Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Vaccine 24 (2006) 6830–6838
Vaccine
www.elsevier.com/locate/vaccine

Safety and efficacy of an infectious bronchitis virus used for chicken embryo vaccination

I. Tarpey [a,*], S.J. Orbell [a], P. Britton [b], R. Casais [b], T. Hodgson [b], F. Lin [a], E. Hogan [a], D. Cavanagh [b]

[a] *Intervet UK, Walton Manor, Walton, Milton Keynes, Bucks MK7 7AJ, UK*
[b] *Division of Microbiology, Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire RG20 7NN, UK*

Received 25 May 2006; received in revised form 19 June 2006; accepted 20 June 2006
Available online 5 July 2006

Abstract

Commercial vaccines for *in ovo* vaccination have not yet been developed for infectious bronchitis virus (IBV), the major coronavirus in the poultry industry. Recombinant IBVs based on the Beaudette strain expressing the Beaudette spike protein (Beau-R) or that from the virulent M41 strain (BeauR-M41(S)) were assessed for their potential as prototype vaccines for application to 18-day-old embryos. Pathogenicity was assessed by observing the effect on hatchability, and/or the production of nasal discharge and/or the effects on ciliary activity in the trachea at various time points post hatch. In contrast to commercial IBV vaccines given *in ovo*, the Beau-R and BeauR-M41(S) strains did not reduce hatchability or cause nasal discharge, and caused minimal damage to the ciliated epithelium of the trachea. The presence of the spike protein from a virulent virus did not increase the pathogenicity of the virus according to the criteria used. Assessment of the BeauR-M41(S) strain for efficacy showed that it protected up to 90% of chicks against challenge with virulent IB virus (M41) in a dose dependent manner. Further egg passage of the BeauR-M41(S) strain (BeauR-M41(S) EP10) did not increase its pathogenicity though it did improve its efficacy, based on serology and protection against a virulent challenge. BeauR-M41(S) EP10 was more efficacious than BeauR-M41(S) protecting more birds against virulent challenge and providing a better serological antibody response. BeauR-M41(S) EP10 induced a serological response similar to that of a commercial vaccine given at day-old though the commercial vaccine provided slightly higher efficacy. These results are promising for the development of embryo safe efficacious IBV vaccines for *in ovo* application.

Keywords: Vaccination; *In ovo*; Infectious bronchitis virus

1. Introduction

Infectious bronchitis virus (IBV) is one of the primary causes of respiratory disease in domestic fowl. IBVs primarily and initially infect the trachea though they can also infect the kidney and oviduct and other epithelial surfaces. Infection with IBV reduces the performance of broilers and in laying birds drops in egg production and egg quality can occur. Live-attenuated vaccines against infectious bronchitis are generally given to chicks at 1 day of age in the hatchery by spray, or later in the field by either drinking water or spray. However, as with any live-attenuated vaccine administered via the respiratory tract, which must replicate in order to be effective, vaccination at an early age can damage the epithelial lining of the trachea and depending on the extent of this damage, secondary bacterial infections can occur. For this reason it is important to assess post vaccinal reactions when preparing vaccine strains.

Vaccination *in ovo* against Marek's disease and infectious bursal disease is commonplace in the USA [1]. *In ovo* delivery gives the advantage that all vaccinations can be done prior to hatching as long as the vaccines are shown to be compatible and safe. Whilst considerable work has been done on

* Corresponding author. Tel.: +44 1908 658206; fax: +44 1908 685623.
E-mail address: ian.tarpey@intervet.com (I. Tarpey).

0264-410X/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2006.06.040

in ovo delivery of Marek and infectious bursal disease vaccines, little work has been done on delivering IBV vaccines *in ovo*. This is due to the fact that most strains of IBV are embryo lethal, both in the 9–12-day-old embryos that are used for producing IB vaccines, and in 18-day-old embryos. One group has shown that *in ovo* vaccination with IBV can be successfully accomplished, although the strain used had residual pathogenicity even at low titres [2]. As uniform mass application of IBV vaccines post hatch can be problematic with regard to obtaining even distribution of spray or ensuring all birds receive the correct dosage from drinking water, delivering IBV *in ovo*, via which a precise dosage can be achieved, could bring significant advantages to the poultry industry.

The Beaudette strain is a highly egg-adapted virus of the Massachusetts serotype that does not cause damage to the ciliated epithelium of the trachea in vivo [3,4]. Previous work has demonstrated that using a reverse genetics approach, the efficacy of the Beaudette strain can be improved by substituting the Beaudette spike protein with the spike protein from the virulent M41 strain, also of the Massachusetts serotype [4]. Interestingly, although this altered the cell tropism of the virus [5], the pathogenicity was not altered. This strain was able to afford significant protection to vaccinated birds against challenge with a virulent M41 strain. The aim of this work was to determine whether Beaudette strain-based IBVs produced by reverse genetics could be used as *in ovo* vaccines. Therefore the safety and efficacy of these strains were investigated.

2. Materials and methods

2.1. Embryonated eggs

Specified Pathogen Free (SPF) White Leghorn chicken embryonated eggs (Lohmann, Germany) were used. All chicks were hatched in a contained environment and transferred to negative pressure isolators for the remainder of the experiments. Chick starter crumbs and water were provided *ad libitum* during the course of the experiments.

2.2. Virus strains

2.2.1. Candidate vaccine strains

The Beau-R and BeauR-M41(S) strains were prepared using reverse genetics as described previously [5,6]. Both viruses were grown on primary chick kidney cells. The BeauR-M41(S) was passaged ten times on 9–12-day-old embryonated eggs to give BeauR-M41(S) EP10. Two commercial vaccines, Nobilis Ma5, (Intervet, referred to as CV1 in the text) and IB MM (Fort Dodge, referred to as CV2 in the text), both of the Massachusetts serotype were used. An M41 strain adapted to chick kidney cells (M41-CK) was also used [4]. The titres of the viruses were established in 9–12-day-old embryonated eggs, calculated using the method of Reed and Muench [7] and expressed as 50% egg infectious doses (EID_{50}). Viruses were diluted in Marek's disease vaccine diluent (Nobilis Marek diluent, Intervet).

