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# Recombinant live attenuated avian coronavirus vaccines with deletions in the accessory genes 3ab and/or 5ab protect against infectious bronchitis in chickens

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ABSTRACT

Avian coronavirus infectious bronchitis virus (IBV) is a respiratory pathogen of chickens, causing severe economic losses in poultry industry worldwide. Live attenuated viruses are widely used in both the broiler and layer industry because of their efficacy and ability to be mass applied. Recently, we established a novel reverse genetics system based on targeted RNA recombination to manipulate the genome of IBV strain H52. Here we explore the possibilities to attenuate IBV in a rational way in order to generate safe and effective vaccines against virulent IBV (van Beurden et al., 2017). To this end, we deleted the nonessential group-specific accessory genes 3 and/or 5 in the IBV genome by targeted RNA recombination and selected the recombinant viruses in embryonated eggs. The resulting recombinant (r) rIBV-Δ3ab, rIBV-Δ5ab, and rIBV-Δ3ab5ab could be rescued and grew to the same virus titer as recombinant and wild type IBV strain H52. Thus, genes 3ab and 5ab are not essential for replication *in ovo*. When administered to one-day-old chickens, rIBV-Δ3ab, rIBV-Δ5ab, and rIBV-Δ3ab5ab showed reduced ciliostasis as compared to rIBV H52 and wild type H52, indicating that the accessory genes contribute to the pathogenicity of IBV. After homologous challenge with the virulent IBV strain M41, all vaccinated chickens were protected against disease based on reduced loss of ciliary movement in the trachea compared to the non-vaccinated but challenged controls. Taken together, deletion of accessory genes 3ab and/or 5ab in IBV resulted in mutant viruses with an attenuated phenotype and the ability to induce protection in chickens. Hence, targeted RNA recombination based on virulent IBV provides opportunities for the development of a next generation of rationally designed live attenuated IBV vaccines.


## 1. Introduction

Infectious bronchitis virus (IBV) is an avian gammacoronavirus that belongs to the family *Coronaviridae* of the order *Nidovirales* [1,2]. It was first discovered in the United States in the 1930s [3] as the causative agent of a highly contagious respiratory disease in chickens, known as infectious bronchitis. Although IBV principally infects the upper respiratory tract, some IBV strains affect the renal tubuli, oviduct and parts of the gastrointestinal tract. Infection with IBV may lead to reduced growth and egg production, and is as such regarded as one of the economically most relevant viral pathogens in the poultry industry worldwide.

Current strategies to prevent IBV in poultry include vaccination with live attenuated IBV vaccines as well as with inactivated vaccines. The widely used live attenuated IBV vaccine, H120, was developed in the 1960s in The Netherlands by serial passaging of a Massachusetts-like IBV strain in embryonated eggs [4]. After 120 passages, the resulting virus H120 had become strongly attenuated as a result of its embryo adaptation and did not cause significant disease in young chicks, while it induced an immune response protective against challenge with wild type Massachusetts IBV [4]. The H120 vaccine has been used successfully for decades. With the worldwide occurrence of IBV in both commercial and backyard chicken in a wide variety of geno-, sero- and protectotypes [5], protection against IBV by vaccination has

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become far more complicated nowadays. Yet, the development of new live attenuated vaccines is still done by serial passaging of virus field isolates in embryonated eggs, which is a laborious and time-consuming process with unpredictable outcome with regards to vaccine safety and attenuation.

In order to investigate functions of IBV proteins in a directed way, several research groups independently developed systems to manipulate the IBV genome [6–10]. These reverse genetics systems (RGS) are based either on the cell-culture adapted and highly attenuated IBV strain Beaudette or on the attenuated IBV vaccine strain H120. Since IBV Beaudette and H120 are highly attenuated [4,11], the ability to study virulence factors of IBV *in vivo* is limited. This would require the introduction, or substitution, of factors contributing to the infection *in vivo*, including (parts of) the replicase genes and for example spikes from other IBV serotypes [12–14]. We have recently established a reverse genetics system based on the more virulent IBV strain H52 [4,15]. Using targeted RNA recombination and performing the rescue and selection of candidate recombinants in embryonated chicken eggs, we solved the bottleneck of the inability to propagate virulent IBV strains to grow in cell culture. This provided a novel way to generate recombinant IBV for *in vivo* studies, including vaccine development.

The IBV genome encodes the nonstructural proteins involved in replication of the viral genome (ORF1ab) and the structural proteins spike S, membrane M, envelope E, and nucleocapsid N. In addition, genes 3 and 5, located between S and M and M and N, respectively, code for the proteins 3a, 3b, and 3c (E), and 5a and 5b [2]. These gammacoronavirus-specific proteins 3ab and 5ab are non-structural and non-essential for virus replication [9,16,17]. Several lines of evidence indicate, however, that they might have a role in viral pathogenesis in chickens. In particular, these genes are conserved across all IBV field strains [18] and recent studies indicate that the 3a and 3b proteins induced a delayed activation of the type I interferon (IFN) response *in vitro*, with protein 3a additionally being involved in resistance of IBV to the cellular antiviral state induced by IFN [19,20]. Accessory protein 5b was found to contribute to host cell shut-off, including amongst others the inhibition of translation of type I IFN [21]. Based on these observations, and on the reported role of the accessory genes of other coronaviruses in pathogenicity [22–25], we reasoned that the deletion of the accessory genes 3 and 5 might attenuate the more virulent phenotype of IBV H52 *in vivo*. Here our RGS [15] was used to generate mutant recombinant IBVs for vaccine development based on IBV H52 BI. We show that recombinant viruses lacking the 3ab and/or the 5ab gene cluster are viable and replicate like rIBV-H52, while showing reduced pathogenicity in chickens. Their ability to protect vaccinated chickens against a homologous challenge demonstrates the feasibility of this approach in creating next generation vaccines against infectious bronchitis.

