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Recombinant Infectious Bronchitis Viruses expressing chimaeric spike glycoproteins induce partial protective immunity against homologous challenge despite limited replication in vivo

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ABSTRACT

Vaccination regimes against *Infectious bronchitis virus*, which are based on a single virus serotype, often induce insufficient levels of cross-protection against serotypes and two or more antigenically diverse vaccines are used in attempt to provide broader protection. Amino acid differences in the surface protein, spike (S), in particular the S1 subunit, are associated with poor cross-protection. Here, homologous vaccination trials with recombinant IBVs, based on the apathogenic strain, BeauR, were conducted to elucidate the role of S1 in protection. A single vaccination of SPF-chickens with rIBV expressing S1 of virulent strains M41 or QX, BeauR-M41(S1) and BeauR-QX(S1), gave incomplete protection against homologous challenge, based on ciliary activity and clinical signs. There could be conformational issues with the spike if heterologous S1 and S2 are linked, suggesting a homologous S2 might be essential. To address this, a homologous vaccination-challenge trial incorporating rIBVs expressing full spike from M41, BeauR-M41(S), and S2 subunit from M41, BeauR-M41(S2) was conducted. All chimaeric viruses grew to similar titres *in vitro*, induced virus-specific partial protective immunity, evident by cellular infiltrations, reductions in viral RNA load in the trachea and conjunctiva and higher serum anti-IBV titres. Collectively, these show that vaccination with rIBVs primed the birds for challenge but the viruses were cleared rapidly from the mucosal tissues in the head. Chimaeric S1 and S2 viruses did not protect as effectively as BeauR-M41(S) based on ciliary activity and clinical signs. Booster vaccinations and a rIBV with improved *in vivo* replication may improve the levels of protection.
IMPORTANCE

Infectious bronchitis virus causes an acute, highly contagious respiratory disease, responsible for significant economic losses to the poultry industry. Amino acid differences in the surface protein, spike (S), in particular the S1 subunit, have been associated with poor cross-protection. Available vaccines give poor cross-protection and rationally designed live attenuated vaccines, based on apathogenic BeauR, could address these. Here, to determine the role of S1 in protection, a series of homologous vaccination trials with rIBVs were conducted. Single vaccinations with chimaeric rIBVs induced virus-specific partial protective immunity, characterised by reduction in viral load and serum antibody titres. However, BeauR-M41(S) was the only vaccination to improve the level of protection against clinical signs and the loss of tracheal ciliary activity. Growth characteristics show all of the rIBVs replicated in vitro to similar levels. Booster vaccinations and a rIBV with improved in vivo replication may improve the levels of protection.

INTRODUCTION

Infectious Bronchitis virus (IBV) is classified as a gammacoronavirus, subfamily Coronavirinae, order Nidovirales (1). IBV is responsible for major economic losses to poultry industries worldwide as a result of poor weight gain, decreased egg production and impaired egg quality. The effect of IBV on the ciliary activity in the trachea and the immune system may predispose infected chickens to secondary infections with opportunistic bacteria, which often increases the mortality rate associated with IBV (2-4).

IBV is an enveloped virus, with a single-stranded, positive sense RNA genome (~28kB), and encodes four structural proteins: nucleocapsid (N), spike glycoprotein (S), small membrane protein...
envelope (E) and integral membrane protein (M) (5, 6). The major surface protein of IBV, S, is a type 1 glycoprotein which oligomerises to form trimers (7) and is thought to be the main inducer of protective immunity (8-12). The S protein is proteolytically cleaved into two subunits, the N-terminal subunit S1 (approx. 500-550 amino acids, 90-kDa) and the C-terminal subunit, S2 (630 amino acids, 84-kDa), which contains the transmembrane domain. The S1 subunit plays a critical role in binding to cellular receptors as it contains the receptor binding domain (13, 14), determines the virus serotype and is responsible for the induction of neutralising antibodies (14-16). Multiple studies have shown that recombinant S1 expressed in adenovirus and Newcastle Disease Virus vectors can induce a certain level of protection in specified-pathogen free (SPF) chickens against challenge with wild-type virus (11, 17, 18).

Vaccine programmes against IBV often include a combination of live or inactivated vaccines which are based on several dominant field serotypes of the virus. The current vaccines often induce insufficient cross-protection, and combinations of antigenically different vaccines are used in an effort to improve levels of protection (19). Alongside this, with the continual emergence of new field strains the control of IBV is persistently a significant problem to the poultry industry.

A reverse genetics system based on the avirulent strain of IBV Beaudette has been developed (20, 21). This system has many potential applications, including; to enhance our understanding of the role of individual genes in pathogenicity and to lead to a new generation of rationally designed live attenuated vaccines (20). Previous work using the reverse genetics approach demonstrated that replacement of the ectodomain of the S glycoprotein of the apathogenic IBV Beaudette strain with the same region from either of two pathogenic IBV strains, M41-CK or 4/91, resulted in two non-virulent rIBVs, BeauR-M41(S) and BeauR-4/91(S), respectively. Notably, both rIBVs based on the BeauR backbone acquired the same cell tropism of that of the donor S, M41-CK or 4/91 (22,
Other work demonstrated that the Beaudette S2 subunit confers the unique ability of Beaudette to replicate in African Green Monkey Kidney (Vero) cells, a continuous cell line licensed for vaccine production (24, 26). Vaccination with BeauR-M41(S) or BeauR-4/91(S) can confer protection against homologous challenge based on ciliary activity, reductions in clinical signs and viral load in the trachea at 5 days post-challenge (dpc), further demonstrating the dominant role of the S glycoprotein in inducing protective immunity (23, 25).

In this study we investigated the protection conferred against homologous challenge by two rIBVs, BeauR-M41(S1) and BeauR-QX(S1), that contain S1 subunits from economically relevant strains, M41 and QX respectively, with the S2 subunit derived from BeauR. Notably both rIBVs have the advantageous ability to replicate in Vero cells (26; Bickerton et al. submitted for publication) due to the presence of the Beaudette S2 subunit. We report here on the first application of rIBV with a chimaeric S gene to be used in a vaccination trial. The rIBV BeauR-M41(S2) was also investigated in order to elucidate the relevant roles of both subunits in protective immunity. Whilst the S1 subunit is considered to be immunodominant, the S2 subunit is highly conserved between strains and contains immunogenic regions (14, 27).