2.2.2. IBV challenge strain

For the IBV challenge a non-CK adapted Massachusetts serotype M41 strain was given by the oculonasal route (0.1 ml/bird) at a dose of $10^{3.0}$ EID_{50} per bird.

2.3. Vaccinations

For embryo vaccination, eggs on the 18th day of embryo development were inoculated into the amniotic fluid using a 20 gauge, 2.5 cm long needle. Virus dilution (0.1 ml) was inoculated. Placebos received 0.1 ml of the diluent. Eggs were hatched in separate incubators. Hatch was assessed after 21.5 days of incubation.

For day-old vaccination each bird received 0.1 ml of the vaccine dilution via the oculonasal route.

2.4. Assessment of clinical signs post hatch

Examination of general health was done daily during the course of the experiments. Nasal discharge was assessed by gently squeezing the nares of the chicks and determining if any fluid was visible.

2.5. Assessment of ciliostasis

Assessment of ciliostasis was done as previously described [8]. Chicks were killed by an intravenous injection of barbiturates and their tracheas removed aseptically. Ten, 1 mm explants of each trachea were observed microscopically and the ciliary activity of each ring was scored according to the percentage of the cilia beating on the luminal surface. Post challenge ciliostasis assessments were done on days 5 and 7, these days coinciding with the peak replication of IBV in the trachea.

2.6. Serology

Serological responses to IBV were studied using an ELISA. Briefly ELISA plates were coated with M41 antigen and incubated with serial dilutions of test chicken serum for 1 h. After washing a rabbit anti chicken antiserum conjugated to alkaline phosphatase was added for 30 min. After washing a *p*-nitrophenyl phosphate substrate was added for 30 min. Titres were calculated in comparison to known negative and positive controls and a positive response was considered to be $\geq 5.64 \log_2$.

2.7. Statistical analyses

A two-tailed *T*-test was used to compare ciliary activity and serological responses. *p*-values of less than 0.05 were considered to be significant.

Table 1
Experimental details

Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5	
Virus *in ovo*	Dose (EID_{50})	Virus *in ovo*	Dose (EID50)	Virus *in ovo*	Dose (EID50)	Virus *in ovo*	Dose (EID50)	Virus *in ovo*	Dose (EID50)
Beau-R	$10^{6.0}$	Beau-R	$10^{4.0}$	Beau-R	$10^{4.0}$	BeauR-M41(S)	$10^{4.0}$	M41-CK	$10^{4.0}$
BeauR-M41(S)	$10^{6.0}$	BeauR-M41(S)	$10^{4.0}$	BeauR-M41(S) EP10	$10^{4.0}$	BeauR-M41(S)	$10^{4.0}$	Placebo	–
CV1	$10^{4.0}$	BeauR-M41(S) EP10	$10^{4.0}$	Placebo		CV2	$10^{4.0}$		
CV1	$10^{2.0}$	Placebo	–			CV2	$10^{4.0}$		
CV1	$10^{1.0}$	CV1 at day-old	$10^{4.0}$				–		
Placebo	–								
Ciliostasis post hatch									
6 days		2, 5 and 8 days		–		–		–	
Examination for nasal discharge									
6 days		–		4 and 8 days		–		–	
Challenge with M41									
4 weeks		6 weeks		4 weeks		–		–	

2.8. Experimental design (refer to Table 1)

2.8.1. Experiment 1

Thirty-three SPF eggs were inoculated with high doses of the Beau-R ($10^{6.0}$ EID_{50}) or BeauR-M41(S) ($10^{6.0}$ EID_{50}) vaccines or three graded doses of the commercial vaccine (CV1; $10^{4.0}$, $10^{2.0}$ or $10^{1.0}$ EID_{50}). A placebo vaccinated group was included. The chicks were allowed to hatch in separate hatchers, live chicks counted at 21.5 days of incubation and groups of at least 10 chicks formed when the hatch rate allowed. At 6 days post hatch the birds in each group were examined for nasal discharge and up to five chicks from each group were euthanized, their tracheas removed and assessed for ciliary activity. The remaining chicks were bled and serum collected at 4 weeks then challenged with virulent M41 IBV. At 5 and 7 days post challenge the remaining birds in each group were euthanized, their tracheas removed and assessed for ciliary activity.

2.8.2. Experiment 2

One hundred SPF eggs were inoculated with a dose of $10^{4.0}$ EID_{50} of the Beau-R, BeauR-M41(S) or BeauR-M41(S) EP10 vaccine candidates or with placebo. The chicks were allowed to hatch in separate hatchers and live chicks counted at 21.5 days of incubation. At 2, 5 and 8 days post hatch five chicks from each group were euthanized, their tracheas removed and assessed for ciliary activity. A group of 20 one-day-old birds were vaccinated with the 10^4 EID_{50} of the CV1 commercial vaccine via the oculonasal route. At 5 and 8 days post vaccination five chicks from this group were euthanized, their tracheas removed and assessed for ciliary activity. The chicks were bled and serum collected at 4 weeks then challenged with virulent M41 IBV at 6 weeks. At 5 and 7 days post challenge 5 birds from each group were euthanized, their tracheas removed and assessed for ciliary activity.

