Recombinant infectious bronchitis viruses expressing heterologous S1 subunits: potential for a new generation of vaccines that replicate in Vero cells

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Abstract

The spike glycoprotein (S) of infectious bronchitis virus (IBV) comprises two subunits, S1 and S2. We have previously demonstrated that the S2 subunit of the avirulent Beau-R strain is responsible for its extended cellular tropism for Vero cells. Two recombinant infectious bronchitis viruses (rIBVs) have been generated; the immunogenic S1 subunit is derived from the IBV vaccine strain, H120, or the virulent field strain, QX, within the genetic background of Beau-R. The rIBVs BeauR-H120(S1) and BeauR-QX(S1) are capable of replicating in primary chicken kidney cell cultures and in Vero cells. These results demonstrate that rIBVs are able to express S1 subunits from genetically diverse strains of IBV, which will enable the rational design of a future generation of IBV vaccines that may be grown in Vero cells.

The avian gammacoronavirus infectious bronchitis virus (IBV) is responsible for an economically important highly contagious respiratory disease of poultry. IBV replicates primarily in the respiratory tract [1, 2], causing nasal discharge, snicking, rales and tracheal ciliostasis in chickens [3], but it is also able to replicate in many other epithelial surfaces, including enteric surfaces [4], oviducts and kidneys [5–7].

Coronaviruses generally exhibit restricted cell and tissue tropism, which is dependent upon the S glycoprotein of individual coronavirus strains [8, 9]. However, some strains of IBV, following adaptation, are able to replicate in primary chicken cells, such as chicken kidney (CK) cells. Interestingly, the Beaudette strain, after several hundred passages in embryonated eggs, was also discovered to have an extended host range, with the ability to replicate in a mammalian cell line, Vero cells [10] and, to a limited extent, baby hamster kidney (BHK-21) cell lines [11]. The IBV Beau-R, a molecular clone of Beaudette [12] strain, replicates in both primary CK cells and in Vero cells, whereas H120, a vaccine strain, only replicates in primary cells, and QX, a virulent field strain, is not able to replicate in cell culture.

The IBV spike (S) glycoprotein comprises S1 and S2 subunits that are cleaved during biosynthesis by a furin-like protease, but which remain non-covalently linked. We have previously demonstrated that the cellular tropism of IBV is determined by the S glycoprotein; replacement of the ectodomain of the IBV Beaudette S glycoprotein with the corresponding region from the pathogenic IBV M41-CK resulted in a recombinant IBV (rIBV), BeauR-M41(S), which had the tissue tropism associated with M41-CK [13]. Further work demonstrated that the S2 subunit of the avirulent Beaudette strain of IBV confers the ability to replicate in Vero cells, particularly the Beaudette-specific motif surrounding the S2’ cleavage site [14].

Vaccines against IBV, both live attenuated and inactivated, are currently produced in embryonated hen’s eggs, a cumbersome and expensive process, due to the fact that most IBV strains do not replicate in cultured cells. Production in a cell line such as Vero cells, derived from African green monkey kidney epithelial cells, would be beneficial, as they can be grown in suspension or flat bed and it is possible to achieve consistent virus yields. Vero cells have already been validated for virus growth and are licensed for use in human vaccine manufacture, including the production of polio and rabies vaccines [15, 16], and several influenza virus vaccines have been developed for growth on Vero cells [17, 18].

In the present study we have investigated the possibility of generating attenuated recombinant IBVs expressing heterologous S1 subunits that can be propagated in Vero cells and may have the additional benefit of being capable of being used as vaccines.
The IBV strains used in this study were: (1) Beau-R [12], a molecular clone of CK cell-adapted Beaudette [19], Beaudette-CK [20]; (2) H120, a vaccine strain of IBV [21]; and (3) QX, a virulent field strain of IBV that was first detected in China in 1996 [22] and has since spread throughout Europe, Asia and the Middle East [23]. The isolate of QX used in this study, L1148, was obtained from Professor Richard Jones, University of Liverpool [24]. The IBV strains were propagated and titrated in CK cells [25] as described previously [26–28].