We have shown here that vaccination with a recombinant IBV expressing a chimaeric S gene can induce a partially protective response against challenge, as assessed by viral load, cellular infiltration, clinical signs and a boost in serum antibody titres post-challenge. Vaccination with rIBV expressing homologous S1 and S2 subunits (i.e. full S gene) in the Beaudette backbone induced partial protection classified by the level of ciliary activity and presence of clinical signs following challenge with wild-type IBV. Comparison of in vitro growth characteristics shows that inclusion of a foreign S gene or a chimaeric S gene in the rIBVs does not impede replication in vitro. However, our data show that despite the ability to induce a degree of virus-specific protective
immunity, the rIBVs are hindered by limited in vivo replication and the attenuated BeauR backbone.

RESULTS

Characterisation of rIBV BeauR-M41(S1) and BeauR-QX(S1) for homologous protection.

To determine if a single vaccination with rIBV expressing the S1 subunit of the S gene (with a Beaudette derived S2 subunit) was sufficient to induce protection against challenge with homologous pathogenic isolates of IBV, a vaccination/challenge trial was conducted with BeauR-M41(S1) and BeauR-QX(S1). No clinical signs nor loss of ciliary activity in the trachea were observed in either of the vaccinated groups following vaccination (data not shown). These results showed that replacement of the BeauR S1 gene with the S1 gene from pathogenic strains did not confer pathogenicity to the resulting BeauR-M41(S1) and BeauR-QX(S1) viruses.

Three weeks after the primary inoculation, chickens were challenged with a homologous wild-type virus strain, M41-CK or QX. Clinical signs were at the highest level in the challenge control groups, with QX more pathogenic than M41-CK (Fig. 2A and 2B). The rIBV vaccines expressing the S1 subunit did not confer full protection against clinical signs associated with IBV, although snicking and rales in the group vaccinated with QX(S1) resolved quicker than the QX challenge control (Fig. 2A and 2B). Vaccination with BeauR-M41(S1) or BeauR-QX(S1) did not prevent the loss of ciliary activity in the trachea following challenge with the homologous wild-type virus (Table 1).

To investigate the tissue tropism of the rIBVs, a range of tissues collected at 2 and 4 days post-vaccination (dpv) were assessed by RT-PCR. BeauR-M41(S1) and BeauR-QX(S1) RNA was not
detected in the conjunctiva, Harderian gland, nasal-associated lymphoid tissue (NALT) or trachea at 2 and 4 dpv (data not shown). Histological analysis of the head-associated lymphoid tissues revealed cellular infiltrates in both the Harderian gland and the conjunctiva-associated lymphoid tissue (CALT) at 2 dpv (Fig. 3A-3D), with areas of CALT more prominent in vaccinated tissues compared to Mock (Fig. 3E). Collectively, these suggest that the recombinant vaccine viruses did infect these tissues but were no longer detectable by PCR at 2 dpv, suggesting rapid clearance from the sites of inoculation and mucosal tissues in the head-associated lymphoid tissues, exerted by a virus-specific protective immune response.

To elucidate if BeauR-M41(S1) and BeauR-QX(S1) were able to confer a degree of protection against homologous challenge, evident by a reduction in viral load of infected tissues post-challenge, qPCR was conducted to assess the level of viral RNA in trachea and CALT. At 2 dpc, IBV viral RNA load in both trachea and CALT were significantly lower in the BeauR-M41(S1) vaccinated groups compared to challenge controls (Fig. 4A and 4C), but at 4 dpc the viral RNA load was only significantly lower in the CALT of the BeauR-QX(S1) vaccinated group (Fig. 4B and 4D). Infectious viral load determined by titration of trachea tissue supernatant in TOCs, showed a reduction in infectious virions recovered from BeauR-M41(S1) and BeauR-QX(S1) vaccinated chickens, although not significant compared to corresponding wild-type controls (BeauR-M41(S1), P=0.961 and BeauR-QX(S1), P=0.999) (Fig. 4E). The wild-type control groups were the only groups to report significantly higher infectious viral loads recovered from the trachea compared to that of the Mock/Mock controls (Fig. 4E).

Serum IBV-specific antibodies were assessed post-vaccination (pre-challenge) at 21 dpv and at 2, 4 and 14 dpc. Compared to the challenge control group, titres were significantly higher in the BeauR-QX(S1) vaccinated group at 2 and 4 dpc (Fig. 5A and 5B) (P<0.05 and P<0.01,
respectively). At 14 dpc, serum titres were higher in both the BeauR-M41(S1) and BeauR-QX(S1) vaccinated groups compared to the challenge control groups, but only the QX vaccinated group was significantly higher compared to the corresponding challenge control group (Fig. 5C and 5D) ($P<0.05$). For both vaccinated groups, antibody titres at 21 dpv (pre-challenge) could be classed as “borderline” positive due to being above the limits of the S/P cut-off (Fig. 5C and 5D).

In summary, results from Trial 1 suggest that although vaccination of the chickens with BeauR-M41(S1) and BeauR-QX(S1) did not confer complete protection against homologous challenge based on clinical signs and ciliary activity, a single vaccination of young chickens induced a partially protective virus-specific immune response as indicated by a significant reduction in viral load in trachea and CALT tissues. Higher IBV-specific serum antibody titres compared to challenge-only controls shows that vaccination with chimaeric rIBVs were able to prime the birds for challenge. Whether the lack of full protection against the loss of ciliary activity and clinical signs was due to the absence of a homologous S2 subunit or an incorrect folding of M41/QX (S1) and BeauR (S2) and therefore lower infectivity could not be answered in this specific study. Therefore, a second trial addressing the issue of whether a homologous S2 is required for protection was conducted.

Relative contribution of S1 and S2 to homologous protection. In Trial 2, the rIBV used were; BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) (described in Fig. 1), with a similar experimental design to that of Trial 1. No clinical signs were observed in any of the vaccinated groups after vaccination (data not shown). Following vaccination, there was no loss of ciliary activity in the trachea, indicating the apathogenicity of the rIBVs (data not shown). In the same manner to Trial 1, at 21 dpv the chickens were challenged with M41-CK. Clinical signs were observed until 7 dpc, BeauR-M41(S) was the only vaccinated group to show less prevalent clinical
signs post-challenge compared to the M41-CK challenge control (Fig. 6A and 6B). There was little difference between the BeauR-M41(S1), BeauR-M41(S2) and M41-CK groups in terms of the presence and severity of clinical signs (Fig. 6A and 6B), but in the vaccinated groups clinical signs resolved more rapidly compared to the M41-CK controls. Ciliary activity was assessed at 4 dpc and the level of protection afforded were assessed according to European Pharmacopeia standards (28). The BeauR-M41(S) vaccinated group retained ~60% ciliary activity, showing an improved level of protection in comparison to groups vaccinated with BeauR-M41(S1) and BeauR-M41(S2), in which 20% protection in each group were evident (Table 2). Noteworthy, assessment on an individual bird level showed that 3 out of 5 birds in the BeauR-M41(S) were classed as “protected against ciliostasis” however, as the group average was 60% this does not translate into protection on a group level (Table 2).

