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 25 (2007) 5558–5562
Vaccine
www.elsevier.com/locate/vaccine

Manipulation of the infectious bronchitis coronavirus genome for vaccine development and analysis of the accessory proteins☆

Dave Cavanagh[a,*], Rosa Casais[a], Maria Armesto[a], Teri Hodgson[a], Sousan Izadkhasti[a], Marc Davies[a], Fengsheng Lin[b], Ian Tarpey[b], Paul Britton[a]

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

Received 23 June 2006; received in revised form 12 January 2007; accepted 16 February 2007
Available online 6 March 2007

Abstract

Infectious bronchitis coronavirus (IBV) is the cause of the single most economically costly infectious disease of domestic fowl in the UK—and probably so in many countries that have a developed poultry industry. A major reason for its continued dominance is its existence as many serotypes, determined by the surface spike protein (S), cross-protection being poor. Although controlled to some degree by live and inactivated vaccines, a new generation of IB vaccines is called for. Reverse genetic or 'infectious clone' systems, which allow the manipulation of the IBV genome, are key to this development. New vaccines would ideally be: genetically stable (i.e. maintain a stable attenuated phenotype); administered *in ovo*; and be flexible with respect to the source of the spike protein gene. Rational attenuation of IBV requires the identification of genes that are simultaneously not essential for replication and whose absence would reduce pathogenicity. Being able to modify a 'core' vaccine strain to make it applicable to a prevailing serotype requires a procedure for doing so, and the demonstration that 'spike-swapping' is sufficient to induce good immunity.

We have demonstrated that four small IBV proteins, encoded by genes 3 and 5, are not essential for replication; failure to produce these proteins had little detrimental affect on the titre of virus produced. Our current molecularly cloned IBV, strain Beaudette, is non-pathogenic, so we do not know what effect the absence of these proteins would have on pathogenicity. That said, plaque size and composition of various gene 3/5 recombinant IBVs in cell culture, and reduced output and ciliostasis in tracheal organ cultures, shows that they are less aggressive than the wild-type Beaudette. Consequently these genes remain targets for rational attenuation. We have recently obtained evidence that one or more of the 15 proteins encoded by gene 1 are also determinants of pathogenicity. Hence gene 1 is also a target for rational attenuation.

Replacing the S protein gene of Beaudette with that from the pathogenic M41 strain resulted in a recombinant virus that was still non-pathogenic but which did induce protection against challenge with M41. We have since made other 'spike-swapped' recombinants, including ones with chimaera S genes. Uniquely, our molecular clone of Beaudette is benign when administered to 18-day-old embryos, even at high doses, and induces immunity after this route of vaccination.

Taken together, our results point to the creation of a new generation of IB vaccines, based on rational modification of the genome, as being a realisable objective.

Keywords: Coronavirus; Vaccines; Poultry; *In ovo*

1. Introduction

Infectious bronchitis virus (IBV) is the single most economically costly infectious disease of domestic fowl in the UK, causing losses in both meat-type and commercial egg layer birds [1,2]. Although live and inactivated vaccines have contributed immensely to the management of IB,

☆ Presented at the 4th International Veterinary Vaccines and Diagnostics Conference, 25–29 June 2006, Oslo, Norway

* Corresponding author. Tel.: +44 1635 578411; fax: +44 1635 577304.
E-mail address: dave.cavanagh@bbsrc.ac.uk (D. Cavanagh).

0264-410X/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2007.02.046

control is suboptimal due to a number of factors, including inefficient application of vaccine and extensive antigenic variation [3]; serotypes induce poor cross-protection [4–6]. The poultry industry is moving towards *in ovo* vaccination where possible. This has been achieved for Marek's disease. To date all IB vaccines have severely reduced hatching. We have two strategic goals. One, to modify the genome of our molecularly cloned cDNA of the Beaudette strain of IBV (non-pathogenic) to achieve optimum stimulation of protection whilst retaining its non-pathogenic phenotype in both chicks and 10-day-old embryos. Two, to swap the gene of the spike protein (S), which induces protective immunity, to make a vaccine appropriate for a prevailing serotype.