Recombinant IBVs with chimaeric S genes were generated using reverse genetics. The resulting viruses, BeauR-H120 (S1) and BeauR-QX(S1), have the S1 subunit of H120 or QX within the genomic background derived from Beau-R, including the Beau-R S2 subunit. Briefly, sequences corresponding to the S1 subunit of H120 (GenBank accession number M21970) or QX (GenBank accession number DQ431199) and the S2 subunit of Beau-R, flanked by 474 nucleotides (nt) of Beau-R replicase sequence and 333 nt of Beau-R gene 3 (GenBank accession number AJ311317), were synthesized and cloned into pGPT-NEB193 [29] by

![Figure 1](image-url)

**Fig. 1.** Recombinant IBVs expressing heterologous S1 subunits replicate in CK cells as wild-type IBV. (a) CK cells were grown on 13 mm coverslips in 24-well plates to approximately 50 % confluency and infected with 0.15 m.o.i of IBV in duplicate. Infected cells were fixed 24 h post-infection with 4 % paraformaldehyde in PBS and permeabilized using 0.5 % Triton-X100. IBV-infected cells were identified by incubation with a 1 : 400 dilution of mouse anti-dsRNA J2 IgG2a monoclonal antibody and detected with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody diluted 1 : 200 (green). Nuclei (blue) were stained with DAPI. Scale bars indicate 50 µm. (b) Confluent monolayers of CK cells in six-well plates were infected with 0.005 m.o.i. IBV in triplicate for 1 h at 37 °C in 5 % CO₂, washed twice with PBS and then 3 ml of fresh 1 x BES medium was added. Cell supernatants were harvested at 1, 12, 24, 48 and 72 h post-infection and assayed for progeny virus by plaque assay using CK cells. Three replicates were performed and the averages were taken. The error bars indicate the standard error of the mean.
GeneArt (Thermo Fisher). The S1 subunits from H120 and QX replaced the Beau-R sequence between the 3’ end of the S gene signal sequence at nt position 20,418 and the 5’ end of the S1/S2 cleavage site (RRFRR) at nt position 21,964. As the C-terminal end of the IBV replicase gene overlaps with the N-terminal end of the S gene by 50 nt, the signal sequence of the chimaeric S gene was derived from Beau-R. The IBV nucleotide and amino acid positions are based on the Beau-R sequence (GenBank accession number AJ311317). The plasmids were pGPT-H120(S1)-BeauR(S2) and pGPT-QX(S1)-BeauR(S2). The PCR products derived from the plasmids, the S gene sequence and adjoining regions of the Beaudette genome were sequenced to authenticate the modified sequences using a variety of oligonucleotides derived from the Beau-CK sequence [30]. Assembly of the sequences was performed using Gap4 of the Staden sequence software programs [31].

The IBV cDNAs contained within plasmids pGPT-H120(S1)-BeauR(S2) and pGPT-QX(S1)-BeauR(S2) were introduced into the Beau-R cDNA genome minus the S gene within the genome of recombinant vaccinia virus (rVV) by homologous recombination using the transient dominant selection system as previously described [14, 29, 32]. Infectious rIBVs were recovered from recombinant vaccinia viruses containing the correctly modified IBV cDNAs and passaged three times in CK cells (P<sup>3</sup>-CK) to generate a stock virus for experimental use. The rIBVs were passaged in Vero cells seven times (P<sup>7</sup>-Vero) and BeauR-QX(S1) was

![Fig. 2. Recombinant IBVs expressing heterologous S1 subunits replicate in Vero cells. (a) Vero cells were grown on 13 mm coverslips in 24-well plates to approximately 50% confluency and infected with 0.15 m.o.i of IBV in duplicate. Infected cells were fixed 24 h post-infection with 4% paraformaldehyde in PBS and permeabilized using 0.5% Triton-X100. IBV infected cells were identified by incubation with a 1:400 dilution of mouse anti-dsRNA J2 IgG2a monoclonal antibody and detected with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody diluted 1:200 (green). Nuclei (blue) were stained with DAPI. Scale bars indicate 50 µm. (b) Confluent monolayers of Vero cells in six-well plates were infected with 0.005 m.o.i of IBV in triplicate for 1 h at 37°C in 5% CO<sub>2</sub>, washed twice with PBS and then 3 ml of fresh 1 BES medium was added. Cell supernatants were harvested at 1, 12, 24, 48 and 72 h post-infection and assayed for progeny virus by plaque assay using CK cells. Three replicates were performed and the averages were taken. The error bars indicate the standard error of the mean.](https://www.microbiologyresearch.org)
further passaged to P11-Vero as little cytopathic effect was observed by brightfield microscopy (data not shown).