Viral RNA loads in the tracheas and CALTs isolated from challenged chickens were determined by qPCR to elucidate whether the S1 and S2 subunits played any further role in conferring protection. At 2 dpc only the CALT from BeauR-M41(S) and BeauR-M41(S2) vaccinated chickens showed any significant reduction ($P<0.001$) in viral RNA load compared to the challenge control (Fig. 7A). However, at 4 dpc all groups had significantly lower viral RNA loads in the CALT (Fig. 7B) ($P<0.001$, BeauR-M41(S) and $P<0.01$, BeauR-M41(S1) and BeauR-M41(S2)). Viral RNA loads in the trachea were only significantly lower at 2 dpc in BeauR-M41(S) vaccinated chickens ($P<0.001$, BeauR-M41(S)) and significantly lower for all vaccinated groups at 4 dpc (Fig. 7C and 7D) ($P<0.05$, BeauR-M41(S) and BeauR-M41(S2), $P<0.01$, BeauR-M41(S1)). Failure to locate the rIBVs in the head-associated lymphoid and respiratory tissues at 2 dpv in Trial 1, lead to the inclusion of the 1 dpv time-point in Trial 2. BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) were detected by RT-PCR in a number of the Harderian glands and tracheas isolated.
from chickens at 1 dpv, however, at 2 and 4 dpv the rIBVs were mainly detected in the nasal turbinates (Table 3), suggesting rapid clearance of rIBVs from the mucosal head tissues and sites of inoculation. Although, the titres of infectious challenge virus recovered from tracheas at 4 dpc were not significantly reduced in BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccinated chickens compared to controls (because of the variation within each group), there was a general trend that vaccination resulted in a reduction in viral infectivity, with no detected infectious virus recovered in 4 out of 5 birds in the (S) group, 3 out of 5 in the S1 group and 1 out of 5 in the S2 group (Fig. 7E). Collectively, this shows that the chimaeric rIBVs are able to induce a degree of local protection against the replication of IBV in the trachea.

To assess if the rIBVs induced humoral antibody responses following vaccination with BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) viruses, IBV-specific serum titres were assessed at 2 and 4 dpc. At 2 dpc, there was clear evidence of a boost in antibody titres in the BeauR-M41(S) and BeauR-M41(S2) vaccinated groups (Fig. 8A), with significantly higher titres compared to Mock/M41 controls ($P<0.001$). IBV induced antibody titres at 2 dpc in BeauR-M41(S) vaccinated chickens were higher than those from BeauR-M41(S1) and BeauR-M41(S2) vaccinated chickens across the dilution series (Fig. 8A). At 4 dpc, serum antibody titres from all vaccinated groups were significantly higher compared to the Mock/M41 titres (Fig. 8B), suggestive of a primed antibody response in the vaccinated chickens. The serum antibody titres at 14 dpc indicated no significant differences between the vaccinated groups and the challenge-only controls (Fig. 8C), suggesting that a boosted response was lacking in response to challenge with wild-type virus.

The virus neutralisation activity of the serum collected at 4 and 14 dpc were assessed and at 4 dpc there was no neutralisation of the virus detected (data not shown). At 14 dpc, only serum from
BeauR-M41(S) and BeauR-M41(S1) vaccinated chickens had significantly higher neutralisation activity of the virus compared to Mock/Mock control ($P=0.002$ and $P=0.0066$, respectively; Fig. 9A). BeauR-M41(S) vaccination induced significantly higher virus neutralisation titres compared to BeauR-M41(S2) vaccination ($P=0.04$), whereas there was no significant difference in titres compared with serum from BeauR-M41(S1) vaccinated or Mock/M41 challenge-only group (Fig. 9A). The levels of virus neutralisation activity detected were moderately positively correlated to the anti-IBV serum titres ($r^2=0.5$, $P=0.002$, Fig. 9B).

**Characterisation of rIBVs in vitro.** Following on from the observation of differences during the *in vivo* vaccination trials, to elucidate whether the inclusion of a chimaeric S gene or a foreign S gene had an effect on viral replication, the replication kinetics of rIBV BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) viruses were investigated *in vitro*. At 12 hpi all viruses had similar titres (Fig. 10A and 10B). This suggests that the inclusion of a foreign S gene, or a chimaeric S gene has not impeded replication *in vitro* in either chicken kidney cells (CKCs) derived from Valo chickens (Fig. 10A) nor CKCs derived from Rhode Island Red (RIR) birds (Fig. 10B). Single-step growth curves performed in CKCs derived from RIR birds show that over the latent period (2-8 hpi), BeauR-M41(S2) had lower virus titres compared to the other viruses, however when the exponential growth was compared there was no statistical difference between the viruses (Fig. 10C). The titres of BeauR-M41(S) and BeauR-M41(S1) are similar over all time points (Fig. 10C).

**DISCUSSION**

We have previously shown that rIBVs expressing the ectodomain of the Spike protein of a pathogenic strain in the context of an apathogenic strain BeauR, could induce increased levels of
protection against homologous and partially against heterologous challenge infection. Here, we extended this work and replaced only the S1 subunit of the ectodomain of BeauR with the S1 domain of M41 or QX, representing two strains that circulate in poultry flocks worldwide. These rIBVs have the advantage of being able to replicate in Vero cells, potentially allowing large scale vaccine production in cell culture rather than in embryonated eggs. In this first vaccination study, using a single dose of BeauR-M41(S1) or BeauR-QX(S1) in 1-week-old chicks, the birds were not protected against homologous challenge based on ciliary activity and clinical signs. Vaccination with BeauR-QX(S1) induced significantly higher serum titres post-challenge and the clinical signs associated with challenge virus, although present, decreased rapidly compared to unvaccinated birds challenged with QX. Together, these data show that vaccination with chimaeric rIBVs are able to induce a degree of virus-specific immunity with partial local protection in the mucosal head tissues and the primary site of replication, the trachea.