## 2. Materials and methods

### 2.1. Cells, eggs & viruses

Murine LR7 cells [26] were cultured in Dulbecco's Modified Eagle Medium (DMEM; BioWhittaker), supplemented with 4 mM l-glutamine (Lonza), 10% Fetal Calf Serum (FCS; BioWhittaker) and 0.05 mg/ml gentamicin (Gibco Invitrogen), at 37.0 °C and 5% $CO_2$.

Fertilized specific pathogen free (SPF) white leghorn eggs (Animal Health Service, Deventer, The Netherlands) were incubated at 37.5 °C and 45–65% relative humidity. Embryonated chicken eggs (ECE) were inoculated into the allantoic cavity at day ten of incubation, unless stated otherwise, and candled twice daily. Eggs were transferred to 4 °C for 16–24 h prior to collection of the allantoic fluid (AF) and the chorio-allantoic membrane (CAM). Virus titration *in ovo* was based on 50% embryonic infectious dose ($EID_{50}$) per ml, as determined at day 7 post inoculation (pi) according to Reed and Muench [27]. For preparing virus stocks, AF of four to ten ECEs inoculated with 100 $EID_{50}$ were pooled after incubation for 24 h.

IBV strain H52 BI (Boehringer Ingelheim, BI, Ingelheim, Germany), recombinant IBV wild-type derived from H52 (rIBV-wt) and murinized (m)IBV (strain #1B3-IIA) were propagated as described before [15].

### 2.2. Immunohistochemistry (IHC)

Immunohistochemistry on CAMs collected from ECEs was performed as described previously [15] using monoclonal antibody (MAb) Ch/IBV 26.1 directed against the IBV S2 protein (Prionics, Lelystad, The Netherlands) [28,29].

### 2.3. RNA isolation, reverse transcription & PCR

RNA was isolated using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to manufacturer's protocol, with random hexamers. PCR was performed with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific) for sequencing and cloning purposes. One-step RT-qPCR was used to semi-quantitatively assess virus load in AF, using primers IBV.RdRp.F41 and IBV.RdRp.R41 as previously described [15].

### 2.4. Construction of p-IBV-Δ3ab, p-IBV-Δ5ab, and p-IBV-Δ3ab5ab

The design of donor plasmid p-IBV has been described previously [15] (Fig. 1A). The constructs p-IBV-Δ3ab, p-IBV-Δ5ab, and p-IBV-Δ3ab5ab, in which the accessory genes 3ab, 5ab, and 3ab5ab are deleted, are shown in Fig. 1B and C. Design of the delta3ab fragment (Δ3ab) was such that the 1 nt overlap between the stop codon of the spike gene and the start codon of the 3a gene was replaced by an overlap between the stop codon of the spike gene and the start codon of the E gene (Fig. 1C-5). For the delta5ab fragment (Δ5ab) the start codon of the 5a gene is now used as start codon of the nucleocapsid gene (Fig. 1C-6). DNA fragments spanning the semi-unique restriction enzyme sites (RES) surrounding the accessory genes with the deletions designed as described (Δ3ab and Δ5ab) were cloned into pUC57-simple by Genscript (Piscataway, NJ, USA). Delta3ab was ligated into p-IBV-5-1b-S-SIR [15] after *Nhe*I-*Pml*I double digestion to remove the 3ab gene, followed by the 3T region to form p-IBV-5-1b-S-SIRΔ3ab-3T, now called p-IBV-Δ3ab. Likewise, Δ5ab was ligated into p-IBV-3T [15] after *Afe*I-*Nhe*I double digestion to remove the 5ab gene. Fragment 3TΔ5ab was subsequently ligated after p-IBV-5-1b-S-SIR or p-IBV-5-1b-S-SIRΔ3ab to form p-IBV-5-1b-S-SIR-3TΔ5ab or p-IBV-5-1b-S-SIRΔ3ab-3TΔ5ab, respectively, now called p-IBV-Δ5ab and p-IBV-Δ3ab5ab. Composition of each of the plasmids was confirmed by PCR, restriction enzyme digestion and sequencing of the inserts (Macrogen, Amsterdam, The Netherlands) (see Table 1).

### 2.5. Targeted RNA recombination and rescue of recombinant IBVs

rIBVs were generated by introducing the IBV spike ectodomain into the mIBV genome by targeted RNA recombination between p-IBV donor RNAs and recipient virus mIBV, as described previously [15]. In short, capped run-off donor transcripts were synthesized from p-IBV-Δ3ab, p-IBV-Δ5ab and p-IBV-Δ3ab5ab using the