Our first goal requires the identification of genes that can be modified without having a negative impact on replication—which would increase vaccine production costs. Coronaviruses have one or more (IBV has two, numbers 3 and 5) genes, interspersed amongst the structural protein genes, encoding proteins (four in the case of IBV) of unknown function (Fig. 1). Using our reverse genetic system [7] we have made recombinant IBVs (rIBVs) of the non-pathogenic strain Beaudette, that are unable to make one or more of these proteins [8,9]. Most recently we have made a chimaera, comprising gene 1 from non-pathogenic Beaudette and all other genes from pathogenic strain M41.

Inactivated IB vaccines are ineffective unless preceded by live attenuated vaccines to prime the protective immune response. The S protein comprises two subunits: S1 (~520 amino acids), derived from the amino-terminal half of the S polyprotein, and the membrane anchoring S2 subunit (~625 amino acids), derived from the carboxy-terminal half. The S1 spike protein subunit is necessary and sufficient to induce protective immunity [10]. Serotypes commonly differ from one another by 20–25% of S1 amino acids [1,2], although differences in as few as 5% of the amino acids in S1 can decrease cross-protection [11]. A new generation of IB vaccines will still require that S protein genes of different serotypes are available. S gene swapping could result in changes in pathogenicity, as has been demonstrated for porcine transmissible gastroenteritis virus (Enjuanes and colleagues [12,13]). Therefore we have investigated this, simultaneously examining the protection-inducing capacity of spike-swapped rIBVs [11].

Finally, we have demonstrated that our molecularly cloned IBV, and derivatives of it, do not have deleterious effects on hatching, following inoculation of embryos at 18 days of age.

2. Proteins 3a, 3b, 5a and 5b are not essential for replication

In order to ablate production of the two proteins encoded by open reading frames 5a and 5b we used two approaches, both of which involved minimal alteration to the genome. The AUG translation initiation codes of each ORF were rendered inoperable by substitution mutation. We did this for each ORF separately, and both together. Growth curves in both primary chick kidney cells and in embryonated chicken eggs revealed that titres were within two to three-fold that of the wild-type virus [8]. The same phenotype was obtained when expression of the gene 5 mRNA was eliminated, by mutation of the transcription-associated sequence (TAS), thus resulting in no production of the 5a and 5b proteins.

Very similar results were obtained when ORFs 3a and 3b of gene 3 were rendered inoperative by mutation of the translation start codons [9]. Modification of the gene 3 TAS could not be done as this would have resulted in non-production of the essential structural protein E, encoded by a third ORF within gene 3.

These results showed that none of 3a, 3b, 5a or 5b were essential for replication. For this reason they have been called 'accessory proteins', whose functions may include antag-

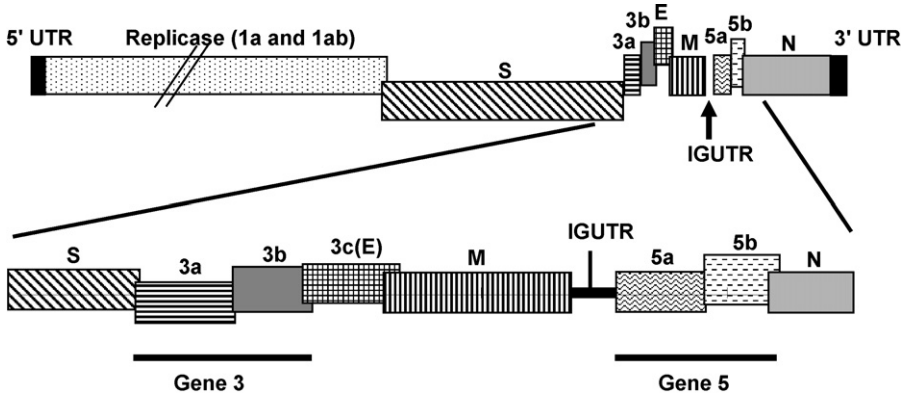

Fig. 1. Schematic diagram showing the genomic positions of the IBV genes 3 and 5. The top part of the diagram shows the overall genome structure of IBV in which the replicase gene represents approximately two thirds of the genome. The lower part of the diagram highlights part of the genome, representing the genes downstream of the replicase gene, showing the overlapping nature and position of genes 3 and 5. IBV gene 3 encodes three proteins, 3a and 3b of unknown function and E(3c) which is a structural protein and has been shown for other coronaviruses to be involved in virus budding. IBV gene 5 encodes two proteins, 5a and 5b, of unknown function.