2.8.3. Experiment 3

Sixty SPF eggs were inoculated with a dose of $10^{4.0}$ EID_{50} of the BeauR-M41(S) or BeauR-M41(S) EP10 vaccine candidates. The chicks were allowed to hatch in separate hatchers and live chicks counted at 21.5 days of incubation. At 4 and 8 days post hatch the chicks were examined for nasal discharge. The chicks were bled and serum collected at 4 weeks then challenged with virulent M41 IBV. At 5 and 7 days post challenge 5 birds from each group were euthanized, their tracheas removed and assessed for ciliary activity.

2.8.4. Experiment 4

Fifty SPF eggs were inoculated with the BeauR-M41(S) ($10^{4.0}$ EID_{50}), Beau-R ($10^{4.0}$ EID_{50}) or two different doses of the CV2 vaccines ($10^{4.0}$ or $10^{1.0}$ EID_{50}) or with a placebo. The eggs were marked, mixed in a single incubator and the hatch assessed by counting the marked eggs remaining.

2.8.5. Experiment 5

Fifty SPF eggs were inoculated with the M41-CK virus ($10^{4.0}$ EID_{50}) or with a placebo. The eggs were marked, mixed in a single incubator and the hatch assessed by counting the marked eggs remaining.

3. Results

3.1. The effect on hatchability of embryo vaccination with IBV

To determine if the candidate vaccines based on the Beaudette strain were safe for embryo vaccination, five experiments were performed in which embryonated eggs were inoculated at day 18 of incubation. In Experiments 1–3 separate hatchers were used therefore the performance of each hatcher may have influenced the results. In Experiments 4

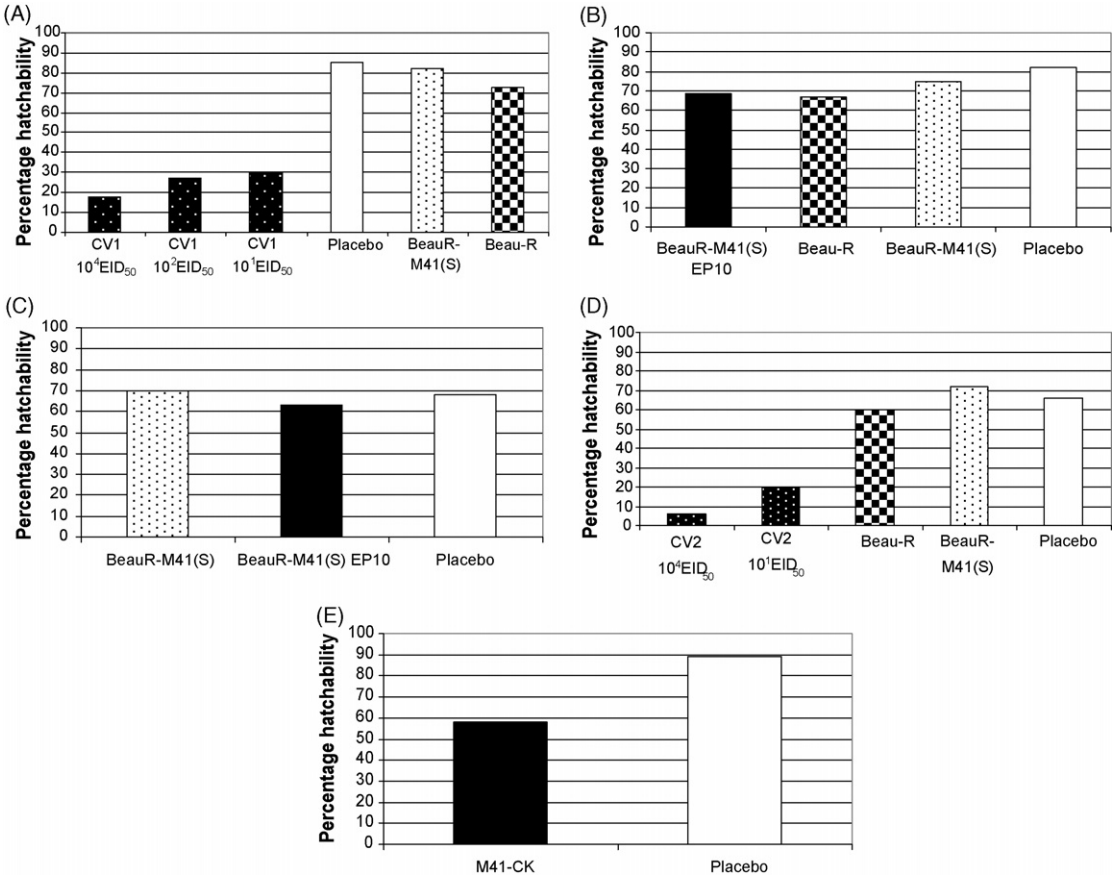

Fig. 1. The effect of IBV *in ovo* vaccination on hatchability. Groups of 33 (Experiment 1, panel A), 100 (Experiment 2, panel B), 60 (Experiment 3, panel C) or 50 (Experiment 4, panel D and Experiment 5, panel E) fertile SPF eggs were inoculated with either a commercial vaccine (CV1, Experiment 1: 10^4, 10^2 or 10^1 EID_{50} or CV2, Experiment 4: 10^4 or 10^1 EID_{50}, panel D), M41-CK (Experiment 5: 10^4 EID_{50} panel E), a Beaudette-derived virus, or a placebo. The Beaudette-derived viruses were Beau-R (Experiment 1: 10^6 EID_{50} panel A, Experiment 2: 10^4 EID_{50} panel B, Experiment 4: 10^4 EID_{50} panel D), BeauR-M41(S) (Experiment 1: 10^6 EID_{50} panel A, Experiment 2: 10^4 EID_{50} panel B, Experiment 3: 10^4 EID_{50} panel C, Experiment 4: 10^4 EID_{50} panel D) or BeauR-M41(S) EP10 (Experiment 3: 10^4 EID_{50} panel C). The percentage of hatched birds at 21.5 days is shown.

and 5 all eggs were hatched from the same machine to avoid the influence of individual hatchers. In Experiments 2 and 4 commercial vaccines were included to determine their effect on the hatchability.