In attempt to address the questions of whether a full homologous S is required for optimal folding, virus replication and protection using an apathogenic recombinant virus, a second vaccination experiment was performed. One-week-old birds were immunised once with BeauR-M41(S), BeauR-M41(S1), or BeauR-M41(S2). Replacement of the apathogenic BeauR-S1 or S2 subunits with a S1 or S2 from a pathogenic strain, allowed BeauR to remain apathogenic, suggesting that the S1 or S2 alone do not play a role in the pathogenicity of IBV. This further expands our previous work showing that spike switching of BeauR-S with M41-S showed no effect on pathogenicity (23). Here, vaccination of chickens with a rIBV based on a BeauR backbone expressing a full S gene from the donor serotype enhanced the level of protection afforded against tracheal ciliostasis, with 3 out of 5 birds classed as fully protected. However, when classified under European Pharmacopeia standards for assessment of IBV vaccines (28), at which 80% protection (at a group
level) against ciliostasis is required, the BeauR-M41(S) vaccinated group was only able to confer partial protection (~60%), and therefore is still not satisfactory for the criteria used for the assessment of IBV vaccines for industrial application. Consistent with previously published work, we show that collectively as a group the chickens vaccinated via ocular-nasal routes with BeauR-M41(S) had ~60% ciliary activity remaining, a reduction in clinical signs and viral load post-challenge (25). The protection seen at the trachea may potentially be improved with assessment of ciliostasis at a later time-point, as Armesto et al. (23) reported that vaccination with BeauR-4/91(S) gave ~60% ciliary activity at 4 dpc, which then improved to 90-100% at 6 dpc. In Trial 1, viral RNA load in the trachea and CALT from the S1 vaccinated groups was reduced at 2 dpc, whereas in Trial 2, all vaccinated groups had a clear significant reduction in viral RNA load at 2 and 4 dpc in trachea and CALT. The qPCR used here is designed to detect the 5’UTR region of the genome (29) and it therefore may be detecting incomplete virions or challenge virus captured in the lumen of the trachea. To further support the viral RNA load data, infectious viral load recovered from the trachea in both Trial 1 and 2 were lower in rIBV vaccinated chickens, indicating a degree of local protection at the site of infection, which was not robust enough to completely protect against viral replication in vivo and the loss of ciliary activity.

The major surface glycoprotein of coronaviruses, spike, is a type 1 glycoprotein and has two structurally distinct conformations, pre-fusion and post-fusion (30-32). In the coronavirus replication cycle the spike mediates the critical steps of receptor binding and membrane fusion. Upon binding of the S1 receptor binding domain to the host cell, an irreversible conformational switch to the post-fusion state allows the S2 subunit to fuse viral and cellular membranes, facilitating entry of the viral genome and therefore downstream viral replication (32-34). Recently, the crystal structure of the pre-fusion spike from Mouse hepatitis virus (MHV) and Human
coronavirus (HCoV HKU1) were resolved, highlighting the critical role that the interaction between the trimers of S1 and S2 plays in stabilisation of the pre-fusion conformation of spike (31, 32). Here, expression of a chimaeric spike in a recombinant IBV backbone with the lack of a homologous S2 possibly resulted in conformational changes either within the S1 subunit or complete S protein, potentially affecting receptor binding and entry, but may have also altered immunogenic epitopes. The S2 subunits of BeauR shares 87% and 97% amino acid sequence similarity with QX and M41-CK, respectively, showing that there are only a few different amino acid residues between them. The interactions between S1 and S2 sub-units are critical for maintenance of conformation, recognition and efficient fusion of the spike to host cells; it has been consistently shown that even a single amino acid change within the S2 subunit of coronavirus spikes may influence the secondary structure of the overall spike or the S1 subunit (35, 36).

The development of a cryo-EM structure of IBV M41 spike, highlighting the evolutionary difference between the pre-fusion spike structures of IBV compared to betacoronaviruses and alphacoronaviruses, nonetheless indicates a high degree of structurally similarity to porcine deltacoronavirus (37, 38). This structural model of pre-fusion IBV spike will significantly aid in addressing the challenges over whether (i) expression of a chimaeric spike in a rIBV backbone causes conformational changes either within the S1 subunit or complete S or (ii) it is vital that homologous “matched” S1 and S2 and their interactions are required to maintain the correct pre-fusion conformation of spike, as suggested in other coronaviruses.

The Beaudette strain, used here in the reverse genetics system, has an extended in vitro tropism, ability to grow in cell cultures and an apathogenic nature, making it an excellent resource for investigation of heterologous genes and growth characteristics of rIBV. During embryo passages however the Beaudette strain may have acquired mutations which are likely to contribute to its
lack of pathogenicity and restrict its in vivo tropism and replication. Replacement of the BeauR S1 or S2 with corresponding subunits from a pathogenic strain did not indicate a significant impairment of in vitro growth of the viruses in comparison to the BeauR virus, showing no indication that BeauR-M41(S1) and BeauR-M41(S2) were unable to enter the cells, fuse with cell membranes or failed to replicate in vitro. Nevertheless, the lack of full protection afforded by BeauR rIBVs against wild-type challenge and the limited in vivo replication, strongly suggest that attenuations have occurred in genes playing an essential role in replication and these are negatively impacting upon its suitability as a vaccine vector. Development of an alternative, less attenuated backbone for expression of heterologous genes in rIBVs may promote the development of these live attenuated vaccines for the control of IBV.

Expression of the IBV S1 subunit alone has been shown to induce virus neutralising antibodies, albeit often requiring repeated vaccination (8, 10). Here, immunisation of chickens with rIBVs based on the Beaudette backbone expressing either M41 S or chimaeric S1/S2 induced virus neutralising antibodies, however the Mock/M41 serum also had a degree of neutralising activity. BeauR-M41(S) vaccinated chickens had significantly higher virus neutralising titres compared to BeauR-M41(S2) group but there was no statistical difference with the BeauR-M41(S1) group, showing that following a single vaccination with rIBV expressing M41(S1) neutralising antibodies are induced.