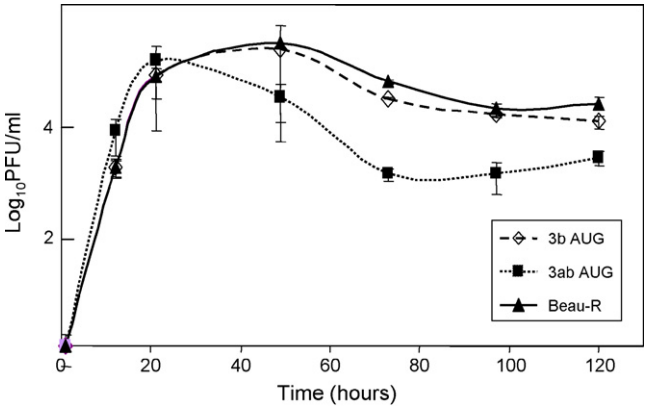

Fig. 2. Early decline in the replication of rIBV Beaudette unable to produce protein 3a, encoded by the first open reading frame (ORF) of gene 3. Groups of chicken tracheal organ cultures (TOCs) were inoculated in triplicate with 10^4 PFU of virus. After 1 h at 37 °C the inoculum was removed, the TOCs washed, and incubation continued. At intervals progeny was titrated by plaque assay in chick kidney cells. rIBV unable to produce the ORF 3b protein (labelled 3b AUG) replicated very similarly to the wild-type virus (Beau-R). Virus unable to produce both 3a and 3b proteins initially replicated normally but then the titre declined earlier than the wild-type virus. This phenomenon was demonstrated in repeated experiments [9].

onism of innate immune responses. Shen et al. [14] and Youn et al. [15] have also demonstrated that the IBV proteins 3b and 5a, respectively, are not required for replication *in vitro*.

More complex results were obtained during growth curve experiments using tracheal organ cultures. (The ciliated epithelium of the trachea is one of the major targets of the virus.) Recombinant IBVs unable to make 3a, 5a or 5b initially replicated to the same titre as wild-type virus, but then the titres declined more rapidly (Fig. 2). If it is the case that one or more of the accessory proteins are antagonists of innate immunity, it may be that the innate immune responses operating within the tracheal organ cultures are more effective than in chick kidney cells and 10-day-old embryos.

Taken together our results show that the four proteins in question are not essential for replication and hint that they have accessory functions, possibly associated with innate immunity. IBV and related avian coronaviruses [1] are in coronavirus Group 3. The coronaviruses of mammals, which occupy Groups 1 or 2, have genes interspersed amongst the structural protein genes, although they have no sequence relationship with those of Group 3 coronaviruses [16]. Inactivation of the production of these proteins had little or no effect on the titres of virus produced in cell cultures [17–19] showing that they, too, were accessory proteins. In some, though not all, cases, failure to produce these proteins resulted in attenuation of pathogenicity. In the latter cases, production of the accessory proteins had been prevented by deletion of the ORFs. It is not clear whether the attenuated phenotype arose from the absence of the proteins or from the perturbation of the genome caused by the deletions.

3. Gene 1 encodes determinants of pathogenicity

Coronaviruses express two polyproteins, pp1a and pp1b, from gene 1, the second polyprotein results from a ribosomal shift mechanism, which encode 16 non-structural proteins (nsp1–nsp16). The products of the polyproteins are associated with RNA replication and transcription [20,21]. However, the IBV gene 1 does not encode a product equivalent to nsp1, expressed from gene 1 of the other coronaviruses, and therefore IBV gene 1 encodes 15 products, named nsp2–nsp16 to be consistent with the gene 1 products encoded by the other coronaviruses. In order to test the hypothesis that one or more of the four structural protein genes and/or accessory protein genes 3 and 5 of the pathogenic M41 strain were determinants of the pathogenicity, we made a rIBV that had gene 1 from Beaudette and all other genes from M41. The recombinant was viable but was not pathogenic and caused little ciliostasis in tracheal organ cultures (Maria Armesto, D. Cavanagh and P. Britton, in preparation). Looked at one way, Beaudette had not been made pathogenic by substitution of its non-gene 1 genes with those from pathogenic M41. An alternative way of looking at it is to say that the pathogenicity of the M41 strain had been attenuated by replacement of its gene 1 by that of Beaudette. Either way, it indicates that one or more of the gene 1-encoded proteins are determinants of pathogenicity. Our result does not negate the possibility that genes 3 and 5 have 'self-defence' roles but it does indicate that their affect can be overcome by proteins encoded by gene 1. Hence gene 1 is another target for rational attenuation for vaccine development.

