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Viral Vectors in Veterinary Vaccine Development

A Textbook

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ISBN 978-3-030-51926-1 ISBN 978-3-030-51927-8 (eBook)
https://doi.org/10.1007/978-3-030-51927-8

Coronaviruses as Vaccine Vectors for Veterinary Pathogens

Ding Xiang Liu, Yan Ling Ng, and To Sing Fung

Abstract

Coronaviruses (CoVs, family *Coronaviridae*) are enveloped, plus-stranded RNA viruses that can cause highly contagious upper respiratory diseases in humans and animals with potentially fatal outcomes. Typical symptoms found in chickens infected with infectious bronchitis coronavirus (IBV) include coughing, sneezing, gasping, nasal discharge and tracheal rales. Animal CoVs also cause local epidemics and pandemics with high infection rates, significantly increasing the economic burden on the poultry and livestock industry. With the realization that animal CoVs can be transmitted to humans, these viruses are now considered a global health threat. Improvement in technologies, such as reverse genetics, has conferred the ability to manipulate coronaviral genomes in the development of antiviral intervention and as vaccine vectors against other veterinary pathogens. This chapter summarizes new information on CoV reverse genetics and advances in vaccine development.

Keywords

CoV · Vaccine vectors · Bacterial artificial chromosome · In vitro ligation · Vaccinia virus · Infectious bronchitis · Vaccination

Learning Objectives

After reading this chapter, you should be able to:

- Explain the basic molecular biology, pathogenesis and replication cycle of coronavirus
- Explain the working mechanisms of the four coronavirus reverse genetics systems and compare and contrast their advantages and limitations
- Use practical examples to demonstrate how coronavirus genomes can be manipulated to serve as vaccine vectors for veterinary pathogens

1 Introduction

Coronaviruses (CoVs) are a family of enveloped viruses with non-segmented, single-stranded and positive-sense RNA genomes. Viruses in this family infect and cause diseases in various domesticated and laboratory vertebrates, including cats, dogs, pigs, chickens and mice. In addition, several CoVs are able to infect humans and cause respiratory diseases with mild to severe outcomes. Three recently emergent zoonotic

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T. Vanniasinkam et al. (eds.), *Viral Vectors in Veterinary Vaccine Development*,
https://doi.org/10.1007/978-3-030-51927-8_10

CoVs, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and 2019 novel coronavirus (SARS-CoV-2), have crossed the species barrier and infected humans directly or via intermediate hosts, causing lethal diseases in a pandemic scale. In terms of animal CoVs, the high mortality rates in infected young animals and the reduction in product quality and yield have imposed heavy economic burdens on the global livestock industry.

Ever since the identification of the first CoV more than 80 years ago, continuous effort has been made to isolate and cultivate CoVs in tissue and cell cultures with some success, but many CoV field isolates still remain unculturable. Meanwhile, research on the molecular virology of CoV has revealed its highly characteristic replication mechanism, featuring by the synthesis of a nested set of subgenomic mRNA species, shared by other viruses belonging to the order *Nidovirales* (*nidus*, nest). While the replication cycle of CoV is relatively simple occurring exclusively in the cytoplasm, the enormous genome size (27–32 kb) has made the initial attempts to establish reverse genetics systems a daunting task. Nonetheless, several approaches have been developed, and the ability to recover recombinant CoVs harbouring precise deletions and mutations proves to be invaluable in the basic and applied research of CoV.

The large genome size and unique transcription strategy of CoVs make them promising candidates for the development of viral vectors expressing heterologous genes. Studies with CoV replicons, the autonomous replicating RNA molecules, have led to the identification of viral genes and the *cis*-acting elements indispensable for efficient CoV replication. Meanwhile, experiments using reporter genes, such as fluorescent proteins and luciferases, have demonstrated the impacts of genomic localization, sequence composition and transgene size on the expression levels of the heterologous genes and the recovery rate and stability of the recombinant viruses. Several pioneering studies have also explored the feasibility of using CoV-based vectors to construct vaccines and evaluated their efficacies against virulent strains of CoVs or other viruses. In this chapter, we first review the basic knowledge of CoV biology, followed by a detailed discussion on the four CoV reverse genetic systems. We then summarize current studies on the construction of CoV replicons and CoV vectors and discuss their potential application to serve as vaccine vectors.

2 Background

2.1 Clinical Importance

Records of diseases attributable to CoVs date back to 1931 in North Dakota, United States, in what was described as “an apparently new respiratory disease of baby chicks” known as infectious bronchitis (IB) [1]. The viral nature of IB was established 2 years later, when Bushnell and Brandly reported a similar disease in chickens [2]. Bacterial and toxin origins were ruled out as the causative agent was filterable. Since then, different CoVs have been discovered and the symptoms they cause extend beyond the respiratory system. In all cases, the viral particles were found to be between 80–150 nm, pleomorphic, membrane-coated and decorated with widely spaced club-shaped surface projections [3–7]. This group of viruses, known as CoVs (*corona* denoting the reminiscent of a solar corona), was officially recognized as a new genus in 1971 [8].

CoV research hit its prime in 2002, when the emergence of the SARS-CoV outbreak resulted in 8096 reported cases worldwide and an approximate 10% mortality rate [9]. The subsequent occurrences of MERS-CoV epidemic in 2012 and SARS-CoV-2 pandemic in 2019 have once again demonstrated that it is highly probable for CoVs circulating amongst bat species and other animals to be introduced into the human population. This prompts the need for more research to elucidate their replication mechanisms to identify potential drug targets and develop vaccines, which can be effective countermeasures against CoVs.

2.2 Taxonomy

CoVs are taxonomically classified under the order of the *Nidovirales*, a large group of enveloped, single-stranded, positive-sense RNA genomes which produce a 3′ co-terminal nested set of subgenomic mRNAs during infection [10]. CoVs were traditionally classified into four groups based on phylogenetic clustering. However, extensive changes were made to the order in the 2018 International Committee on Taxonomy of Viruses (ICTV) taxonomy, in which the nidoviruses are reorganized into seven suborders (Fig. 1). The *Coronaviridae* family is now under the suborder of *Cornidovirineae* and further divided into two subfamilies, *Letovirinae* and *Orthocoronavirinae*. The introduction of a new subgenus rank also redistributes the *alpha-*, *beta-*, *delta-* and *gammaCoVs* across 12, 5, 4 and 2 subgenera, respectively. Almost all *alpha-* and *beta*CoVs have mammalian hosts, whereas *gamma-* and *delta*CoVs have been isolated mainly from avian hosts.

2.3 Morphology

On negative contrast electron microscopy (EM), CoV virions appear roughly spherical and pleomorphic (Fig. 2), with an average diameter of 80–150 nm [11, 12]. The virion surface is covered with a fringe of 20 nm crown-like surface projections consisting of trimers of spike (S) glycoprotein. In *betaCoVs*, there exists a second type of protein projections on the surface, the homodimeric hemagglutinin-esterase (HE) glycoprotein, which measures about 5–7 nm in length [13, 14].

2.4 Genome Structure and Organization

CoVs possess the largest and most complex RNA genome amongst established RNA viruses, with genome sizes typically ranging between 26 and 32 kb. These viruses maintain a well-conserved genome organization, with the essential genes occurring in the order 5′-replicase-S-E-M-N-3′. The genomic RNAs (gRNAs) contain a methylated cap structure at the 5′ termini of its viral genomic and subgenomic RNAs (sgRNAs) [15] and a 3′ terminal polyadenylated tail [16]. The replicase-transcriptase constitutes about two-thirds of the virus genome and is the only gene directly translated from the genomic RNA upon entry into the host cell. Products of their downstream open reading frames (ORFs) are derived from subgenomic mRNAs.