In Experiment 1, 33 SPF eggs were inoculated with high doses of Beau-R or BeauR-M41(S) or graded doses of the commercial vaccine (CV1). As shown in Fig. 1A, Beau-R (73%) and BeauR-M41(S) (82%) gave slightly lower hatch than the placebo vaccinates (85%). The lowest hatch in the candidate vaccines was in the Beau-R group which had four fewer chicks hatch than the placebo group. This reduction in hatch may have been due to the pathogenicity of Beau-R but could also been an effect of hatching the different groups in separate hatchers. In contrast to the candidate vaccines, the CV1 commercial vaccine gave a very poor hatch in all three groups even though 100 (18% hatch), 10,000 (27% hatch) or 100,000 (30% hatch) fold lower doses than Beau-R and BeauR-M41(S) were inoculated.

In Experiment 2, 100 SPF eggs were inoculated with 100-fold less BeauR-M41(S) or Beau-R ($10^{4.0}$ EID_{50}) than in Experiment 1. In addition the BeauR-M41(S) EP10 strain was included to determine if egg passage could improve the immunogenicity of this strain without affecting its pathogenicity. Inoculation with any of the candidate vaccines caused a lower hatch than the placebo (82% hatch) in this experiment with the BeauR-M41(S) (75% hatch) vaccine candidate giving the best result (Fig. 1B). Though slightly lower than the placebo vaccinates the difference in hatch with these vaccine candidates may again have been due to the different hatchers used.

In the third experiment 60 SPF eggs were inoculated with $10^{4.0}$ EID_{50} of BeauR-M41(S) or BeauR-M41(S) EP10. Inoculation with BeauR-M41(S) gave a higher hatch (70% hatch) than the placebo (68%) whilst inoculation with BeauR-M41(S) EP10 caused a slightly lower hatch (63%) than the placebo (Fig. 1C).

In the fourth experiment 50 SPF eggs were inoculated with BeauR-M41(S) or Beau-R ($10^{4.0}$ EID_{50}). A second commercial vaccine (CV2) was included at doses of $10^{4.0}$ EID_{50} and $10^{1.0}$ EID_{50}. To exclude any variability in hatchers all eggs in this experiment were placed in the same hatching unit. BeauR-M41(S) gave a higher hatch (Fig. 1D, 72% hatch)

than the placebo (66%) in this experiment, whilst Beau-R vaccine gave a slightly lower hatch (60%) than the placebo. The CV2 vaccine gave very low hatchability at both doses (6% at 10^4 EID_{50} and 20% at 10^1 EID_{50}).

In the fifth experiment 50 SPF eggs were inoculated with M41-CK virus (10^4 EID_{50}), the donor of the M41 spike gene inserted into BeauR-M41(S) [5], or with placebo. To exclude any variability in hatchers all eggs in this experiment were placed in the same hatching unit. M41-CK gave 58% hatch in comparison to 89% hatch in the control group.

3.2. Clinical observations post hatch

As the birds were held in isolators during each trial it was difficult to examine some of the clinical parameters associated with IBV (tracheal rales, coughing and sneezing). In Experiment 1 *in ovo* inoculation with Beau-R or BeauR-M41(S) did not cause any morbidity during the post hatch observation period. Nasal discharge was not detected in birds vaccinated *in ovo* with either Beau-R or BeauR-M41(S) (Fig. 2A) suggesting that these viruses were not pathogenic. In contrast, 38% to 88% of the hatched CV1 *in ovo* vaccinates had nasal discharge when examined, the occurrence of nasal discharge not being dose dependent. In the third experiment none of the birds vaccinated with BeauR-M41(S) or BeauR-M41(S) EP10 had nasal discharge when tested at 4 and 8 days post hatch (data not shown).

Overall these results suggest that the Beau-R, BeauR-M41(S) and BeauR-M41(S) EP10 vaccine candidates are safe for embryo vaccination and the presence of the spike gene from virulent M41 did not have an effect on hatchability, nor cause clinical signs post hatch.

3.3. Assessment of ciliary activity post hatch

Analysis of ciliary activity post vaccination can be used as a measure of the safety of an IBV vaccine. In Experiment 1, three birds from the CV1-vaccinated groups and five in each of Beau-R and BeauR-M41(S) *in ovo*-vaccinated groups were euthanized 6 days post hatch and their ciliary activity assessed. The results are shown in Fig. 2B. CV1 inoculated *in ovo*, although only assessed in three birds, gave very high ciliostasis scores independent of the dose given. Ciliostasis scores for BeauR-M41(S) and Beau-R vaccinates were not significantly different from each other though both were significantly higher than the placebo vaccinates (BeauR-M41(S), $p = 0.00006$, Beau-R, $p = 0.00004$). These scores are acceptable on the grounds of safety [9], again suggesting that neither of these viruses were pathogenic and that the inclusion of the M41 spike gene does not increase pathogenicity.