4. Spike-swapping for serotype-specific vaccines

When we first replaced the S protein gene of our molecularly cloned Beaudette strain with that of the M41 strain, we did it by rebuilding our full-length cDNA [22]. We have since achieved this by a quicker process [23], and have subsequently replaced the S gene of Beaudette with that from two other strains of IBV.

The rIBV with the S protein of M41 had a phenotype like that of M41, i.e. its host range *in vitro* was restricted, like that of M41 *in vitro*, and unlike Beaudette, which can grow in some mammalian cell lines [22]. This demonstrated that the S protein is a determinant of host range.

To establish the principle that spike-swapped rIBVs had potential for vaccine development, we inoculated chickens, by eye-drop and intranasally, and challenged them 3 weeks later using the M41 strain. Using prevention of snicking (analogous to sneezing) and retention of tracheal ciliary activity as measures of protection, Beaudette did not induce protection against M41 whereas the spike-swapped recombination did do so [11] (Fig. 3). Firstly, this demonstrated that spike-swapped rIBVs were effective at inducing protective immunity. Secondly, the result suggested that relatively few differences between S proteins could result in poor cross-

Table 1
Embryo vaccination with recombinant IBVs, analysis of hatch, clinical signs, serological responses and protection from virulent challenge

Virus	Hatch rate (%)	Mean ciliary activity posthatch (%)	Serological response (log_2) ELISA	Protection (based on ciliary activity postchallenge)	
				Day 5	Day 7
Beau-R	73	71	6.30	0	30
BeauR–M41(S)	82	77	7.05	80	100
CV1	18	5	10.5	100	100
Placebo	85	98	≤5.64	0	0

Fertile SFP eggs were inoculated with either a commercial vaccine (CV1; 10^4 EID_{50}); the molecularly cloned Beaudette strain Beau-R (10^6 EID_{50}); Beau-R in which the S protein gene had been replaced by that from the pathogenic M41 strain, to make BeauR–M41(S) (10^6 EID_{50}); or a placebo. Hatch was assessed at 21.5 days of incubation. Ciliary activity was assessed in five birds at day 6 posthatch, serological responses by ELISA at 4 weeks posthatch. All birds were challenged with virulent M41 at 4 weeks posthatch and the ciliary activity determined at 5 and 7 days postchallenge by microscopic observation of tracheal rings. A high percentage of ciliary activity was indicative of protection.

protection: the S proteins of the Beaudette and M41 strains differ by only 5% of S1 and of S2 amino acids.

We have replaced the S gene of Beaudette with that of the UK/4/91 strain (a.k.a. 793/B). IBV strain UK/4/91 grows very poorly in CK cells, presumably from lack of adaptation to theses cells. Therefore the rIBVs based on Beaudette but expressing the UK/4/91 S protein had to be recovered using 10-day-old embryonated eggs. We have also replaced the Beaudette S protein gene with the S protein gene of the Begian BE/B1648/87 strain. In this case, recombination between the S genes of B1648 (in a plasmid) and Beaudette (within the full-length IBV genome cDNA within the vaccinia virus genome) was allowed to happen randomly, resulting in recovery of several rIBVs with different cross-over points within the S gene, i.e. producing rIBVs with chimaeric S genes (S. Izadkhasti, D. Cavanagh and P. Britton, in preparation). This demonstrated that recombination within the S gene can give rise to viable recombinant IBVs, at high frequency (∼50% in our experiments), providing hard evidence to support the circumstantial evidence (sequence comparison of field strains) that recombination is a feature of the evolution of IBV.