2.5 CoV Replication Cycle

2.5.1 Attachment and Entry

Viral infection of host cells begins with receptor recognition by CoVs, which is initiated by the binding interaction between the S protein and its cognate receptors (Fig. 3). Host range and tissue tropism are mainly determined by the interaction between the S protein and its receptor. The cellular receptor for *gammaCoVs* is currently unknown, although sialic acids are thought to serve as non-specific attachment factors [17–19].

Following receptor binding, membrane fusion between the viral and host membrane is mediated by conformational changes of the S protein, largely accomplished by the S2 subunit of the S protein. Cleavage of the S protein occurs at two sites within the S protein, firstly at the (S1/S2) site for separating the receptor-binding domain (RBD) and fusion domain of the S protein and secondly at the (S2′) site for exposing the fusion peptide [20]. The insertion of the fusion peptide into the host membrane upon S2′ cleavage then triggers the joining of two heptad repeats (HRs) in S2, forming an antiparallel six-helix bundle [21]. The formation of this bundle permits membrane mixing to eventually deliver the viral genome into the cytoplasm.

2.5.2 Replicase Gene Translation and Processing

The next step of the CoV life cycle involves the translation of the replicase gene from the incoming virion genomic RNA. The replicase gene

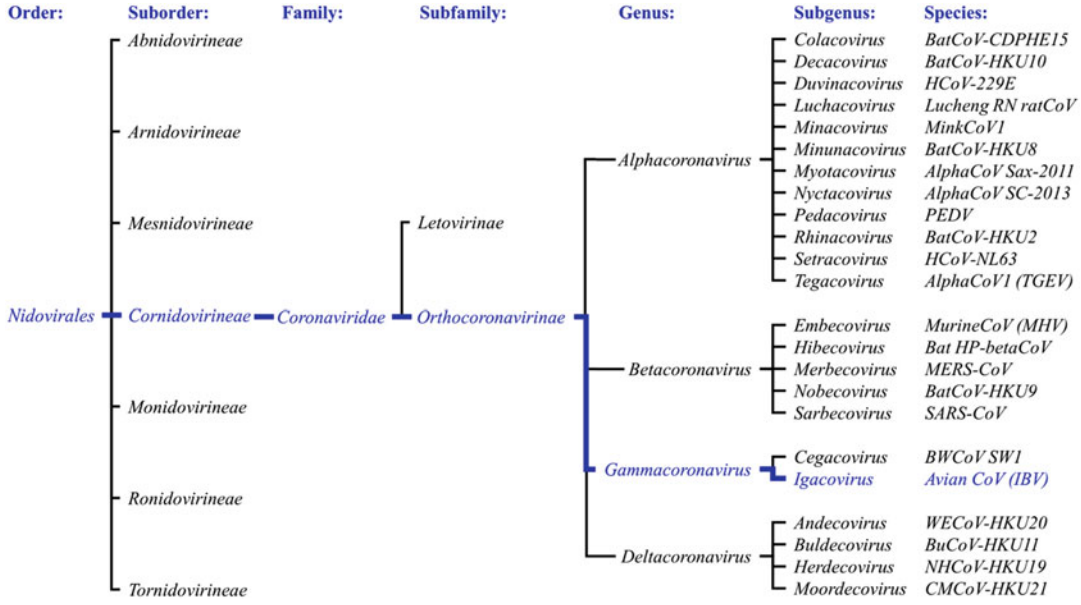

Fig. 1 Current taxonomy of the order *Nidovirales* according to the International Committee on Taxonomy of Viruses (ICTV). Coronaviruses are classified under the subfamily of *Coronavirinae* in the family *Coronaviridae*

The following virus name abbreviations were used: *BtCoV* bat coronavirus, *PEDV* porcine epidemic diarrhoea virus, *HCoV* human coronavirus, *TGEV* transmissible gastroenteritis virus, *PRCV* porcine respiratory coronavirus, *FCoV* feline coronavirus, *MiCoV* mink coronavirus, *CiCoV* civet severe acute respiratory syndrome CoV, *RatCoV* rat coronavirus, *MHV* murine hepatitis virus, *PHEV* porcine hemagglutinating encephalomyelitis virus, *BCoV* bovine coronavirus, *CaCoV* canine respiratory coronavirus, *ECoV* equine coronavirus, *DrCoV* dromedary camel coronavirus, *WtDCoV* white-tailed deer coronavirus, *GCoV* giraffe coronavirus, *AnCoV* sable antelope coronavirus, *WBkCoV* waterbuck coronavirus, *SdCoV* sambar deer coronavirus, *RabCoV* rabbit coronavirus, *HeCoV* hedgehog coronavirus, *IBV* infectious bronchitis virus, *CMCoV* common moorhen coronavirus, *WECoV* wigeon coronavirus, *BuCoV* bulbul coronavirus, *ThCoV* thrush coronavirus, *MuCoV* munia coronavirus, *PCoV* porcine coronavirus, *WiCoV* white-eye coronavirus, *NHCoV* night heron coronavirus

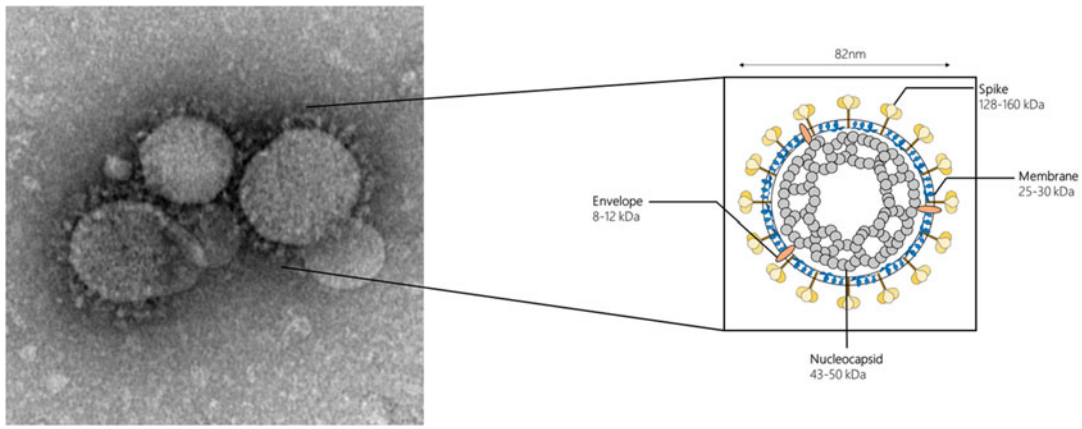

Fig. 2 Coronavirus morphology. An electron micrograph image of Middle East respiratory syndrome coronaviruses (MERS-CoV). The structural proteins of coronaviruses that comprise the spike, membrane, nucleocapsid and envelop protein. The molecular weight of each structural protein monomer is as shown. The size of an infectious bronchitis virus virion is 82 nm. (Image source: Cynthia Goldsmith/Maureen Metcalfe/Azaibi Tamin, from https://www.cdc.gov/coronavirus/mers/photos.html)

