Emerging animal viruses in China, from discovery to control measures

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Propositions

1. Virus-induced mitochondrial antiviral-signaling protein (MAVS) degradation, mediated by a viral protein, benefits virus replication.

2. There is a bright future for virus-like particle based vaccines.

3. mRNA vaccine technology is not worthwhile for the veterinary field.

4. Exogenous expression by transfection of plasmids exaggerates protein's natural function.

5. More professional expectations for women from society inhibit the growth of the birth rate.

6. The frequent outbreaks of animal viruses lead to vegetarianism.

Propositions belongs to the PhD thesis entitled,

Emerging animal viruses in China, from discovery to control measures.

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Table of contents

Chapter1

General introduction

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1.1 Introduction

With the discovery of the tobacco mosaic virus in 1892 (Iwanowski 1903), the discipline of "virology" was born. The virus definition comes from a Latin word that means "slimy liquid" or "poison". Dr. Martinus Beijerinck is recognized as "Pioneer of Virology" because he summarized the virus as "a living, reproducing organism (contagium vivum fluidum, in Latin)" (W 1898). Since then, viruses have become more known to the public due to frequent outbreaks. Emerging and re-emerging viruses nowadays are regarded as a big threat and burden for public health and cause considerable socio-economic problems globally (Morens *et al.* 2004). Likewise, animal viruses, which cause many diseases that significantly affect the veterinary industry often lead to high fatalities and notable economic losses (Bloom and Cadarette 2019). In addition, these infected animals can act as potential zoonotic reservoirs in the ecosystem, which may lead to more virus outbreaks in the future.

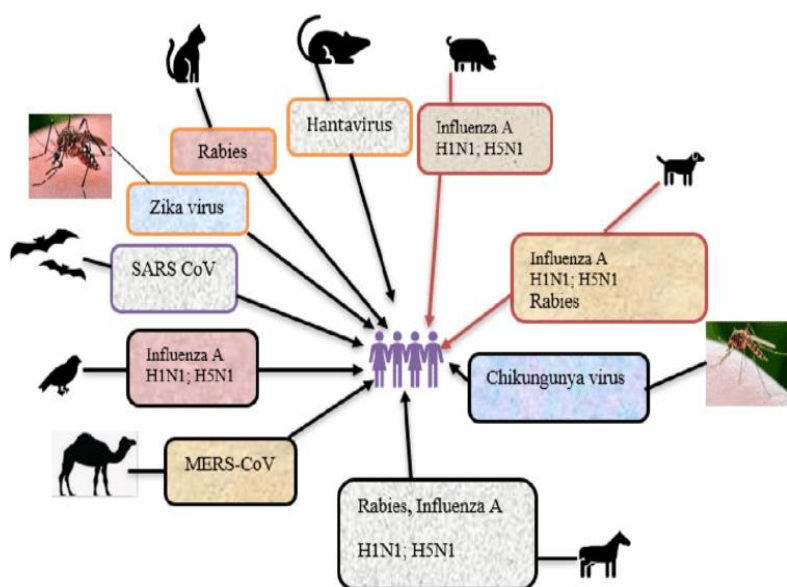**Figure 1.1 Examples of zoonotic viruses that can be transmitted from animals or insects to humans**

Picture used with permission from Abebe G, 2020

Animal viruses are classified as viruses not infecting humans but only animals, including livestock, companion animals and wild animal species. RNA viruses are a major cause of morbidity and mortality in animals, responsible for substantial economic losses worldwide with

notable disease outbreaks such as Avian influenza virus (AIV), Newcastle disease virus (NDV), Bluetongue virus (BTV), Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine epidemic diarrhea virus (PEDV), etcetera. Most of these RNA viruses affect mammalian and avian species, however, the mammalian viruses are believed to have a higher potential capacity of cross-species transmission to other mammals (Parrish *et al.* 2008). The high mutation rates of RNA viruses, compared to DNA viruses, is considered beneficial for these viruses for enhanced virulence, immune evasion, and adaptation to new tissues and hosts (Duffy 2018). Furthermore, the close contact between farmed animals with wild animals in natural ecosystems, the booming veterinary industry with intensive farming, and the global trade of the animals, leads to emergence of veterinary viral diseases (Rohr *et al.* 2019).

