antigens at 30 days of age. Group I=d0:H120, d14:CR88; group II=d0:H120+CR88, d14:CR88; group III= control. Bars represent the mean±SD for eight birds in each group. Different superscript letters in the bar indicate significant difference (P<0.05), while data with same letters indicate that there were no significant differences (P>0.05) (within same antigen). The cut-off titre=$3\log_2$

TABLE 1: Ciliostasis test and gross lesions of infectious bronchitis virus (IBV)-vaccinated and unvaccinated chicks following challenge with IS/885 or IS/1494 isolates

| Groups | IS/885 | | | IS/1494 | | |
| | Ciliostasis test Protection score† | Gross lesions* | | Ciliostasis test Protection score | Gross lesions | |
		Trachea	Kidney		Trachea	Kidney
I	60	0 (0/1)[a]	1 (0/1)[b]	80	0 (0/1)[a]	0 (0/1)[a]
II	83	0 (0/0)[a]	0 (0/0)[a]	94	0 (0/0)[a]	0 (0/0)[a]
III	0	1 (0/2)[b]	1 (0/2)[b]	0	1 (0/1)[b]	1 (0/1)[b]

Group I=d0:H120, d14:CR88; group II=d0:H120+CR88, d14:CR88; group III=unvaccinated control
*Severity of gross lesions induced by virulent IBV infection and data are expressed as the median (minimum/maximum) (Number of samples=10 per group). Different superscript lowercase letters within same columns indicate significant difference (P<0.05), while data with same letters indicate that there were no significant differences (P>0.05)
†Protection score=1, the mean score for vaccinated and challenged group/mean score for challenge control group ×100; the higher the score, the better the protection

H120+CR88, d14:CR88) as compared with group I (d0: H120, d14:CR88), could be due to the higher levels of local and cellular immunity at the tracheal site which may have prevented the virulent virus from reaching the kidneys (Lambrechts and others 1993). In an another experiment, cellular and local immunity induced by administration of combined live Massachusetts and 793B-type vaccines at day-old showed a significant increase in the expression of CD4+, CD8+ and IgA-bearing B cells in the trachea compared with single H120 alone or unvaccinated groups (Awad and Ganapathy, unpublished). These results reinforce the importance of optimising local and cell-mediated mucosal immunity at the respiratory lining through strategic heterologous day-old vaccination for enhanced protection from variant viruses.

Humoral antibody responses following vaccination as measured by ELISA and HI are often used for monitoring vaccine-take (Raj and Jones 1997). In this study, at 30 days of age (challenge day) the mean ELISA titre and the mean 793B HI titre in group II were significantly higher than the other vaccinated group. This reflects that combined live IBV vaccination at day-old and revaccination at 14 days-old has provided an immunological boost compared with the other vaccination schedule (group 1). Although it has been demonstrated before that circulating humoral antibody levels are of minor importance in the protection from IBV infection (Raggi and Lee 1965, Darbyshire and Peters 1985, Endo-Munoz and Faragher 1989, Pensaert and Lambrechts 1994), in the present study the group with higher levels of humoral antibodies had better protection from both of the Middle East isolates. Cytotoxic T cell plays a vital anti-viral activity during early stages of IBV infection while serum IgG is critical at the late stage of IBV infection (Collisson and others 2000).

Despite the low level of genetic identity in the S1 protein between the vaccine and challenge strains used in this study, the protection achieved with the vaccination programme employed in the present study was high. The genetic relationship of the hypervariable region of the S1 gene between IS/885 and H120 or

CR88 was 80 per cent and 79 per cent, respectively, while the genetic relationship between the IS/1494 and H120 or CR88 was 81 per cent and 80 per cent, respectively (Awad and others 2014a). Despite the 19–21 per cent variation in the S1 gene, the successful protection conferred by vaccination programmes employed in this study may be attributable to the shared characteristics of the S1 protein of the vaccine and the challenge IBVs (Cavanagh and others 1986).

Based on the data presented in this study, it appears that a combination of live H120 and CR88 vaccines given at day-old followed by CR88 vaccine at day 14 of age confer an excellent protection from virulent variant IS/885 and IS/1494 viruses. More work is needed to establish the underlying immune mechanisms for such higher and broader protection conferred by this vaccination programme.

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