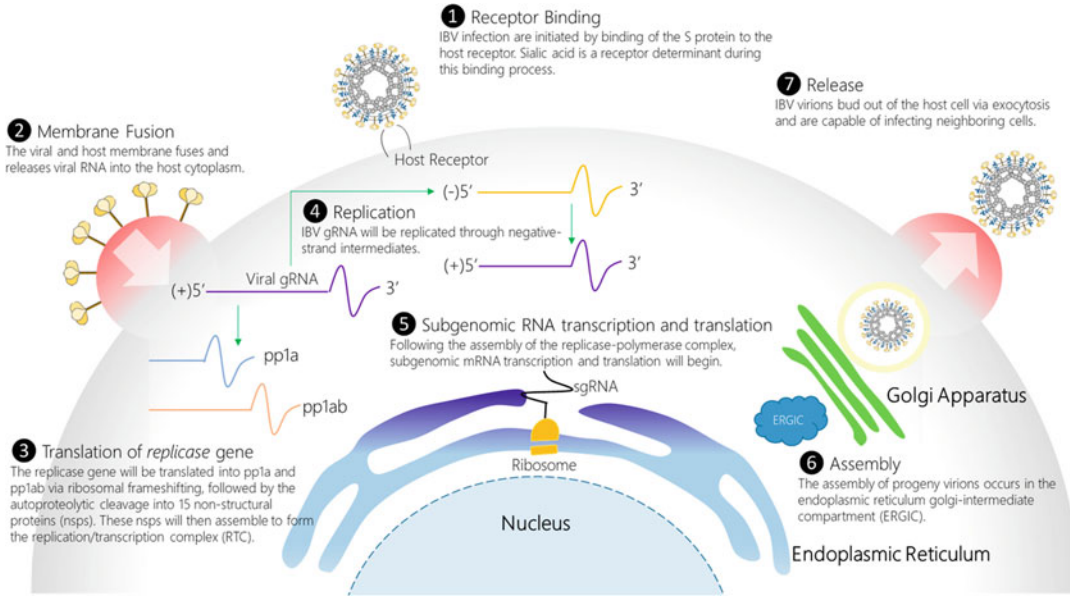

Fig. 3 Infectious bronchitis virus (IBV) replicase gene and processing scheme of replicase protein products. (**a**) Ribosomal frameshifting elements of IBV replicase gene. Pseudoknot stems are indicated as S1 and S2, and loops are indicated as L1 and L3. (**b**) Translation of the replicase genes ORF1a and ORF1b begins following the release of the viral genome into the host cytoplasm via ribosomal frameshifting, into polyprotein (pp) 1a and 1ab. Pp1a and pp1ab will then be autoproteolytically cleaved at cleavage sites by papain-like protease (PLpro) (in red triangles) and main protease (Mpro) (in orange triangles) into 15 non-structural proteins

encodes two large ORFs, ORF1a and ORF1ab, which share a small overlap and encode two large polyproteins, pp1a and pp1ab, respectively. Polyprotein1ab is translated by a ribosomal frameshifting event (Fig. 4a). As demonstrated by in vitro studies, the incidence of ribosomal frameshifting is as high as 25%. This alternate translation mechanism can help either to regulate the ratio of pp1a and pp1ab proteins or to postpone the translation of products from the 1b coding region until sufficient products of ORF1a are synthesized, thereby creating a suitable environment for RNA replication [22].

Following the production of pp1a and pp1ab, the polyproteins are autoproteolytically processed by its encoded viral proteases to form mature protein products, termed non-structural proteins (nsps) 1–16, except for *gammaCoVs,* which do not encode nsp1 [23] (Fig. 4b). These processed nsps will assemble to form the replication-transcription complex (RTC), creating an environment suitable for RNA synthesis, RNA replication and transcription of subgenomic RNAs.

2.5.3 Replication and Transcription

The expression and assembly of the RTC set the stage for viral RNA synthesis, a process which results in the replication of both gRNA and the transcription of multiple sgRNAs. Each sgRNA contains a leader RNA of 70–100 nucleotides (nt) identical to the 5′ end of the genome joined to the body RNA identical to the 3′ portions of the genome. The fusion of the leader and body RNA happens at short motifs on the genome, known as transcriptional regulatory site (TRS).

There is now general consensus that this fusion takes place through discontinuous extension of the negative-stranded RNA [24]. As a result, the negative-stranded sgRNAs in a partial duplex with the positive-stranded gRNA are now used as templates for the synthesis of the corresponding multiple positive-stranded RNAs. In addition to the formation of gRNA and sgRNAs, CoVs can undergo homologous and non-homologous RNA recombination [25, 26]. The ability of these CoVs to recombine may play a prominent role in viral evolution and

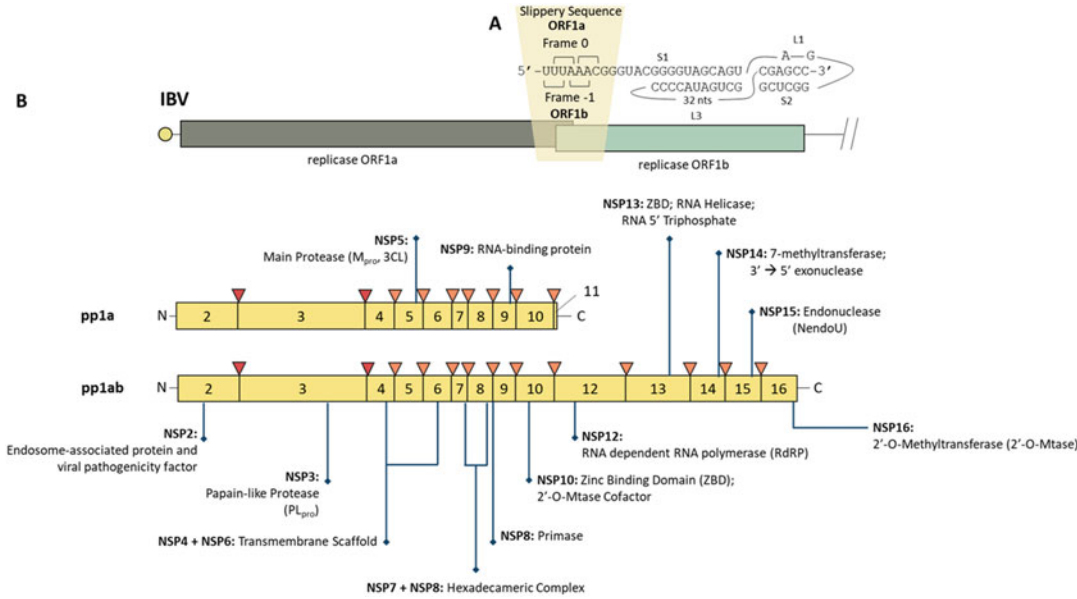

Fig. 4 Coronavirus replication cycle

in preventing the accumulation of deleterious mutations.

2.5.4 Assembly and Egress

With the replication and transcription of the gRNA and sgRNAs, the next step of the replication cycle is to enable the translation of the structural and accessory proteins, which help to direct the assembly of progeny viruses. The S, membrane (M) and envelope (E) proteins are translated and inserted into the endoplasmic reticulum (ER), where they transit along the secretory pathway into the site of virion assembly – the ER-Golgi intermediate compartment (ERGIC) [27, 28]. Here, the viral genomes encapsulated by the nucleocapsid (N) protein coalesce, in addition with the envelope components, will bud into the ERGIC and translocated to the Golgi to form mature virions [29].