Live attenuated vaccines against IBV need to induce a good level of mucosal immunity with local tracheal and cell-mediated immunity also playing an important role in prevention of IBV infection (39, 40, 42). As discussed earlier, the BeauR backbone is impeded by poor in vivo replication and the lack of protection shown against ciliostasis indicates that there is a poor level of local immunity induced in the trachea by vaccination with BeauR rIBVs. Cytotoxic responses can also play a key
role in the early control of IBV as indicated by previous studies showing; NK cell activation (41), IBV-specific cytotoxic T-cell lymphocyte (CTL) activity of splenocytes isolated from IBV-infected chickens (42) and higher CTL proportions in respiratory tissues following IBV infection (43). Cellular infiltrates in the head-associated lymphoid tissues as well as a reduction in viral load in the trachea and CALT also implies that the rIBVs infected the chickens and suggests a possible role for the cell-mediated response. However, as we were unable to consistently detect the recombinant S1 viruses at 2 dpv, it raises possibilities that the viruses were either rapidly cleared from the tissues, replicate poorly at these sites of inoculation or have limited replication in a few cells which are below detectable limits of the assays. In the BeauR-M41(S) vaccinated group, over 50% of the chickens were positive for vaccine virus as assessed by RT-PCR, in the Harderian gland and nasal turbinates at 1 and 2 dpv, respectively. The primary site of IBV infection is thought to be the ciliated epithelium lining the trachea, however following ocular-nasal vaccination the virus has been detected in the nasal turbinates (44) and Harderian gland (45).

One possible explanation for poor protection of ciliary activity afforded by the recombinant S1 viruses could be that we only administered one single vaccine dose to the SPF chicks. Previous studies using baculovirus expressed IBV recombinant proteins or IBV purified proteins have required multiple injections to achieve a degree of protection in SPF chickens (10, 17). There is also evidence of an impaired humoral response in young chicks with regards to IBV vaccination; vaccination of 1-day and 7-day-old chicks showed a delay in both systemic and local IgA and IgG levels compared to vaccination of older chicks (14, 21 or 28-day-old) (46). Here, in an attempt to improve the protection against respiratory signs and ciliostasis with the recombinant S1 viruses, a prime/boost approach may aid in overcoming these potential issues.
In summary, we have previously generated recombinant IBV based on a BeauR backbone expressing a heterologous S1 from M41 or QX, and in the present study we have shown that a single vaccination in young chicks with these rIBVs although not adequate to completely prevent ciliostasis and clinical signs, they can induce a degree of virus-specific protective immunity. This was characterised by reduction in viral load recovered from trachea and CALT, cellular infiltrations at head mucosal and inoculation sites, higher serum anti-titres in vaccinated groups and induction of virus neutralising activity. Vaccination with BeauR-M41(S), despite expressing the homologous full S to attempt to overcome any issues with heterologous S1 and S2 subunits and suboptimal folding, only induce a partially protection against the loss of ciliary activity. As in vitro growth characteristics shows that inclusion of a foreign S gene or a chimaeric S gene in the rIBVs does not impede replication in vitro it suggests that the attenuated Beaudette backbone has hindered the in vivo replication of these rIBVs and to improve protection, multiple vaccinations or an alternative backbone may be required.

MATERIALS AND METHODS

Ethics statement. All animal experimental protocols were carried out in strict accordance with the UK Home Office guidelines and under licence granted for experiments involving regulated procedures on animals protected under the UK Animals (Scientific Procedures) Act 1986. The experiments were performed in The Pirbright Institute (TPI) Home Office licensed (X24684464) experimental animal house facilities and were approved by TPI animal welfare and ethical review committee under the terms of reference HO-ERP-01-1. Trial 1 used SPF Rhode Island Red (RIR) chickens obtained from TPI Poultry Production Unit in Compton. Trial 2 used the same chicken breed but obtained from The National Avian Research Facility in Edinburgh.
**Cells and viruses.** Tracheal organ cultures (TOCs) were prepared from 19-day-old SPF RIR chicken embryos (47-49). Primary Chicken Kidney (CK) cells were prepared by The Central Services Unit, TPI from kidneys extracted from either 2 to 3-week-old SPF RIR chickens or 2-week-old SPF derived Valo chickens (49). The pathogenic M41 strain (50) used in this study had previously been adapted in CK cells to produce M41-CK (Accession number X04722) (25). The pathogenic strain, QX (QX L1148 strain, Accession number KY933090) (51), was donated by Prof. Richard Jones, University of Liverpool. The rIBVs BeauR-M41(S), BeauR-M41(S1), BeauR-M41(S2) and BeauR-QX(S1) used herein are described in a schematic illustration (Fig. 1) and constructed using the backbone of Beau-R, which is the molecular clone of Beau-CK (Accession number AJ311317) (21, 26). All isolates of IBV and rIBV were propagated in 10-day-old RIR SPF embryonated eggs. Allantoic fluid was clarified by low speed centrifugation, 24 to 48 hours post infection (hpi). Titrations to determine virus infectivity were either performed in TOCs as described by (25), or in CK cells (49); titres are expressed as 50% (median) ciliostatic doses (CD50) per ml or plaque forming unit (PFU) per ml, respectively.

**Analysis of growth kinetics in CK cells.** Confluent CK cells seeded in either 6-well or 12-well plates were inoculated with 10^4 PFU rIBV or IBV for multi-step growth curves or 10^5 PFU rIBV or IBV for single-step growth curves in 0.5 ml serum-free N,N-Bis(2-hydroxyethyl)-2aminoethanesulphonic acid (BES) medium and incubated for 1 h at 37°C, 5% CO_2. Cells were washed with phosphate buffered saline a (PBSa) to remove residual virus and 2 ml of serum-free BES medium was added per well. Extracellular virus was harvested at defined intervals and assayed by titration in CK cells.