In Experiment 2, a more extensive analysis of the ciliostasis caused by the candidate vaccines was undertaken (Fig. 2C). In this experiment an egg-passaged vaccine candidate, BeauR-M41(S) EP10 was included. The BeauR-

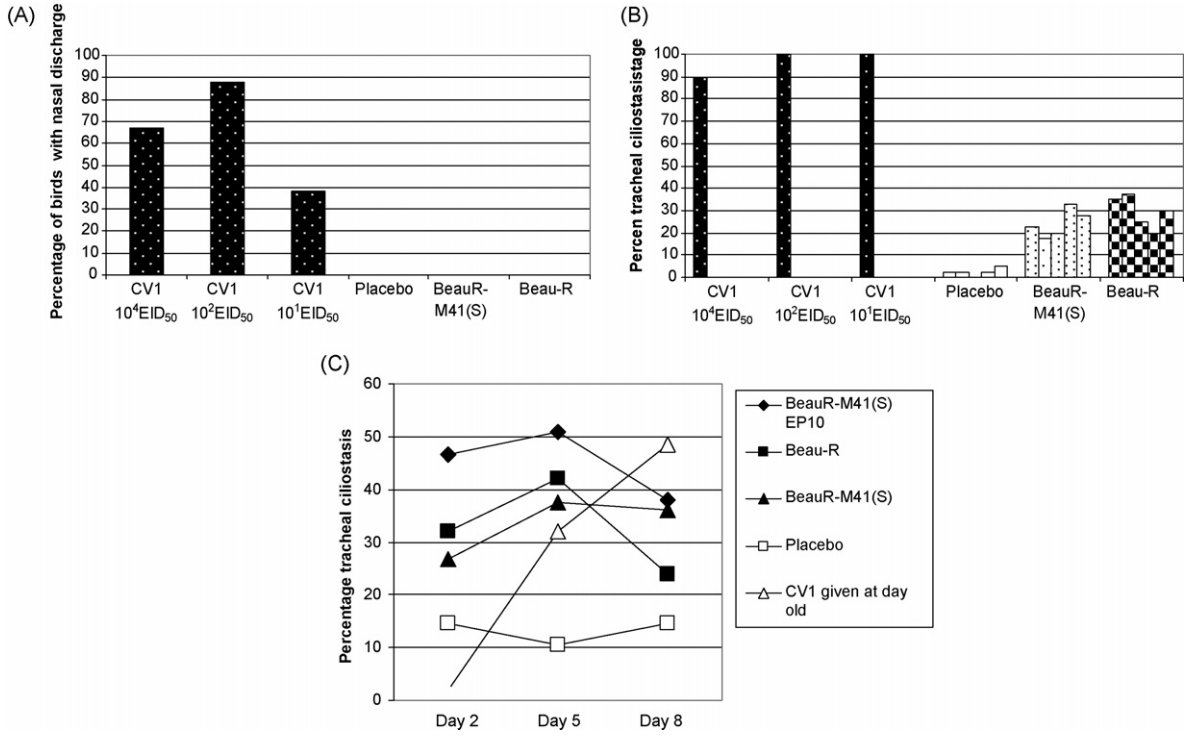

Fig. 2. Analysis of post hatch clinical signs and ciliary activity. The hatched chickens following *in ovo* vaccination were assessed for nasal discharge 6 days post hatch (Experiment 1, panel A: Beau-R and BeauR-M41(S) 10^6 EID_{50}, graded doses of a commercial vaccine as shown). The hatched chickens were euthanized and assessed for tracheal ciliary activity on day 6 post hatch (Experiment 1, panel B: Beau-R and BeauR-M41(S) 10^6 EID_{50}, graded doses of a commercial vaccine as shown) or on days 2, 5 and 8 (Experiment 2, panel C: Beau-R, BeauR-M41(S) and BeauR-M41(S) EP10 10^4 EID_{50}. A commercial vaccine CV1 was given at day-old 10^4 EID_{50} and tracheal ciliary activity assessed on days 5 and 8 (Experiment 2, panel C).

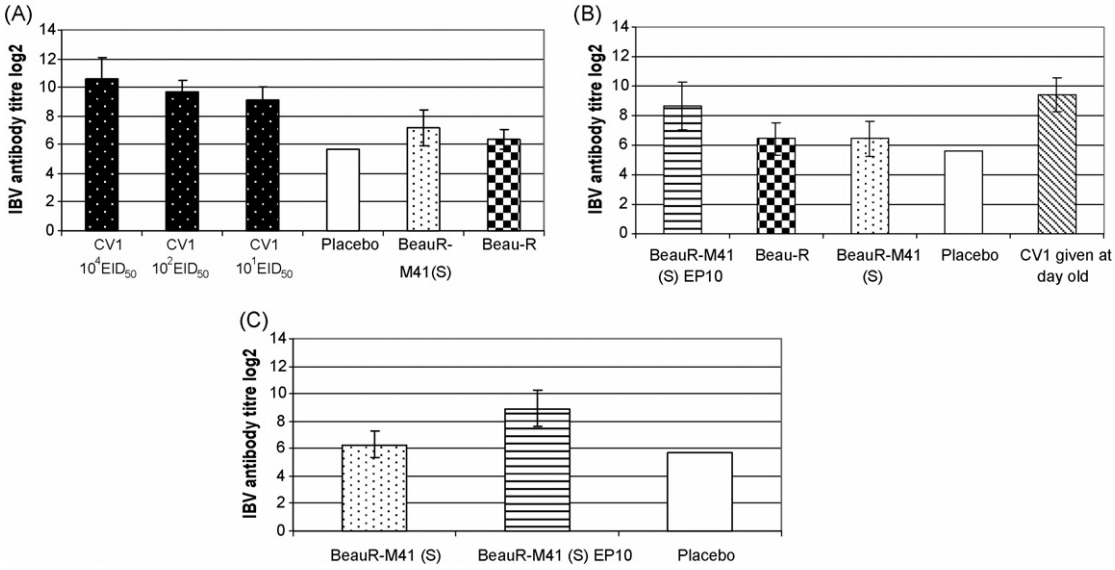

Fig. 3. Analysis of the serological antibody responses of hatched chickens following *in ovo* vaccination. The hatched chickens were bled after 4 weeks (Experiment 1, panel A and Experiment 3, panel C) or 6 weeks (Experiment 2, panel B) and sera analysed by ELISA for IBV-specific antibodies. Panel A: Beau-R and BeauR-M41(S) 10^6 EID$_{50}$, graded doses of a commercial vaccine as shown; Panel B: Beau-R, BeauR-M41(S), BeauR-M41(S) EP10 10^4 EID$_{50}$ *in ovo* or CV1 commercial vaccine at 10^4 EID$_{50}$ given at day-old; and Panel C: BeauR-M41(S) or BeauR-M41(S) EP10 10^4 EID$_{50}$ *in ovo*.