Following assembly and budding, the virions are exported from the infected cells in vesicles and are released by exocytosis. A portion of the S protein, which is not assembled into virions, will translocate to the plasma membrane, where it mediates cell-cell fusion between neighbouring uninfected cells. This leads to the formation of large, multinucleated cells, which promote virus spread within an organism without being detected by virus-specific antibodies.

2.6 CoV Genetics and Reverse Genetics

In the past, CoV genetics are broadly restricted to the analysis of three types of mutants. The first were naturally arising deletion mutants, which offered clues to some of the phenotypic changes found in various pathogenic strains. The second were defective RNA templates, which depend on replicase proteins provided *in trans* by a helper virus [30–33]. The last were temperature-sensitive (*ts*) mutants isolated from MHV and IBV using chemical mutagenesis and by adaptation to different culture temperatures [34–37]. Reversible and easy to use, *ts* mutants quickly became powerful tools for studying gene function after natural mutants. However, a great deal of efforts must be expended to produce a comprehensive collection of mutants representing the possible complementation groups of cistrons encoded in the CoV genome, consequently limiting their use in CoV genetics.

Reverse genetics for CoVs were developed as early as the 1990s, when the large size of the CoV genome and expression of specific CoV cDNA sequences in bacterial cloning systems became major impediments in the generation of recombinant CoVs using classical genetic techniques [38]. Here, we review four approaches developed for building CoV infectious cDNAs using IBV, FIPV and TGEV as models and how each of these systems has been extended to the generation of CoV replicon RNAs and CoV-based vaccine vectors.

3 Construction of the CoV Vectors

3.1 Reverse Genetics Systems for CoV

3.1.1 Homologous RNA Recombination

Targeted RNA recombination was the first reverse genetics method developed when it was not clear if the construction of full-length infectious cDNA clones would ever be feasible [39]. This system was originally designed for MHV, which harnesses on the intrinsic property of the CoV replication machinery to recombine RNA molecules. It involves generation of a chimeric RNA donor bearing the desired mutations being transfected into cells, which have been infected with a parental virus presenting certain characteristics (i.e. *ts* or host range-based selection) that can be selected against [40–42]. Recombinant CoVs generated by targeted RNA recombination can then be isolated by counter-selection of the parental virus and purified.

Despite the existence of RNA recombination since the 1990s, attempts at recombining IBV RNA molecules have proved to be unsuccessful until recently, when van Beurden and colleagues established a targeted RNA recombination reverse genetics system on IBV H52 (Fig. 5) [43]. Using this approach, the IBV donor plasmid, pIBV, was constructed as stepwise ligation of fragments derived from five plasmids. Each of these five plasmids contains gene fragments of various IBV structural and accessory genes. To construct a recombinant chimeric murinized (m) IBV intermediate (mIBV) donor plasmid, the ectodomain of MHV A59 spike gene was amplified by PCR and ligated into a plasmid to produce p-MHV-S [44–48]. Targeted RNA recombination of IBV proceeds in two steps. Construction of the recombinant chimeric mIBV was the first step. In the next step, the recombinant IBV (rIBV) was generated by exchanging the IBV S ectodomain back into the mIBV genome.

With this method, manipulating the 3′ one-third of the CoV genome, which consists of the structural and accessory genes as well as the 3′ UTR, has been successful in FIPV [49], TGEV [50] and most recently, in IBV [43]. However, despite its value, there are clear drawbacks in this system. For technical reasons, this method cannot be expanded to the 5′ two-thirds of the genome, where the replicase is located due to the requirement of these gene products for virus passage. As such, to get around the barriers presented by the huge size of the replicase gene and the instability of key regions propagated in bacterial clones, three other methods were developed to overcome these challenges, namely, construction of the full-length cDNA clones in bacterial artificial chromosomes (BACs), in vitro ligation and propagation of full-length cDNAs in vaccinia virus-based vectors.

3.1.2 Bacterial Artificial Chromosomes (BACs)

The BAC system is based on *Escherichia coli* and its single-copy F vector, which follows a strictly controlled replication to produce only one or two copies per cell. Amongst bacterial cloning systems, BAC has been chosen for reverse genetics as it has been shown to be capable of maintaining large DNA fragments from various genomic sources with high structural stability even after generations of serial growth [51]. The first full-length cDNA-based reverse genetics system for CoVs using the BAC approach was developed for TGEV in 2000 (Fig. 6) [52, 53]. In this system, the full-length cDNA copy of the TGEV genome was assembled in a synthetic low-copy-number BAC pBeloBAC11, downstream of a

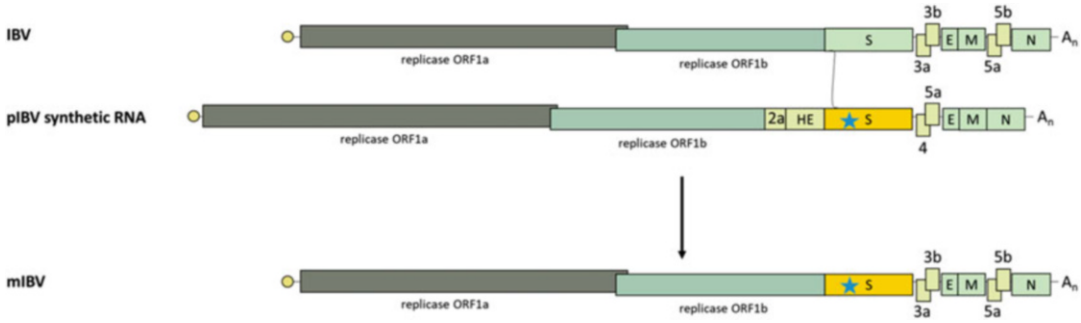

Fig. 5 Targeted RNA recombination. An interspecies chimeric murinized IBV containing an MHV S domain (mIBV) is generated via a single recombination event between the IBV genomic RNA with the synthetic donor plasmid (p-IBV synthetic RNA) bearing a mutation (star) in the spike (S)

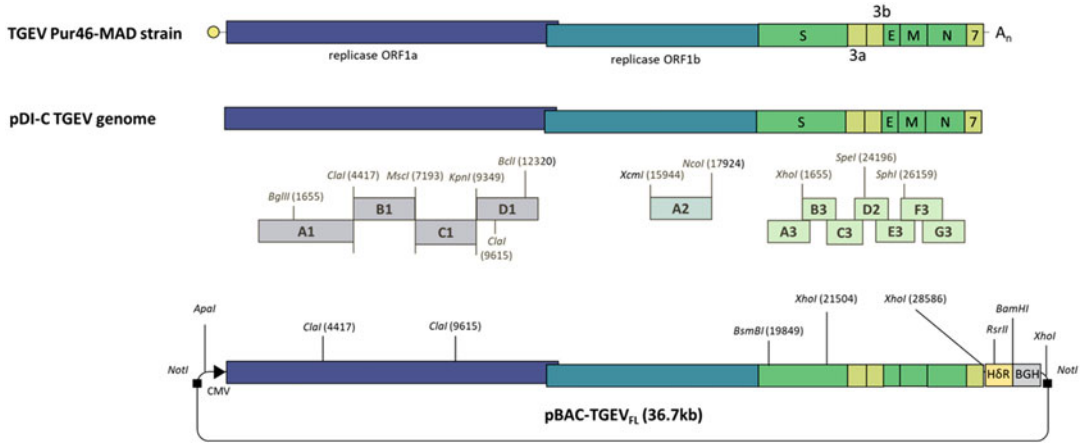

Fig. 6 Assembly of full-length TGEV cDNA clone using bacterial artificial chromosome (BAC) method. To construct the complete cDNA of the TGEV genome, a plasmid containing a cDNA encoding TGEV-derived DI (pDI-C) was used. As pDI-C RNA contains three deletions across the genome, a set of cDNAs encoding the fragments of the missing regions were generated by RT-PCR (highlighted in yellow boxes). The final TGEV cDNA was flanked by a CMV promoter at the 5′ end and a 24 bp poly(A) tail at the 3′ end, followed by the hepatitis delta virus ribozyme (HδR) and bovine GH termination (BGH) and polyadenylation sequences (Adapted from [52])