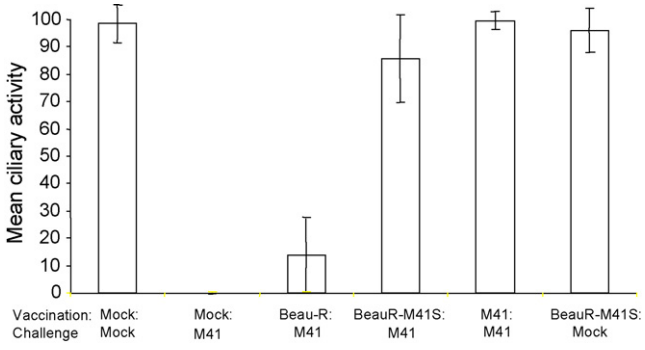

Fig. 3. Better induction of protection, against challenge with IBV M41, by vaccination with IBV Beaudette R expressing the spike protein of M41 (BeauR–M41S) than by expression of the Beaudette spike protein (Beau-R) [11]. Chicks were vaccinated or not as indicated, and challenged 3 weeks later with M41, or not challenged. The trachea was removed from groups of three chicks at days 4–6 after challenge. Protection was assessed by observing the tracheal rings by low power microscope, and recording the percentage of the lumenal surface that exhibited ciliary activity.

5. Vaccination *in ovo*

Embryo vaccination against Marek's disease is common in the USA and South America. Embryo vaccination saves on labour charges so can be cost effective for large hatcheries. As most strains of IBV are lethal to embryos little work on embryo vaccination with IBV has been done. One group has shown that *in ovo* vaccination with IBV can be successfully accomplished, although the strain reduced hatch at relatively low titres [24].

We tested rIBVs based on the Beaudette strain expressing the Beaudette S protein (Beau-R) or the M41 S protein (BeauR–M41(S)) for their potential as vaccines for 18-day-old embryos. A high dose of virus was inoculated *in ovo* and the pathogenicity was assessed by observing the effect on hatch, plus clinical signs and effect on the tracheal ciliary activity posthatch. Neither of the two rIBVs strains reduced hatchability or caused nasal discharge, and caused minimal damage to the ciliated epithelium of the trachea (Table 1). In contrast, a commercial vaccine (refereed to as CV1) drastically reduced the hatch and caused substantial clinical signs (Table 1). The rIBVs induced a serological response and the spike-swapped rIBV gave a high level of protection of the embryos against virulent M41 challenge (Table 1). These results are promising for the development of embryo-safe efficacious IBV vaccines for *in ovo* application [26].

6. Final remarks

Genetic manipulation of coronaviruses of Groups 1–3 have demonstrated that the small non-structural protein genes interspersed amongst the structural protein genes encode mostly accessory proteins: not essential for replication, and presumably having a function *in vivo*. Deletion of all the non-gene 1 accessory proteins of murine hepatitis virus (MHV) produced virus that replicated in mice but which, unlike the wild-type virus, was non-lethal [17]. Deletion of some, but not all, of the accessory protein genes of feline coronavirus [18] reduced pathogenicity. Removal of accessory protein gene 3 of transmissible gastroenteritis coronavirus [19] did

not reduce its enteropathogenicity. Thus the accessory protein genes of coronaviruses are targets for modification for the purpose of vaccine development, although each gene has to be investigated in its own right. It is becoming increasingly likely that one or more proteins encoded by gene 1 also influence pathogenicity. This stems from our own observations with chimaeric IBV, and those of Sperry et al. [25] with MHV; a single amino acid substitution in nsp14 encoded by gene 1 attenuated pathogenicity for mice. Gene 1, therefore, is another target for manipulation. As we have demonstrated, the extensive antigenic variation of IBV can be addressed by spike-swapping, and our molecularly cloned IBV has potential for the development of a vaccine that can be applied *in ovo*.

Acknowledgements

We acknowledge the support of the following organisations: Department of the Environment, Food and Rural Affairs (Defra); Biotechnology and Biological Sciences Research Council (BBSRC); Intervet International; British Egg Marketing Board Research and Education Trust.