We have previously shown that it is possible to express the S1 subunit from M41, a genetically similar strain of IBV, within the genomic background of Beau-R [14]. M41, H120 and Beaudette all belong to the GI-1 lineage, previously known as the Massachusetts serotype, and share 98% (M41 and Beau-R) or 97% (H120 and Beau-R) identity within the S1 subunit [33]. The results of this study demonstrate that Beau-R is capable of expressing the S1 subunit from a more genetically diverse strain of IBV; the QX strain of IBV is of the GI-19 lineage [33], and the S1 subunit of QX shares only 79% identity with Beaudette.

The growth characteristics of the P3-CK and P7- or P11-Vero rIBV isolates were analysed by confocal microscopy with indirect immunofluorescence and the replication kinetics were assessed in CK cells (Fig. 1) and in Vero cells (Fig. 2). CK and Vero cells were infected with 0.15 multiplicity of infection (m.o.i) of rIBV and fixed 24h post-infection with 4% paraformaldehyde in phosphate-buffered saline (PBS) before being permeabilized using 0.5% Triton X-100. IBV-infected cells were identified by incubation with mouse anti-dsRNA J2 IgG2a monoclonal antibody (English and Scientific Consulting Bi), which is a marker for IBV replication, and detected with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Immunolabelled cells were imaged using a Leica TCS SP5 DM6000 confocal microscope using the Leica Microsystems LAS AF software.

Both recombinant IBVs with chimaeric S genes are able to replicate in CK cells as well as the parent virus, Beau-R (Fig. 1). This is somewhat surprising as the S1 subunit has been shown to possess the receptor-binding domain [34], which is responsible for interacting with the virus receptor on susceptible host cells. Whilst the H120 strain is able to replicate in CK cells, the QX strain is unable to (Fig. 1a). Our previous study introducing the M41 S1 subunit to the Beau-R genome indicated that the S2 subunit of Beau-R conferred the ability to replicate in Vero cells [14], however, as M41 is also able to replicate in CK cells, there was no difference in the tropism of the rIBV for CK cells. The present study indicates that Beau-R’s ability to replicate in both CK and Vero cells may be conferred by the S2 subunit.

The growth characteristics of the P3-CK and P7- or P11-Vero rIBV isolates in Vero cells were analysed alongside the S1 donor isolates, H120 and QX (Fig. 2). Both S1 donor isolates, H120 and QX, were unable to sustain replication in Vero cells. The recombinant IBVs expressing the H120 S1 subunit were able to replicate as well as Beau-R in Vero cells, but the replication of those expressing the QX S1 subunit were reduced by 1–2 log_{10} over the 72 h period. This may have been due to the increased sequence diversity between the S1 and S2 subunits between Beau-R and QX leading to conformational differences affecting attachment or fusion, although no such reduction in titre was observed in CK cells (Fig. 1b).

The growth kinetics of the rIBVs were very similar between the P3-CK and P7- or P11-Vero isolates, indicating that little adaptation was required for growth in either CK or Vero cells. The S gene sequences of each isolate were determined to investigate whether any mutations occurred during the rescue in CK cells, or passaging in Vero cells. A single-nucleotide substitution occurred in the S gene of BeauR-H120(S1) isolates, C_{22257}T, resulting in an amino acid change P_{630}L within the S2 subunit. Two nucleotide substitutions occurred in the S gene of BeauR-QX(S1) isolates, T_{21822}C and C_{22955}A, resulting in amino acid changes L_{466}P and A_{863}D in the S1 and S2 subunits, respectively. All of these mutations were present upon rescue in CK cells and maintained during passage on Vero cells, indicating that they may be required to compensate for structural changes in the chimaeric spike glycoproteins.

These results demonstrate that the S1 subunit of a vaccine strain or a field strain, the most relevant region for the generation of neutralizing antibodies, can be incorporated into an attenuated virus that is able to grow in Vero cells, enabling IBV vaccines to be grown in cell lines rather than embryonated eggs. Further work will be required to establish whether these recombinant IBVs will be able to stimulate a protective immune response in chickens.

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