cytomegalovirus (CMV) promoter [54]. Downstream of the 3′ end of the genomic RNA are also a poly(A) tail, the hepatitis delta virus (HDV) ribozyme and a bovine growth hormone (BGH) termination and polyadenylation sequence to ensure that the synthetic RNAs produced contain the genuine 3′ end sequence of the genome. The infection is initiated from the transcription of the transfected cDNA by using the host RNA polymerase II [55]. This method is advantageous in avoiding the potential limitations of in vitro capping and transcription of genomic RNA. More recently, modified BAC approaches have been used to generate the full-length cDNA clone of SARS-CoV Frankfurt-1 under the control of a T7 RNA polymerase promoter instead of the CMV promoter, to prevent mRNA splicing after transcription of the full-length cDNA by host RNA polymerase II in the nucleus [56].

BAC clones can also be modified into E. coli cells by homologous recombination, using the red recombination system and homing endonuclease I-SceI, for counter-selection [57]. This method employs a sequence bearing the desired

modifications and allows an accurate and efficient introduction of modifications to a BAC clone in a "scarless" fashion.

The BAC system poses several advantages. First, manipulation of the BAC clones follows standard cloning protocols, such as selecting suitable restriction sites and ligating cDNA fragments into the vector, making it relatively easy and essentially similar to molecular manipulation of a conventional plasmid [51]. Likewise, the high stability of the exogenous DNA sequences provided by the BACs permitted infinite cDNA production in the cell. Together with the high transfection efficiency of BACs in mammalian cells, this system quickly becomes an attractive tool for CoV reverse genetics [58] and has been successfully employed to study the role of various viral proteins in replication and pathogenesis as well as for the production of genetically attenuated viruses that are potential vaccine candidates for SARS-CoV [59–61] and MERS-CoV [62].

3.1.3 In Vitro Ligation of Full-Length Genomic cDNA

The third system constructs a full-length genomic cDNA through the systematic assembly of smaller cloned cDNA fragments via in vitro ligation [44]. Using this approach, each smaller clone was ligated in a directed manner using asymmetric restriction sites and then transfected to permissive host cells. The full-length cDNA was in vitro transcribed to generate a capped, full-length RNA transcript, from which infectious viruses can be rescued after co-transfection with a capped N gene RNA transcript. This method has been successfully used to recover clones of many CoV, including TGEV [44], MERS-CoV [63], MHV [46], SARS-CoV [47] and IBV [64–66].

This in vitro cDNA assembly approach is proven to be simple and straight forward, as it allows rapid mutagenesis of independent cDNA fragments in parallel using conventional techniques and is compatible with other reverse genetics methods, such as BAC and vaccinia vectors. However, the initial application of this approach met two potential caveats: one was the generation of premature T7 transcription termination signals during in vitro transcription of the full-length viral RNA, and another was the lack of restriction enzymes that provide a unique overhang that does not randomly self-assemble. To overcome the first problem, several mutations were inserted into the genome to avoid potential T7 transcription termination signals. To overcome the second caveat, a variation of the approach was initially developed to engineer the MHV infectious cDNA by utilizing type IIs restriction enzymes, such as *BsaI* and *SapI* at the ends of each cDNA fragments [46]. These enzymes recognize asymmetrical sites and leave behind 1–4 nt overhangs that could be ligated in vitro using standard protocols without changing the viral sequence. Through cleavage and ligation of these fragments, the added restriction sites are removed, leaving the exact viral sequence at the junction, allowing one to generate cDNA clones without introducing mutations to the viral genome sequence. Additionally, it is possible to introduce mutations to the CoV genome at any position by designing primers, which incorporate a type IIs restriction site and mutation of interest to the viral genome [46, 67].

By using this strategy, the construction of a full-length cDNA clone derived from a Vero cell-adapted IBV Beaudette strain was obtained by in vitro assembly of five cDNA fragments spanning the entire IBV genome (Fig. 7) [64]. Each of these fragments was generated by RT-PCR using specific primers that introduced *BsmBI* or *BsaI* into the 5′ and 3′ ends of the fragments. These cDNA fragments were digested and systematically and unidirectionally assembled into a full-length cDNA by in vitro ligation. Following in vitro transcription, the genome-length transcripts were electroporated into Vero cells and used to recover the infectious viruses [63, 68].

This approach has been used with success at identifying and evaluating promising zoonotic vaccine candidate strains for SARS-CoV [69] and developing a recombination safe vaccine platform by rewiring the viral TRSs [70].

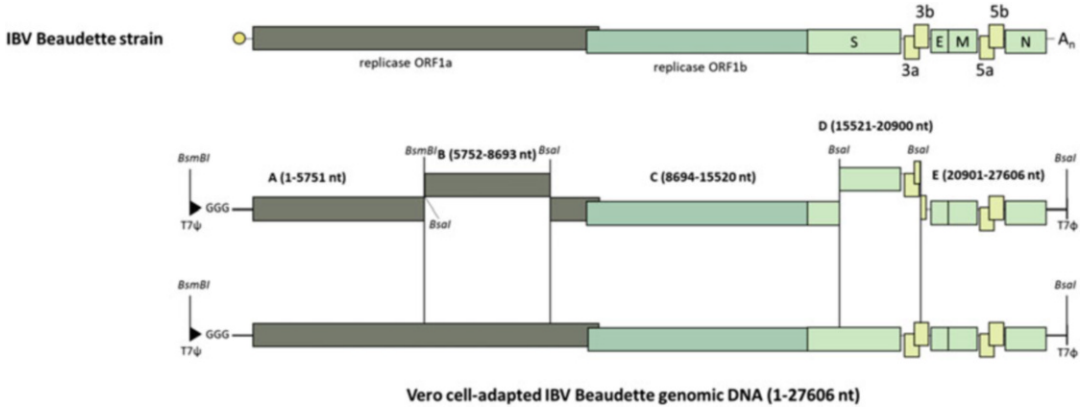

Fig. 7 Assembly of an IBV full-length cDNA clone by in vitro ligation. A full-length cDNA of Vero cell-adapted IBV Beaudette strain was assembled by in vitro ligation of five contiguous cDNA fragments (A to E) spanning the entire viral genome, which were flanked by native or engineered BsmBI and BsaI restriction sites. The assembled full-length cDNA contained a T7 RNA polymerase promoter (T7) at the 5′ end and poly(A) tail at the 3′ end, allowing for the in vitro transcription of a full-length, capped polyadenylated transcripts. The viral genes and relevant restriction sites are indicated (Adapted from [64])

3.1.4 Vaccinia Virus-Based Vectors

The last system involves the use of vaccinia virus cloning vector to generate full-length CoV genome [71, 72]. This system represents a basic approach to CoV genetics and was first reported for the generation of human CoV 229E (HCoV-229E) and, soon after, extended to FIPV [73, 74], IBV [45] and MHV-A59 [75]. Vaccinia virus vectors were attractive reverse genetics tools for several reasons. First, vaccinia virus has the capacity to accommodate at least 25 kb of foreign DNA sequence, allowing recombinant vaccinia viruses to replicate in cell culture without compromising on viral titer when compared to non-recombinant virus [76]. Second, vaccinia virus vectors have been designed for foreign DNA insertion by in vitro ligation, negating the need to generate plasmid intermediates to carry the entire cDNA insert [77]. Finally, the cloned cDNA insert is open to mutagenesis through vaccinia virus-mediated homologous recombination [78, 79].