M41(S) EP10 vaccinate caused the highest levels of ciliostasis of the Beaudette based strains, though there was no significant difference between the ciliostasis caused by any of the Beaudette vaccine candidates at any time point (data not shown). Peak ciliostasis of the Beaudette based strains was detected at 5 days post hatch and the ciliostasis levels decreased after this time. The ciliostasis caused by the commercial CV1 vaccine given at day-old was assessed on days 5 and 8 post vaccination. The highest ciliostasis was detected on day 8 though this was not significantly different to any of the candidate vaccines given *in ovo* ($p = 0.059$).

3.4. Serological response to vaccination

In Experiment 1 the serological responses were compared at 4 weeks post hatch (Fig. 3A). The CV1 vaccine given *in ovo* at all doses gave the highest antibody responses and all birds seroconverted regardless of dose. The lowest dose of the CV1 vaccine gave a significantly higher response than BeauR-M41(S) ($p = 0.004$) and Beau-R ($p = 0.0004$). Beau-R gave a lower antibody response than BeauR-M41(S) but this was not significant ($p = 0.1$). Sixty percent of the Beau-R vaccinates and 80% of the BeauR-M41(S) vaccinates had seroconverted at 4 weeks post hatch.

In Experiment 2 BeauR-M41(S) EP10 vaccination *in ovo* gave the highest antibody response of the Beaudette based strains with all birds in the group seroconverting (Fig. 3B). The response to BeauR-M41(S) EP10 was significantly higher than both BeauR-M41(S) vaccine ($p = 0.002$) and Beau-R ($p = 0.002$) which gave approximately equal responses with only 40% of birds seroconverting in each of these groups (data not shown). The CV1 vaccine given at day-old gave the highest antibody response in this experiment with 90% of the birds responding. However this response was not significantly different to that induced by BeauR-M41(S) EP10 ($p = 0.88$).

In Experiment 3, BeauR-M41(S) EP10 vaccination *in ovo* gave the highest antibody response with 90% of birds in the group seroconverting (Fig. 3C). The response to BeauR-M41(S) EP10 was significantly higher than vaccination *in ovo* with BeauR-M41(S) ($p = 0.0006$) and only 40% of the birds vaccinated with BeauR-M41(S) seroconverted (data not shown).

3.5. Efficacy of the candidate vaccines

In Experiment 1 (vaccination dose for the Beaudette-based viruses was 10^6 EID$_{50}$) birds were challenged 4 weeks post hatch with virulent M41 virus and the ciliary activity tested on days 5 and 7 post challenge (Fig. 4A). For this purpose birds vaccinated with different doses of the CV1 vaccine were pooled to give a group of 10 birds. Based on ciliary activity of 50% or less being indicative of protection [9] all of the CV1 vaccinated birds were protected, 90% of the BeauR-M41(S) *in ovo*-vaccinated birds were protected and 30% of the Beau-R *in ovo*-vaccinated birds were protected. All non-vaccinated birds had at least 95% ciliostasis. This result indicates that the expression of the spike protein of virulent M41 increases the protective capacity of the BeauR-M41 (S) vaccine in comparison to Beau-R.

In Experiment 2 (vaccination dose for the Beaudette-based viruses was 10^4 EID$_{50}$) birds were challenged at 6 weeks post hatch with virulent M41 virus and the ciliary activity tested on days 5 and 7 post challenge (Fig. 4B). All non-vaccinated