References

[1] Cavanagh D. Coronaviruses in poultry and other birds. Avian Pathol 2005;34:439–48.
[2] Britton P, Cavanagh D. Avian coronavirus diseases and infectious bronchitis vaccine development. In: Thiel V, editor. Coronaviruses: molecular biology and diseases. UK: Horizon Scientific Press; 2007, in press.
[3] Cavanagh D, Picault JP, Gough R, Hess M, Mawditt K, Britton P. Variation in the spike protein of the 793/B type of infectious bronchitis virus, in the field and during alternate passage in chickens and embryonated eggs. Avian Pathol 2005;34:20–5.
[4] Gelb Jr J, Weisman Y, Ladman BS, Meir R. S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). Avian Pathol 2005;34:194–203.
[5] Ladman BS, Loupos AB, Gelb Jr J. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol 2006;35:127–33.
[6] Mardani K, Noormohammadi AH, Ignatovic J, Browning GF. Typing infectious bronchitis virus strains using reverse transcription-polymerase chain reaction and restriction fragment length polymorphism analysis to compare the 3′ 7.5 kb of their genomes. Avian Pathol 2006;35:63–9.
[7] 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.
[8] Casais R, Davies M, Cavanagh D, Britton P. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. J Virol 2005;79:8065–78.
[9] Hodgson T, Britton P, Cavanagh D. Neither the RNA nor the proteins of open reading frames 3a and 3b of the coronavirus infectious bronchitis virus are essential for replication. J Virol 2006;80:296–305.
[10] Johnson MA, Pooley C, Ignjatovic J, Tyack SG. A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. Vaccine 2003;21:2730–6.
[11] 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.
[12] Ballesteros ML, Sanchez CM, Enjuanes L. Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology 1997;227:378–88.
[13] Krempl C, Ballesteros M, Zimmer G, Enjuanes L, Klenk H, Herrler G. Characterization of the sialic acid binding activity of transmissible gastroenteritis coronavirus by analysis of haemagglutination-deficient mutants. J Gen Virol 2000;81:489–96.
[14] Shen S, Wen ZL, Liu DX. Emergence of a coronavirus infectious bronchitis virus mutant with a truncated 3b gene: functional characterization of the 3b protein in pathogenesis and replication. Virology 2003;311:16–27.
[15] Youn S, Leibowitz JL, Collisson EW. In vitro assembled, recombinant infectious bronchitis viruses demonstrate that the 5a open reading frame is not essential for replication. Virology 2005;332:206–15.
[16] Gonzalez JM, Gomez-Puertas P, Cavanagh D, Gorbalenya AE, Enjuanes L. A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. Arch Virol 2003;148:2207–35.
[17] de Haan CA, Masters PS, Shen X, Weiss S, Rottier PJ. The group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host. Virology 2002;296:177–89.
[18] Haijema BJ, Volders H, Rottier PJ. Live attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. J Virol 2004;78:3863–71.
[19] Ortego J, Sola I, Almazan F, Ceriani JE, Riquelme C, Balasch M, et al. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. Virology 2003;308:13–22.
[20] Gorbalenya AE, Enjuanes L, Ziebuhr J, Snijder EJ. Nidovirales: evolving the largest RNA virus genome. Virus Res 2006;117:17–37.
[21] Pasternak AO, Spaan WJ, Snijder EJ. Nidovirus transcription: how to make sense...? J Gen Virol 2006;87:1403–21.
[22] 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.
[23] Britton P, Evans S, Dove B, Davies M, Casais R, Cavanagh D. Generation of a recombinant avian coronavirus infectious bronchitis virus using transient dominant selection. J Virol Meth 2005;123:203–11.
[24] Wakenell PS, Sharma JM. Chicken embryonal vaccination with avian infectious bronchitis virus. Am J Vet Res 1986;47:933–8.
[25] Sperry SM, Kazi L, Graham RL, Baric RS, Weiss SR, Denison MR. Single-amino-acid substitutions in open reading frame (ORF) 1b-nsp14 and ORF 2a proteins of the coronavirus mouse hepatitis virus are attenuating in mice. J Virol 2005;79:3391–400.
[26] Tarpey I, Orbell SJ, Britton P, Casais R, Hodgson T, Lin F, et al. Safety and efficacy of an infectious bronchitis virus used for chicken embryo vaccination. Vaccine 2006;24:6830–8.