The generation of a reverse genetics system for IBV will be described to illustrate the vaccinia virus-based system (Fig. 8) [45]. In principle, the procedure can be divided into two parts: generation of a full-length IBV cDNA and the recovery of the infectious rIBV. Firstly, three plasmids (pFRAG-1, pFRAG-2 and pFRAG-3) which contain the contiguous regions of the IBV genome were constructed for the final assembly of the full-length cDNA. The assembly of the full-length cDNA clone, which encompasses the entire IBV genome downstream of the T7 promoter, was achieved via a two-step in vitro ligation method. Secondly, the in vitro ligation products, comprising the full-length IBV cDNA with dephosphorylated Bsp102I ends, were ligated to the *NotI* arm present in the genomic DNA of the vaccinia virus vector vNotI/tk. The ligation products were then transfected into CK cells, which were previously infected with a fowlpox virus (FPV) expressing T7 polymerase as a helper to recover the recombinant IBV [80]. A second plasmid, pCi-Nuc under the control of both the CMV and T7 RNA polymerase promoter, was also co-transfected to express IBV N [81]. FPV was selected as a helper virus, because of the abortive FPV infection in the mammalian cells together with the rarity of recombination events between two poxviruses [71, 82].

Two recombination methods have also been developed based on the sequential use of *E. coli* guanine-phosphoribosyl transferase gene (gpt) as both a positive and negative selection marker

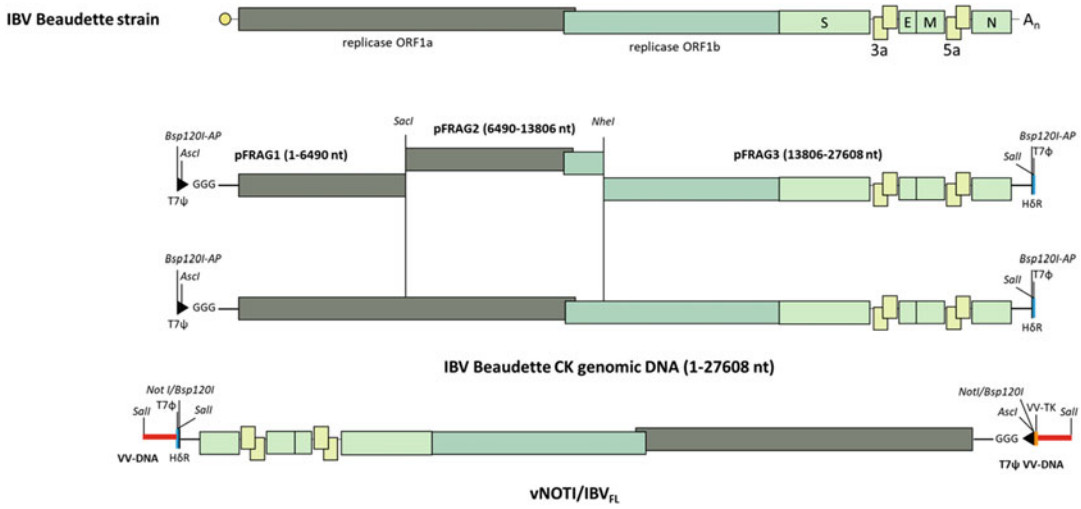

Fig. 8 Assembly of an IBV full-length cDNA clone in the vaccinia virus genome. Three contiguous cDNA fragments (pFRAG1 to pFRAG3) spanning the entire viral genome of IBV Beaudette CK strain was cloned in vaccinia virus DNA (V) by in vitro ligation. pFRAG1 contained a T7 RNA polymerase promoter (T7) at the 5′ end and pFRAG3 fragment a 28 nt poly(A) tail at the 3′ end. The assembly of the full-length IBV cDNA and its insertion into the vaccinia virus genome is shown with the final orientation of the cDNA in the vaccinia genome. (Adapted from [45]). *AP* dephosphorylated ends, *T7ψ* T7 promoter, *T7φ* T7 terminator, *HδR* hepatitis delta antigenome ribozyme

[72, 83]. In the first method, the CoV region of interest in the recombinant vaccinia virus was substituted with the gpt gene in a plasmid, and the gpt gene was flanked by CoV sequences to facilitate a double recombination event. The second method, known as transient dominant selection [79, 83], requires the modified CoV cDNA region to be inserted into a plasmid containing the gpt selective marker under the control of a vaccinia virus promoter. The complete sequence of the plasmid (including the CoV genome) was then transiently integrated into the vaccinia virus by homologous recombination with a single crossover event. The recombinant vaccinia virus containing the modified CoV cDNA region was then gpt-positively selected.

Collectively, it is possible to genetically modify CoV genomes at desirable positions using these approaches. Recombinant CoVs with gene inactivation, deletions or attenuation modifications can now be generated to study the role of specific gene products in viral replication and pathogenesis. Most importantly, attenuated viruses can be potential vaccine candidates, and modified CoV genomes have been developed as eukaryotic, multigene expression vectors [84].

3.2 Construction of CoV Replicons

In line with the development of viral vectors based on CoVs, previous studies have also explored the possibilities of constructing CoV replicons. Replicons are autonomous replicating RNAs encoding all viral proteins and *cis*-acting elements required for RNA replication and are considered as a safe alternative to full-length viral genomes as they lack structural genes to produce infectious virus particles. In order to construct CoV replicons, it is important to identify viral genes and sequence elements that are indispensable for efficient CoV replication. By analogy to other positive-stranded RNA viruses, it was assumed that the replicase gene and *cis*-acting elements at the 5′ and 3′ termini would be sufficed for efficient CoV replication and transcription. However, reverse genetics analyses revealed a role of CoV N proteins for efficient CoV replication [85, 86]. Therefore, the basic

units of a CoV replicon include (i) the replicase gene, (ii) the 5′ and 3′ genomic termini containing the *cis*-acting elements required for replication and transcription and (iii) the nucleocapsid gene.

CoV replicons have been constructed for many animal CoVs using reverse genetics approaches [85, 87] and are currently being used as a non-infectious system to analyze CoV replication and transcription [88–90]. This system is particularly useful if the corresponding virus grows poorly in tissue culture or if the pathogenicity of the virus is a matter of concern. Several replicon versions have also been reported to include a variety of reporter genes such as green fluorescent protein (GFP), firefly luciferase (FLuc) or Renilla luciferase (RLuc) alone or in combination with antibiotic resistance genes [91–93]. In the case of SARS-CoV, these replicons are particularly useful in the identification of viral and host factors involved in CoV RNA synthesis [94] and antiviral drug testing [89].