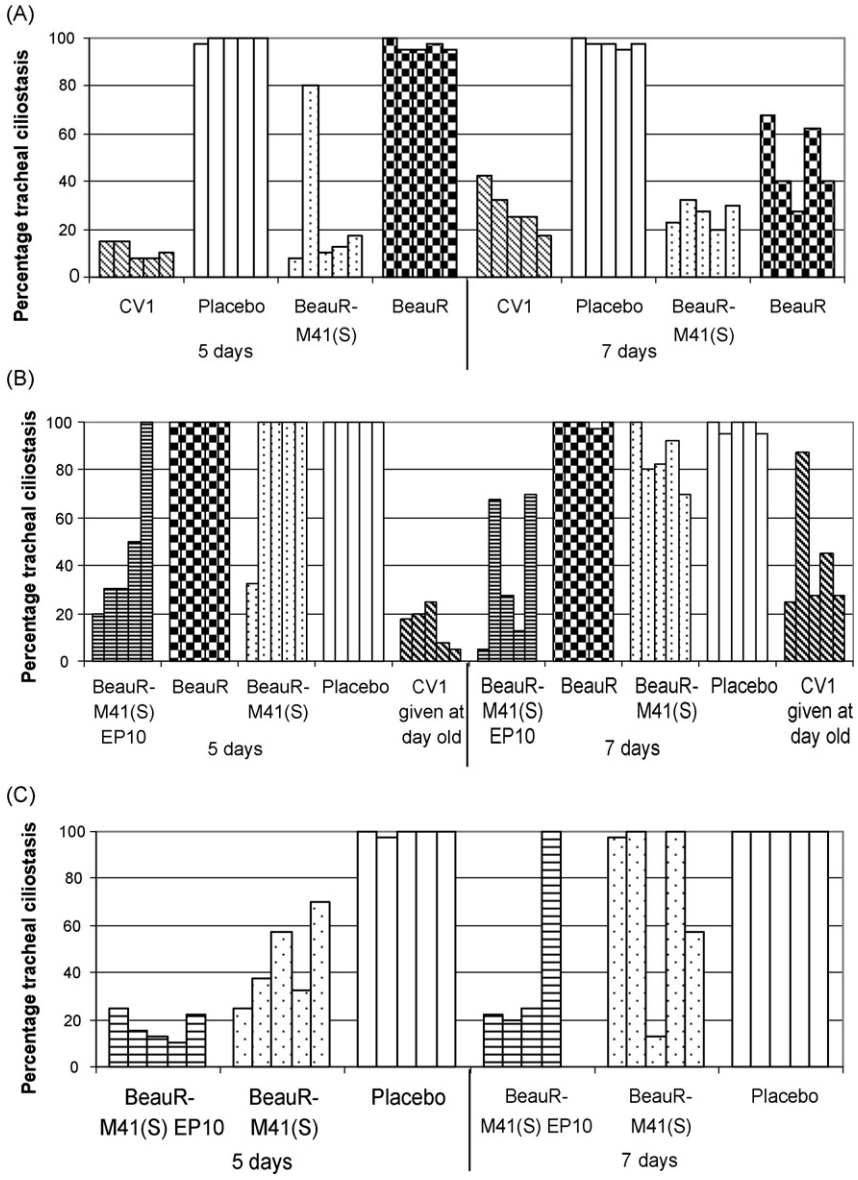

Fig. 4. Analysis of the efficacy of the candidate vaccines. Groups of 10 birds were challenged 4 weeks (Experiment 1, panel A and Experiment 3, panel C) or 6 weeks (Experiment 2, panel B) after hatch with virulent M41 via the oculonasal route. Birds were euthanized 5 and 7 days after challenge, the tracheas removed and assessed for ciliary activity over 10 areas. The scores are presented as percentage ciliary activity. Panel A: represents Beau-R and BeauR-M41(S) vaccinated *in ovo* at a dose of 10^6 EID_{50} and birds pooled from the 3 groups vaccinated with the commercial vaccine at graded doses. Panel B: represents Beau-R, BeauR-M41(S), BeauR-M41(S) EP10 10^4 EID_{50} inoculated *in ovo* or CV1 commercial vaccine at 10^4 EID_{50} given at day-old. Panel C: BeauR-M41(S) or BeauR-M41(S) EP10 10^4 EID_{50} inoculated *in ovo*.

birds had at least 95% ciliostasis. BeauR-M41(S) EP10 inoculated *in ovo* protected 70% of the birds whilst none of the Beau-R *in ovo* vaccinates were protected and only 10% of the birds vaccinated *in ovo* with BeauR-M41(S) were protected. The CV1 vaccine administered at day-old provided 90% protection. In comparison to Experiment 1 this result suggested that efficacy may be dose and/or time dependent for the Beau-R and BeauR-M41(S) vaccines with better protection given by a high dose (10^6 EID_{50}) with a 4-week challenge (Fig. 4B) than a lower dose (10^4 EID50) and a 6-week challenge.

In Experiment 3 birds were challenged at 4 weeks post hatch with virulent M41 virus and the ciliary activity tested on days 5 and 7 post challenge (Fig. 4C). All non-vaccinated birds had at least 95% ciliostasis. One of the birds in the BeauR-M41(S) EP10 *in ovo*-vaccinated group had to be euthanized due to a genetic defect. BeauR-M41(S) EP10 inoculated *in ovo* protected 89% of the vaccinates whilst only 40% of the BeauR-M41(S) *in ovo*-vaccinated birds were protected.

Overall the results indicated that IBV Beaudette strain expressing the spike gene from the M41 strain was safe for

delivery to embryos and had potential as an efficacious vaccine.

4. Discussion

As the poultry industry is moving towards greater use of *in ovo* vaccination, the purpose of this study was to determine if IBV vaccines based on the Beaudette strain could be delivered via this route. Surprisingly, even at high doses, the Beaudette based strains tested did not significantly affect the ability of the chick to hatch (Fig. 1A–D) or cause clinical signs post hatch (Fig. 2A and Experiment 3, data not shown) when compared to vaccination with a placebo. This is in contrast to work of Wakenell and Sharma [2] whose IBV *in ovo* vaccine candidate showed residual pathogenicity at a dose of 500 pfu. As expected the embryo adapted commercial vaccines greatly reduced hatchability (Fig. 1A and D) and gave clinical signs post hatch (Fig. 2A). The reason why the recombinant Beaudette strains are safe for *in ovo* vaccination is unknown though could be associated with the passage history of these particular strains. The parent Beaudette virus has been passaged on embryos more than the commercial vaccines and also passaged on CK cells, the precise passage history being unknown. Subsequently the recombinant viruses have been passaged further on CK cells. In line with the results of Hodgson et al. [4], with hatched chicks, we found that that the substitution of the Beaudette spike protein with that of the virulent M41 strain did not increase the pathogenicity of the BeauR-M41(S) strain over Beau-R in 18-day-old embryos or post hatch. This result suggests that the ability of M41-CK virus to reduce hatchability (to 58% in comparison to 89% with the placebo in Experiment 5) is not associated with the spike protein but may be associated with differences in other genes. It is interesting to note that further egg passages of the BeauR-M41(S) strain did not increase pathogenicity based on hatching criteria.

The ciliostasis test is used to determine the damage to the trachea following growth of IBV in this tissue. The Beaudette-based vaccine strains caused tracheal ciliostasis following *in ovo* inoculation but at an acceptable level with respect to vaccine safety [9]. This indicates that the Beaudette strains were able to replicate in the trachea of the *in ovo*-vaccinated chicks. In contrast, virtually no tracheal ciliostasis was caused by the Beaudette strains following inoculation of chicks by the oculonasal route [4], although Beaudette does cause ciliostasis when inoculated onto tracheal organ cultures. This suggests that delivery of IBV *in ovo* enables it to grow better in the trachea than post hatch delivery possibly due to the embryos ingesting amniotic fluid containing the viral inoculum, introducing the virus directly to the trachea. In contrast the commercial vaccine tested caused very high ciliostasis scores post hatch after delivery *in ovo* (Fig. 2B). This is in contrast to the results for the commercial vaccine delivered at day-old by nose and eye-drop application (Fig. 2C), again suggesting that delivery of IBV *in ovo* enables it to grow better in the trachea than delivery at day-old.