**Experimental design of in vivo vaccination/challenge trials.** SPF RIR chickens were housed in positive-pressure, HEPA-filtered isolation rooms in which each group was housed in a separate
room. In two separate experiments, birds were randomly divided into 5 groups of 30 birds for Trial 1 and 5 groups of 40 birds for Trial 2. Eight-day-old chicks were inoculated (classified as primary inoculation) with $10^5$ PFU of BeauR-M41(S1) or BeauR-QX(S1) (Trial 1) or $10^4$ PFU BeauR-M41(S), BeauR-M41(S1) or BeauR-M41(S2) (Trial 2) in a total of 0.1 ml of PBS via conjunctival (eye drop) and intranasal routes. A challenge dose, equal to the primary inoculation, $10^5$ PFU (Trial 1) and $10^4$ PFU (Trial 2) of the corresponding wild-type viruses were administered in the same manner 21 days after the primary inoculation to the appropriate groups. Of note, the IBV QX strain used here could not be propagated in CK cells, so a CD$_{50}$ dose of $10^{2.73}$ was used. Mock-infected controls were inoculated via the same route with 0.1 ml of PBS and mock/challenge control groups were inoculated with 0.1 ml PBS and challenged with the same dose of wild-type virus. Birds were euthanised by cervical dislocation at specific times post-infection and a panel of tissues sampled to allow for downstream analysis. Blood samples were collected and processed for the collection of serum. Clinical signs used to determine pathogenicity were snicking, rales and ciliary activity of the trachea (a bird was considered protected if 50% or more ciliary activity was retained in 9 out of 10 tracheal rings, this must be in 80% of the group) (28, 52).

**Isolation of tissues: Virus isolation and ciliostasis assay.** Tissues collected were divided into two parts; one part was stabilised in RNAlater® (Ambion) for RNA extraction and the other in 20% sucrose/PBS (0.22µM filtered) at 4°C overnight before snap freezing in OCT (Thermo Scientific) for histology. Tissues collected included: Harderian gland, CALT, NALT and trachea. Tissues were removed at 2 and 4 days post-vaccination (dpv), and at 2, 4 and 14 dpc. Tracheas were removed from five randomly selected chickens from each group at 4 dpv and 4 dpc for assessment of ciliary activity as described previously (25). Part of the trachea and CALT tissues were stored in PBS for virus isolation.
Detection of viral RNA. For virus isolation and RNA extraction, tissues stored in PBS and RNA-later, respectively, were freeze-thawed and homogenised using the TissueLyser II (Qiagen), as described in (23). Total RNA was isolated using the RNeasy® Mini Kit and DNase treated following manufacturer’s instructions (Qiagen). cDNA was synthesised from 1µg of tRNA using Superscript IV Reverse Transcriptase (Life Technologies) with a random oligo primer as per manufacturer’s instructions. To quantify infectious viral load in trachea, tissue derived supernatant was titrated in TOCs. To determine whether infectious virus was present, 10-day-old SPF embryonated eggs were inoculated with 100 µl Allantoic fluid, at 24 – 48 hpi they were assessed for viral presence by RT-PCR using primers specific for the 3’UTR, as described by (53). For quantification of viral load, qPCR was performed using the Taqman Universal PCR Master Mix (Applied Biosystems) with primers and probes specific to the 5’ UTR region, as described by (29). Serial dilutions of M41 cDNA (generated from 1µg tRNA) were included to generate a standard curve and data expressed in terms of the cycle threshold (Ct) value, were normalised using the Ct value of 28S cDNA product for the same sample (54).

Infectious Bronchitis Virus ELISA. Serum samples collected at 21 dpv (pre-challenge), 2, 4 and 14 dpc were assayed with the commercial IDEXX IBV antibody test kit (IDEXX laboratories). To determine the end-point titre the serum samples were two-fold serially diluted in the range 1:20 – 1:2560 prior to incubation. After sample incubation, the remaining steps were followed directly according to the manufacturer’s instructions. The sample/positive (S/P) ratio was determined by the following equation = (Mean sample – Mean Kit Negative)/(Mean Kit positive – Mean Kit Negative). S/P ratios above 0.2 were considered to be positive for IBV antibodies. Polyclonal chicken serum raised against M41 and QX serum were included on each independent test plate (GD Animal Health).
**Immunocytochemistry.** For fluorescent microscopy, cryostat sections (5µm) were fixed in acetone, washed in PBS, and blocked for 1 h at RT with 10% normal goat serum and 0.5% bovine serum albumin in PBS (blocking buffer). Slides were washed and incubated for 1 h with optimally diluted primary antibodies (anti-Bu-1 (clone AV-20, AbD Serotec), anti-CD8α (clone 3-298, AbD Serotec); anti-CD8β (clone EP42, AbD Serotec) and anti-CSF1R (55) or isotype controls, all diluted in blocking buffer. Sections were washed and incubated with an Alexa Fluor 488-labeled goat anti-mouse IgG1/IgG2a or Alexa Fluor 568-labeled goat anti-mouse IgG1/IgG2b according to the appropriate isotype, diluted in blocking buffer for 1 h. Nuclei were visualized using DAPI (Invitrogen). Images were captured with a Leica DMLB fluorescence microscope with a coupled device digital camera and analysed using ImageJ analysis software. For light microscopy, cryostat sections (5µm) were fixed in acetone and stained with Harris’ Haematoxylin (Sigma-Aldrich) and 1% Eosin (Sigma-Aldrich). Sections were dehydrated through graded ethanols and xylene and mounted in a xylene-based medium (DePex, Gurr-BDH Chemicals). Images were captured with a Hamamatsu Nano-zoomer-XR digital slide scanner.

**Analysis of neutralising antibody.** Virus neutralisation tests were performed by GD Animal Health (56). Briefly, two-fold serial dilutions of serum were made in a 1:1 mixture of Medium-199 and Ham’s F10 in 96-well plates. To each well an equal volume of CEK cells (in medium supplemented with 10% FCS) were added. After culture with M41 for 3-4 days at 37°C with 5% CO2, cell monolayers were examined for CPE. All individual titres were expressed as log2 of the reciprocal of the highest serum dilution that showed complete inhibition of CPE.

**Statistical analyses.** Viral load qPCR data were tested for normality through residual plots and the difference between the mean corrected 40-Ct values were statistically evaluated by the parametric one-way ANOVA test adjusted for post-hoc analysis, Tukey’s pairwise comparison.
Serum antibody levels, viral isolation titres, ciliary activity and virus neutralisation titres were tested for normality and non-parametric analyses conducted. Differences between the groups were statistically evaluated by the non-parametric Kruskal-Wallis test adjusted for post-hoc analysis, Mann Whitney U pairwise comparison. The relationship between anti-IBV serum and virus neutralisation titres were compared by Spearman rank correlation analysis. Analysis of the viral growth curves was conducted by fitting a polynomial curve to the exponential phase of viral growth (57), growth rates were then compared between groups by the non-parametric Kruskal-Wallis test adjusted for post hoc analysis. For all statistical analyses, P values of less than 0.05 were considered significant. All statistical analysis was conducted in MiniTab version 17 or GraphPad Prism 7.