With reverse genetics, the generation of CoV replicons can be derived from replication-competent, propagation-defective viruses, as in the case for TGEV which lacked an E gene [87, 95] as well as MERS-CoV [62]. The rationale behind this approach is to generate CoV replicons through deletion of one or more structural genes and to express the missing structural gene(s) *in trans*. In a study reporting the construction of a replicon for HCoV-229E, all the structural genes (including N) and accessory genes were removed, while three reporter genes (chloramphenicol acetyltransferase (CAT), FLuc and GFP) were incorporated, each located downstream of an HCoV-229E TRS [84]. Expression of the reporter genes could be detected in BHK-21 cells transfected with the replicon, but the packaging of the replicon genome into a virus-like particle (VLP) required simultaneous transfection of the replicon RNA, a helper HCoV-229E full-length genomic RNA, and the mRNA for the N gene. However, the availability of VLPs produced in this study was low, and a substantial amount of helper virus was found in VLP stocks [84].

In order to improve the biosafety standard of such vectors, safety guards must be introduced to work in a BSL2 containment facility. Reconstitution of wild-type constructs using vectors containing rearranged structured genes [96] or rewiring of the CoV transcription circuit [70] may be used. In this line, SARS-CoV replicons contain changed TRS elements, which resulted in an incompatibility of recombinant and wild-type TRS elements [70].

3.3 Expression of Heterologous Genes by Recombinant CoV or CoV Replicons

Several approaches to incorporate heterologous genes into the genomes or replicons have been tested for various animal coronaviruses. These include (1) insertion of a transcriptional cassette containing a TRS upstream of the gene of interest, (2) replacement of the coding sequence for one or more accessory genes with that of a heterologous gene, (3) expression of heterologous genes fused with coronavirus structural gene, and (4) insertion of heterologous genes between non-structural proteins encoded by the replicase gene.

Using reporter genes, such as EGFP and FLuc, the expression efficiency of a heterologous gene and the genetic stability of various recombinant viruses were investigated using IBV as a vector. When the EGFP preceded by a TRS from IBV ORF5 was inserted after the M or N gene or when EGFP was fused to the C-terminal region of the S gene, infectious recombinant viruses expressing EGFP could be recovered [97]. However, these recombinant viruses were only genetically stable up to passage 5, after which the inserted EGFP gene was lost [97]. On the other hand, when the accessory genes 3a and 3b were replaced with FLuc gene, the recombinant IBV exhibited stable expression of luciferase activity up to passage 15 [97]. In sharp contrast, the insertion of TRS-FLuc cassette after the M or N gene resulted in highly unstable viruses. Using the same system, two viral proteins (SARS-CoV ORF6 and DENV1 core) and a host protein (eIF3f) were also expressed [97]. While the genomic location is a critical determinant for successful heterologous gene insertion in the CoV vector, the sequence

composition and size of the inserted gene also have major impacts on the expression level and stability of the recombinant virus.

It is also possible to express heterologous genes by modifying the replicase gene. This has been demonstrated for HCoV-229E-based replicon RNA, where a selectable marker, the neomycin resistance (neo) gene, has been inserted between nsp1 and nsp2 [88]. Since the C-terminus of nsp1 and the N-terminus of nsp2 are released by proteolytic processing in the wild-type context, a "2A-like" autoprocessing peptide has been used to liberate a slightly modified nsp1 C-terminus. The neo gene was cloned downstream of the 2A-like element followed by an IRES that drives the translation of nsp2-nsp16. This selection cassette, composed of the 2A-like element, the neo gene and the IRES element, has been used to generate stable replicon-containing cell lines using conventional G418 selection after transfection of the replicon RNA into eukaryotic cells. An attractive application for these non-infectious replicon cell lines is the identification and evaluation of CoV replicase inhibitors [88, 89].

The nsp2 is dispensable for the replication of MHV and SARS-CoV [98]. In one study, the coding sequence for nsp2 was deleted in the MHV genome, and a reporter gene (GFP or FLuc) was fused to nsp3 at the N-terminus, resulting in the recovery of recombinant virus MHV-Δ2-GFP/FFL3 [99]. Meanwhile, the coding sequence for GFP or FLuc was fused at the N-terminus of nsp2, and a cleavage site was introduced to allow proteolytic cleavage between nsp1 and GFP/FFL-nsp2, resulting in the recovery of recombinant virus MHV-GFP/FFL2 [96]. Compared with wild type control, replication of MHV-Δ2-GFP/FFL3 was delayed and the virus titer reduced by 1-$\log_{10}$, whereas replication of MHV-GFP/FFL2 was not significantly affected. MHV-GFP/FFL2 was genetically stable up to passage 5 [96]. Subsequent studies have also used the same approach to express nsp2 proteins fused with APEX2 ascorbate peroxidase for electron microscopy or $BirA_{R118G}$ biotin ligase for proximity labelling studies [100].

4 Application of Recombinant CoVs as Vaccine Vectors

4.1 Recombinant CoVs as Vaccine Vectors

The observation that accessory genes are dispensable for coronavirus replication allows for the replacement of these genes by heterologous genes. In principle, CoV vectors have room for the insertion of large heterologous genes and expression of multiple genes is feasible [84]. CoV reverse genetic systems are therefore used to assess the efficacy of CoV-based expression vectors for producing large amounts of heterologous proteins or as immunogenic vectors in the context of vaccine development and immunotherapy. The extraordinary large genome and unique transcription strategy make CoVs promising candidates for the development of multigene expression vectors [101, 102].

Based on the following three facts, CoVs would be promising vectors for vaccine development [103]. First, some of CoV accessory genes are amenable to deletion and can be used to produce attenuated viruses. Second, CoV host tropism can be altered by manipulating the S protein. Finally, heterologous genes can be expressed by inserting into CoV genomes with appropriate CoV transcription signals.

4.2 CoV Vaccines Against Virulent Strains by the Replacement of S Gene

S protein is the major determinant of host and tissue tropisms for coronaviruses. Using the vaccinia virus approach, Godeke and colleagues identified 64 residues comprising the endodomain and transmembrane domain of the MHV S protein to be critical in packaging into MHV VLPs [104]. This information was then used to generate recombinant viruses expressing a chimeric S protein made up of the C-terminal 64 residues of MHV fused to the ectodomain of FIPV S protein [105]. The recombinant virus, fMHV, lost the

ability to infect murine cells but can now infect feline cells, demonstrating that the chimeric spike protein conferred a switch in species tropism. Similar studies have been conducted on TGEV and IBV, providing the evidence that the cell tropism of these viruses is correlated with the S protein expressed by the recombinant viruses [50, 106–108].

In one earlier attempt, the ectodomain of the S protein of an apathogenic Beaudette strain of IBV (Beau-R) was replaced with that from the pathogenic M41 strain to produce a recombinant IBV BeauR-M41(S) [106]. The replacement changed the cell tropism of the virus in vitro, but no significant differences in pathogenicity were observed between the recombinant BeauR-M41 (S) and the apathogenic parent Beau-R. Remarkably, BeauR-M41(S) induced much greater protection (77%) against challenge with M41 compared with Beau-R (11%). Therefore, the S gene exchange between apathogenic and pathogenic strains may be a new direction in IBV vaccine development.