1.2 Zoonotic veterinary RNA viruses

According to the reports from the World Health Organization (WHO), 60% percent of infectious diseases in humans originate from animals, and more than 70% percent of these are coming from wild animals. Within the animal pathogens, 75% percent is potentially zoonotic, meaning able to transmit from animals to human beings. These zoonotic viruses can be easily transmitted through domesticated or wild animals, sometimes with the help of arthropod vectors like mosquitoes, or via the digestive or respiratory tract, or skin contact (**Figure 1.1**). For example, the currently circulating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can also infect feline species such as cats (Shi *et al.* 2020), and moreover, infected cats and dogs have been discovered in SARS-COV2 infected mink farms (van Aart *et al.* 2021), indicating the possibility that infected companion animals might serve as "silent intermediate hosts" to spread SARS-CoV-2 to humans. However, during virus transmission cycles, a "spillover" of animal viruses to human hosts is also incidentally happening. Pseudorabies virus (PRV) which mainly infects pigs has been reported to lead to human cases of endophthalmitis and viral encephalitis in China (Ai *et al.* 2018, Yang *et al.* 2019, Guo *et al.* 2021). In the USA, the majority of human infections with influenza serotypes H1N1, H3N2, and H1N2 are mainly associated with infected pigs (Rajao *et al.* 2019). In addition to increasing their numbers through virus reservoirs or in susceptible hosts in natural systems, viruses may also expand their host range by adapting to human beings. All activities involved in wildlife hunting, international trading, close contact between domestic or wildlife animals facilitate the transmission of animal viruses to humans, thus threatening human health (Johnson *et al.* 2020). In another way, human movements such as human migration, urbanization, and globalization promote transmission of zoonotic viruses. Insects, such as mosquitoes which serve as vectors for transmission of a number of infectious agents, also play a crucial role in increasing the

chance of cross-species transmission, which is influenced by human activities, especially when multiple intermediate hosts might be used (Martina *et al.* 2017).

In human history, we have witnessed increasing incidences of virus outbreaks all over the world. Historically, the 1918 influenza pandemic (Spanish flu) infected one third of the world population only within two years, causing at least 50 million deaths; the spreading of human immunodeficiency virus (HIV) ranked as second, with a death toll of 30 million so far. The still ongoing SARS-CoV-2 pandemic, caused 3,92 million deaths worldwide so far. Even though not every virus outbreak reaches pandemic levels, the impact of virus outbreaks on economic and social aspects of society is likely underestimated.

The world population is expected to reach more than 9 billion by 2050 (Van Bavel 2013). To meet with the huge food consumption of the increasing population, farmers constantly intensify the use of the land for agricultural production, which leads to ecological unbalance. Livestock animals are the major protein source currently used to meet the demand of the growing population. Globally, livestock constitutes more than 30% of the agricultural gross domestic product (GDP), and in developing countries, it is even more, so domestic animals are believed to be one of the most important and rapidly expanding commercial agricultural sectors worldwide (Thornton 2010).

1.3 Veterinary vaccines

The terms 'vaccine' or 'vaccination' was derived from "Variolae vaccinae" (latin word: smallpox of the cow), which was first used by Edward Jenner, who discovered in 1796 that people could become immune against smallpox by inoculation with material from a cowpox lesion. Louis Pasteur proposed to use the term "Vaccine" in 1881. Four years later, his first rabies vaccine was generated by attenuating virus through culturing in rabbit spinal cords. Louis Pasteur had applied a similar method in immunizing chickens against chicken cholera in the 1870s, which is believed to be the first veterinary vaccine (Plotkin and Plotkin 2011).