ACKNOWLEDGEMENTS

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We would like to thank all animal services staff at The Pirbright Institute (TPI) for their excellent assistance in running the animal experiments, and all members of the Coronavirus group at TPI and Dr. Dominika Borowska for their help with collecting and processing samples during the animal experiments.
REFERENCES


**Figure legends**

**Figure 1.** Design of rIBV constructs. Schematic of wild type BeauR and rIBV genomes generated by reverse genetics to display homologous spike genes in Beaudette backbone. The rIBVs generated expressed either the S1 and/or S2 ectodomain and transmembrane domain (TM) from M41 and QX wild-type virus; with M41 derived genes represented by red boxes and QX derived genes represented by green boxes. In all rIBVs the Beaudette backbone is represented by solid blue boxes and the endodomain (E) of S2 from Beaudette is represented by shaded blue boxes. *BeauR-M41(S) displays the full ectodomain of M41 spike, as previously described (22).*

**Figure 2.** Assessment of clinical signs associated with BeauR-M41(S1) and BeauR-QX(S1) infected chickens following challenge with M41-CK or QX. (A) Snicking and (B) Rales (n=10-20 per group).

**Figure 3.** Cellular infiltrates of head associated lymphoid tissues following vaccination with BeauR-M41(S1) and BeauR-QX(S1). (A and B) Harderian gland at 2dpv (C-E) CALT tissue at 2dpv. (A and C) Cryosections were stained with monoclonal antibodies to detect CSF-1R⁺ (red) and CD8β⁺ (green) cells or (B and D) to detect Bu-1⁺ (green) and CD8α⁺ (red) cells. Nuclei were labelled with DAPI (blue). The scale bars represent 50 µm. (E) H and E stained cryosections of the lower conjunctiva, inset images depict CALT regions detected in (A) BeauR-M41(S), (B) BeauR-QX(S1) tissues which were not clearly evident in (C) Mock lower conjunctivas. The scale bars represent 250 µm. Representative images are shown for all.
Figure 4. Viral load in CALT and Trachea in BeauR-M41(S1) and BeauR-QX(S1) vaccinated chickens following challenge with M41-CK or QX. (A-D). Relative viral RNA load (expressed as corrected 40-Ct) at specific time-points: (A and C) 2 dpc, (B and D) 4 dpc. (E) Infectious viral load titres in trachea at 4 dpc. Data points are shown as individual animals and lines represent mean and standard error of mean (SEM). Statistically significant differences between groups are highlighted: *, P < 0.05; **, P < 0.01.

Figure 5. Measurement of serum anti-IBV titres of BeauR-M41(S1) and BeauR-QX(S1) vaccinated groups. Serum titres were assessed by commercial ELISA at (A) 2 dpc, (B) 4 dpc, (C) M41 groups at 14 dpc and (D) QX groups at 14 dpc. Pre-challenge titres (i.e. 21dpv) are included in (C) and (D). The mean S/P (±SD) from each group (n=5-10) and includes four technical replicates/animal. Dashed line shows the cut-off for positive samples (S/P=0.2). Solid bars denote a trend in statistical significance across dilutions in comparisons with Mock/challenge only group e.g. BeauR-QX(S1)/QX compared to Mock/QX and BeauR-M41(S1)/M41 compared to Mock/M41; *, P<0.05.

Figure 6. Assessment of clinical signs associated with BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccination following challenge with M41-CK. (A) Snicking and (B) rales (n=10-20 per group).

Figure 7. Viral load in CALT and trachea in BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccinated chickens following challenge with M41-CK. (A-D) Relative viral RNA load (expressed as corrected 40-Ct) at (A and C) 2 dpc and (B and D) 4 dpc. (E) Infectious viral load...
titres in trachea (4 dpc). Data points are shown as individual animals and lines represent mean 
(±SEM). Statistically significant differences between groups are highlighted; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

**Figure 8.** Measurement of serum anti-IBV titres of BeauR-M41(S), BeauR-M41(S1) and BeauR-
M41(S2) vaccinated groups. Serum titres were assessed by commercial ELISA at (A) 2 dpc, (B) 4
dpc and (C) 14 dpc. The mean S/P ratio (±SEM) from each group (n=10) includes four technical 
replicates/animal. Dashed line shows the cut-off for positive samples (S/P=0.2). Solid bars denote a trend in statistical significance across dilutions in comparisons with Mock/challenge only group e.g. BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) compared to Mock/M41; *, $P < 0.05$.

**Figure 9.** Measurement of virus neutralisation antibody titres of BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccinated and Mock groups at 14 dpc. (A) Virus neutralisation titres were determined by titration of serum in CK cells. Virus neutralisation titres expressed as log$_2$ of the reciprocal of the highest serum dilution that showed complete inhibition of CPE (n=5 or 10). Lines represent mean (±SEM). Statistically significant differences between groups are highlighted; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B) Relationship between virus neutralisation activity and 14 dpc anti-IBV serum titres. Data points represent the S/P ratios from individual serum samples 
(n=39) plotted against virus neutralisation titres, compared by Spearman rank correlation analysis.

**Figure 10.** Comparison of the growth curves of BeauR-M41(S), BeauR-M41(S1) and BeauR-
M41(S2). (A) Multi-step growth curve in chicken kidney (CK) cells derived from Valo chickens,
(B) 24 h growth curve and (C) single-step 12 h growth curves in CK cells derived from RIR 726 chickens. Supernatant was harvested at various time-points post-infection and titres of progeny 727 virus were determined by a plaque titration assay on CK cells. Data points represent mean of three 728 independent experiments and error bars represent SEM.
Table 1. Assessment of protection against ciliostasis associated with BeauR-M41(S1) and BeauR-QX(S1) vaccination following challenge with M41-CK or QX.