Interestingly the spike protein did not appear to affect the amount of ciliostasis caused by the Beau-R and BeauR-M41(S) strains as these strains showed similar results in two experiments (Fig. 2B 10^6 EID_{50} & 2C 10^4 EID_{50}). In contrast in a single experiment (Fig. 2C, 10^4 EID_{50}) the egg-passaged BeauR-M41(S) EP10 virus gave higher, but not significantly higher, ciliostasis scores. It is difficult to draw conclusions from a single experiment but this result suggests that egg passage may have increased the ability of the BeauR-M41(S) EP10 virus to grow in the trachea.

Although a serological response against IBV has not been correlated with protection against virulent IBV respiratory challenge, it is a useful criterion with which to assess the immunogenicity of the virus strain in the host. No significant differences were seen in the serological responses induced by Beau-R and BeauR-M41(S) in two experiments (Fig. 3A and B) and it is of interest to note that there was no dose dependency regarding the serological response or rate of seroconversion, a 100-fold more virus giving a similar response and seroconversion rate (Fig. 3A versus B). However, egg-passaged BeauR-M41(S) EP10 induced significantly higher responses than BeauR-M41(S) (Fig. 3B and C). This response was not significantly different to that induced by a commercial vaccine given at day-old (Fig. 3B) indicating a high level of immunogenicity of this virus. The reasons for the difference in immunogenicity of the egg-passaged virus requires further investigation though one could speculate that as the virus may have replicated more in the trachea, it subsequently induced a better immune response.

The efficacy of the Beaudette-derived viruses as potential vaccines was studied using a respiratory challenge with the virulent IBV M41 strain, using tracheal ciliary activity as the criterion of protection. A high dose (10^6 EID_{50}) of BeauR-M41(S) induced levels of protection comparable to that induced by the commercial vaccine given *in ovo* (Fig. 4A), though the commercial vaccine was not safe as determined by the criteria described above. The level of protection (90%) resulting from *in ovo* vaccination with BeauR-M41(S) was higher than that induced by Beau-R (30%), suggesting a positive effect of the M41 spike protein. As these viruses appeared to grow equally well in the trachea (Fig. 2B and C) it can be speculated that the increase in protection observed with the BeauR-M41(S) was due to the M41 spike protein which is homologous to the spike protein of the challenge virus. Alternatively, as a cytotoxic T cell response is thought to be important in protection against IB challenge [10] it is possible that the 5% amino acid difference between Beau-R and BeauR-M41(S) spike proteins [4] contain relevant CTL epitopes for protection against M41 challenge. The results presented herein are in agreement with the finding that vaccination with BeauR-M41(S) given at 8-days-old gave higher protection against virulent M41 challenge than vaccination with Beau-R [4].

We observed a dose and/or time dependency of the protection afforded by BeauR-M41(S) as at a 100-fold lower dose and challenge at 6 weeks the protection fell from 90% (Fig. 4A) to 10% (Fig. 4B). In contrast, the egg-passaged virus, BeauR-M41(S) EP10, at the lower dose (10^4 EID_{50}, Fig. 4B and C) provided 70–89% protection in two experiments suggesting that this vaccine was more adapted to growth in the embryo or was more immunogenic.

In summary, vaccine candidates based on the Beaudette strain were safe for delivery, by *in ovo* vaccination, to embryos as determined by their effects on hatchability, nasal discharge post hatch, and ciliary activity. The expression of the M41 spike protein by BeauR-M41(S) did not alter the pathogenicity of the rIBV on the basis of these criteria. Expression of the M41 spike protein, rather than the Beaudette spike protein, and further egg passage of the virus expressing the M41 spike protein improved the efficacy of the virus. To confirm the potential use of these candidate vaccines in the field, future work will concentrate on the effect of maternally derived antibody on the efficacy of these viruses as vaccine candidates following *in ovo* vaccination.

References

[1] Ricks CA, Avakian A, Bryan T, Gildersleeve R, Haddad E, Ilich R, et al. In ovo vaccination technology. Adv Vet Med 1999;41:495–515.

[2] Wakenell PS, Sharma JM. Chicken embryonal vaccination with avian infectious bronchitis virus. Am J Vet Res 1986;47: 933–8.

[3] Geilhausen HE, Ligon FB, Lukert PD. The pathogenesis of virulent and avirulent avian infectious bronchitis virus. Arch Gesamte Virusforsch 1973;40:285–90.

[4] Hodgson T, Casais R, Dove B, Britton P, Cavanagh D. Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces protective immunity. J Virol 2004;78:13804–11.

[5] Casais R, Dove B, Cavanagh D, Britton P. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol 2003;77:9084–9.

[6] Casais R, Thiel V, Siddell SG, Cavanagh D, Britton P. Reverse genetics system for the avian coronavirus infectious bronchitis virus. J Virol 2001;75:12359–69.

[7] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoint. Am J Hyg 1938;27:493–7.

[8] Cook JKA, Orbell SJ, Woods MA, Huggins MB. Breadth of protection of the respiratory tract provided by different live attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotype. Av Pathol 1999;28:477–85.

[9] European Pharmacopoeia. Avian infectious bronchitis vaccine (live) 2004, vol. 1. 5th ed. 01/2005:0442. p. 720–1.

[10] Seo SH, Wang L, Smith R, Collisson EW. The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. J Virol 1997;71:7889–94.