In a more recent study, the S ectodomain of a Vero cell-adapted Beaudette strain of IBV (p65) was replaced with that from the H120 vaccine strain [109]. The resulting recombinant IBV retained the ability to replicate in Vero cells and induced the production of specific antibodies for S, M and N proteins in the immunized chickens. It also induced protection (80%) against challenge with the virulent M41 strain. In a separate study, the entire S gene of H120 strain was replaced with that of the cell-adapted p65 strain [110]. The recombinant R-H120-p65(S) virus thus acquired the ability to grow in Vero cells, while in vivo pathogenicity remained similar to the parental H120 strain. The R-H120-p65 (S) also induced protection (80%) against challenge with the virulent M41 strain. These studies demonstrated that by partial or complete replacement of the S gene, recombinant coronaviruses can be generated that exhibit desirable neutralizing epitopes and/or acquire cell culturability, providing new insights for the development of novel recombinant vaccines.

4.3 CoV as Vaccine Vectors Expressing Heterologous Viral Proteins

The potential to express heterologous genes in CoV-based vaccine vectors has been well demonstrated in studies using TGEV [111]. To increase the cloning capacity, ORF3a and ORF3b were deleted in the parental virus (rPUR-MAD-SC11), resulting in the generation of the rTGEV-Δ3 vector. The heterologous GFP gene, followed by TRS of 3a, was then inserted to replace the deleted ORF3a and ORF3b, yielding rTGEV-Δ3-TRS3a-GFP. Both rTGEV-Δ3 and rTGEV-Δ3-TRS3a-GFP showed similar replication kinetics to those of the parental virus in cell culture, although slightly attenuated in infected animals. Importantly, rTGEV-Δ3-TRS3a-GFP stably expressed GFP (>40 μg/10^6 cells) for up to 20 passages. Immunization of pregnant sows with recombinant virus demonstrated that GFP-specific antibodies were detected in the immunized sows and their progeny, as well as in the colostrum from day 1 of lactation. This study was amongst one of the early attempts to demonstrate that promising potential of using CoV as vaccine vectors.

Using the same system, engineered TGEV vectors expressing antigens from porcine reproductive and respiratory syndrome virus (PRRSV) were constructed. Currently, live PRRSV vaccines only provide partial protection against clinical disease and sometimes revert to virulence, while killed PRRSV vaccines are generally less effective. Previous studies have shown that GP5 and M proteins are involved, respectively, in the induction of neutralizing antibodies and cellular immune response during PRRSV infection [112]. To express PRRSV GP5 and M, a bicistronic expression cassette was adopted to replace the ORF3a/ORF3b expression cassette in TGEV. Both proteins were efficiently expressed in the infected cells and tissues from infected piglets. When 1-week-old piglets were immunized with this recombinant TGEV (rTGEV), antibodies specific for PRRSV GP5 and M were produced, but the immune response

was limited against challenge with a virulent European PRRSV strain [109]. This is likely due to the low level of neutralizing antibodies induced by the rTGEV vector.

Presumably, due to the toxicity of GP5, rTGEV vectors expressing PRRSV GP5 and M were not fully stable, and GP5 expression was lost after 8–10 passage in cell culture. In order to promote induction of neutralizing antibodies, a point mutation was introduced into a glycosylation site near the epitope critical for neutralization, resulting in the generation of rTGEV-GP5-N46S-M virus. Piglets immunized with killed or live virus of this rTGEV vector produced a higher level of anti-GP5 and neutralizing antibodies and exhibited less severe disease upon challenge, although the immune response was not strong enough for full protection.

Because the full-length GP5 protein was not stably expressed, rTGEV expressing a truncated version of GP5 consisting of its N-terminal domain without the signal peptide was constructed [113]. Additionally, rTGEVs expressing a smaller domain containing neutralizing epitopes of the minor envelope protein GP3 and GP4 were also created. In yet another rTGEV, the PRRSV M protein was used as a scaffold to express the GP3 neutralizing epitope in the N-terminal region. The truncated PRRSV GP3, GP4 and GP5 fragments were stable up to passage 16 of the rTGEV vectors. These four rTGEVs together with one that expresses PRRSV M protein were used to immunize 12-days-old piglets. Compared with the non-immunized group inoculated with empty rTGEV vector, the immunized group exhibited less severe clinical symptoms and lower levels of lung inflammation when challenged with the virulent PRRSV Olot91-like strain. PRRSV titre was also slightly lower in the immunized group with a higher humoral response against GP5, suggesting that the rTGEV vectors expressing PRRSV antigens indeed provided partial protection against PRRSV infection.

5 Future Directions and Other Potential Applications

The high mortality posed to livestock and domestic animals by animal CoVs and the lack of specific antivirals and vaccines have greatly motivated investigators to understand this family of viruses at the molecular level, in order to decipher its interactions with the host and to develop strategies to prevent and control CoV infections. To this end, structural and molecular genetics analyses of the CoV genome have been enabled by reverse genetics. Previous caveats faced in the development of infectious cDNA clones, such as the large size of the CoV genome and the instability of its cDNA sequences in bacterial systems have now triumphed over, thanks to new creative approaches in four reverse genetics systems. These reverse genetics approaches based on homologous recombination, full-length cDNA clones in BACs, in vitro ligation and vaccinia virus vectors made CoV full-length infectious clones available for the study of CoV replication, virus-host interactions and pathogenesis, as well as for antiviral drug screening. It would accelerate the development of vaccines and may be used as a vaccine vector for veterinary pathogens without the need for manipulating infectious viruses. Nevertheless, it should be kept in mind that reverse genetics has its own limitations. For instance, full elucidation of viral gene functions that are essential for RNA synthesis by reverse genetics has proved to be difficult. For example, research aimed at investigating the functions of replicase genes by introducing mutations/insertion at certain positions of the viral genome, rendering no recovery of infectious virus. To overcome these pitfalls, individual gene functions may be studied using bioinformatics or structural means. Knowledge deduced from these studies may give insights into the CoV RNA synthesis while exploiting the full potential of CoV reverse genetics.

An important prerequisite for viral vaccine vectors is the delivery efficiency of genetic material to specific target cells, such as targeting of viral vaccine vectors to antigen-presenting cells

(APCs). In the case of MHV, its cognate receptor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is expressed on murine DCs, and given the abundance of CEACAM1 on the DC surface, MHV-based VLPs containing MHV-based vector RNAs may be used to transduce murine DCs [114]. Along with well-established immunological techniques in inbred and transgenic mice, recombinant MHV vectors in the murine model may guide the development of CoV vaccine vectors and pave the way for CoV-based vaccine in livestock and domestic animals [114, 115].

Self-replicative mRNA vaccines, which have been developed with certain RNA viruses, would offer an alternative strategy. For instance, recombinant alphavirus replicon particles are created exclusively from the structural proteins of the donor alphavirus, but the genomic RNAs contained in these particles are chimeric. In this case, the structural proteins of alphavirus are replaced by those from heterologous viruses. Using a similar strategy, a PEDV vaccine was developed using the Venezuelan equine encephalitis virus replicons expressing the PEDV S gene [116]. With a better functional understanding of the CoV replicase gene, a similar approach may be viable to establish mRNA vaccine vectors based on CoV.

6 Summary

The large genome size and unique transcription strategy of CoVs make them promising candidates for the development of vaccine vectors. Other characteristics that make them ideal for use as vaccine vectors include the ability to manipulate their genome leading to the generation of attenuated viruses and change their cell tropism as well as the ability to use them to create multigene expressing vectors. Further understanding of genes such as the replicase gene is required to further develop CoV as an efficacious veterinary vaccine vector.

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