<table>
<thead>
<tr>
<th>Vaccination/Challenge</th>
<th>Mean ciliary activity (±SD)¹</th>
<th>Number of birds with 90% ciliary activity²</th>
<th>Percentage of group protected³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock/Mock</td>
<td>92% (±8.2%)</td>
<td>5/5</td>
<td>N/A</td>
</tr>
<tr>
<td>Mock/M41</td>
<td>2% (±1.4%)</td>
<td>0/5</td>
<td>0%</td>
</tr>
<tr>
<td>BeauR-M41(S1)/M41</td>
<td>9% (±16.3%)</td>
<td>0/5</td>
<td>0%</td>
</tr>
<tr>
<td>Mock/QX</td>
<td>1% (±0%)</td>
<td>0/5</td>
<td>0%</td>
</tr>
<tr>
<td>BeauR-QX(S1)/QX</td>
<td>1% (±1.4%)</td>
<td>0/5</td>
<td>0%</td>
</tr>
</tbody>
</table>

¹Mean ciliary activity per group calculated from ciliostasis scores for 10 tracheal rings per individual bird using formula = ((total ciliostasis score of tracheal rings)/40)*100.

²Ciliary activity assessed according to European Pharmacopeia standards (27) where bird is deemed protected against ciliostasis if no fewer than 9 out of 10 tracheal rings per bird showed normal ciliary activity (>50% ciliary activity retained).

³The vaccine is considered to be efficacious at conferring protection against ciliostasis when 80% or more of the birds in a group were protected.

Table 2. Assessment of protection against ciliostasis associated with BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccination following challenge with M41-CK.

<table>
<thead>
<tr>
<th>Vaccination/Challenge</th>
<th>Mean ciliary activity (±SD)¹</th>
<th>Number of birds with 90% ciliary activity²</th>
<th>Percentage of group protected³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock/Mock</td>
<td>96% (±5.2%)</td>
<td>5/5</td>
<td>N/A</td>
</tr>
<tr>
<td>Mock/M41</td>
<td>0% (±0%)</td>
<td>0/5</td>
<td>0%</td>
</tr>
<tr>
<td>BeauR-M41(S)/M41</td>
<td>65% (±36.2%)</td>
<td>3/5</td>
<td>60%</td>
</tr>
<tr>
<td>BeauR-M41(S1)/M41</td>
<td>19% (±33%)</td>
<td>1/5</td>
<td>20%</td>
</tr>
<tr>
<td>BeauR-M41(S2)/M41</td>
<td>23% (±43.4%)</td>
<td>1/5</td>
<td>20%</td>
</tr>
</tbody>
</table>

¹Mean ciliary activity per group calculated from ciliostasis scores for 10 tracheal rings per individual bird using formula = ((total ciliostasis score of tracheal rings)/40)*100.

²Ciliary activity assessed according to European Pharmacopeia standards (27) where bird is deemed protected against ciliostasis if no fewer than 9 out of 10 tracheal rings per bird showed normal ciliary activity (>50% ciliary activity retained).

³The vaccine is considered to be efficacious at conferring protection against ciliostasis when 80% or more of the birds in a group were protected.
Table 3. Detection of IBV-derived RNA by RT-PCR in head associated lymphoid tissues and trachea samples following vaccination with BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2).

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>Days post-vaccination</th>
<th>Number of virus positive tissues per group</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Harderian gland</td>
<td>Trachea</td>
<td>Nasal turbinates</td>
</tr>
<tr>
<td>Mock</td>
<td></td>
<td>0/5</td>
<td>0/5</td>
<td>N/A</td>
</tr>
<tr>
<td>BeauR-M41(S)</td>
<td>1</td>
<td>3/5</td>
<td>0/5</td>
<td>N/A</td>
</tr>
<tr>
<td>BeauR-M41(S1)</td>
<td>1</td>
<td>1/5</td>
<td>0/5</td>
<td>N/A</td>
</tr>
<tr>
<td>BeauR-M41(S2)</td>
<td>1</td>
<td>1/5</td>
<td>2/5</td>
<td>N/A</td>
</tr>
<tr>
<td>Mock</td>
<td>2</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>BeauR-M41(S)</td>
<td>2</td>
<td>1/5</td>
<td>0/5</td>
<td>3/5</td>
</tr>
<tr>
<td>BeauR-M41(S1)</td>
<td>2</td>
<td>0/5</td>
<td>0/5</td>
<td>3/5</td>
</tr>
<tr>
<td>BeauR-M41(S2)</td>
<td>2</td>
<td>0/5</td>
<td>0/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Mock</td>
<td>4</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>BeauR-M41(S)</td>
<td>4</td>
<td>3/5</td>
<td>0/5</td>
<td>2/5</td>
</tr>
<tr>
<td>BeauR-M41(S1)</td>
<td>4</td>
<td>1/5</td>
<td>0/5</td>
<td>2/5</td>
</tr>
<tr>
<td>BeauR-M41(S2)</td>
<td>4</td>
<td>1/5</td>
<td>2/5</td>
<td>3/5</td>
</tr>
</tbody>
</table>

The results are depicted as “number of positive samples/number of birds per group” (total of 5 birds/group). All positive results were confirmed by sequencing of PCR products (data not shown).
Fig 1

BeauR

BeauR M41(S)*

BeauR M41(S1)

BeauR M41(S2)

BeauR QX(S1)
Fig 3

A

B

C

D

E
Fig 6

![Graph A](https://via.placeholder.com/150)

- **A**: Spike shedding
- **B**: % of chickens with mass

Legend:
- Mock
- Mock/MA41
- Boca/R-M41/S1/MA41
- Boca/R-M41/S2/MA41

Days post-challenge from 2 to 14.
Fig 9

A

B

$r^2 = 0.5$

$p = 0.002$
Fig 10

A

B

C

Virus titre (log_{10} PFU/ml)

Time (hours post-infection)

M41-CK
BeauR
BeauR-M41(S)
BeauR-M41(S1)
BeauR-M41(S2)
Fig 2
Fig 8

A

B

C

Dilution factor

S/P ratio

- ModvM41
- BeauR-M41(S)M41
- BeauR-M41(S1)M41
- BeauR-M41(S2)M41
Fig 9

A

B

$r^2 = 0.5$

$p=0.002$
Fig 10

![Graph A](https://example.com/graph-a.png)

![Graph B](https://example.com/graph-b.png)

![Graph C](https://example.com/graph-c.png)

- **Graph A**
  - Time (hours post-infection)
  - Virus titre (log10 PFU/ml)
  - Data points for different samples

- **Graph B**
  - Time (hours post-infection)
  - Virus titre (log10 PFU/ml)
  - Data points for different samples

- **Graph C**
  - Time (hours post-infection)
  - Virus titre (log10 PFU/ml)
  - Data points for different samples

Legend:
- M41-CK
- BeauR
- BeauR-M41(S)
- BeauR-M41(S1)
- BeauR-M41(S2)