In the 140 years since the first development of vaccines by Pasteur, great technological breakthroughs have been implemented to generate different types of vaccines. In 1886, Daniel Elmer Salmon and Theobald Smith initiated the concept of inactivated or killed vaccines by applying heat-killed hog cholera "virus" to immunized pigeons, which is believed to be the first report of virus inactivation for vaccine purposes (Sanders *et al.* 2015). Now there are various types of vaccines on the market, including inactivated vaccines, live attenuated vaccines (LAVs), and genetically-engineered vaccines. Veterinary vaccines greatly improve animal

health by reducing animal disease outbreaks and limiting the suffering of animals. Furthermore, these vaccines ensure food production, and indirectly also protect public health. With efficient control of disease through veterinary vaccines, the high production of food to feed the growing human population is enabled and the need for using antibiotics is reduced. The most remarkable example of the impact of veterinary vaccines is the "Global Rinderpest Eradication", which was achieved by a large-scale vaccination campaign (Roeder *et al.* 2013). Currently, the majority of the vaccines used for immunizing animals are either traditional inactivated or live-attenuated ones. However, the use of traditional vaccines such as inactivated vaccines can have clear drawbacks, such as a need for a large scale production of active virus in a suitable platform like animals or cells, with a potential risks of virus spill-over during the manufacturing process. In addition, inactivated vaccine efficiency also can be compromised by the inactivation process or by other viruses present during the application of vaccination (Sanders *et al.* 2015). Attenuated vaccines, on the other hand, may potentially accumulate mutations or recombination leading to reversion to a virulent status, with the risk of causing vaccine-associated disease, such as the recently outbreaks of PRV in China (Jiang 2015), and in US, re-assortment of LAVs vaccine virus circulating was reported (Sharma *et al.* 2020). Taken together, these traditional vaccines have important biological safety risks and are therefore less optimal for emerging virus control, compared to a number of novel methods that involve genetic-modification strategies, such as the development of virus-like particle (VLP) vaccines. VLPs are non-replicating particles that are structurally similar to the outer shell of the virion, and that can induce both humoral and cell-mediated immune responses without the usage of adjuvants (Roldao *et al.* 2010). Moreover, VLP vaccines can safely be produced on a large-scale in industry. The Baculoviruses expression vector system (BEVS) is an often used system to produce VLPs, with not only high yield but also proper post-translational modifications which is also important for immunogenicity. BEVS-based recombinant protein and VLP production technology is being implemented to produce several human and veterinary vaccines that are currently on the market (van Oers *et al.* 2015). Important examples are subunit or VLP vaccines against human papillomavirus (Cervarix, GlaxoSmithKline), influenza virus (FluBlok, Fluzone), or Porcine circovirus type 2 (PCV2) (Porcilis, MSD), and there are also some vaccine candidates in clinical development based on BEVS (https://clinicaltrials.gov/). The future of VLP vaccines is promising because there is no possibility of incomplete inactivation or reversion to virulence associated with such vaccines, in contrast to traditional inactivated or live attenuated vaccines.

1.4 Emerging animal viruses in China studied in this thesis

In spite of successful control of a number of known viral diseases, we have witnessed quite a number of newly emerging animal viruses in the Chinese veterinary industry, causing significant losses and concerns (**Table 1.1**), such as the African swine fever virus (ASFV), Tembusu Virus (TMV), Peste des petits ruminants virus (PPRV), Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine epidemic diarrhea virus (PEDV) and Porcine circovirus 2 (PCV2). Moreover, virus discovery efforts have detected hundreds of new animal viruses with unknown zoonotic risks. China has a current population of 1.47 billion people, consumes an increasing amount of meat and has an economy that highly depends on animal products. Thus, the Chinese live-stock industry is very vulnerable to emerging animal virus outbreaks. Food animals in the Chinese market include major species such as pigs, chickens, sheep, goats, rabbits, and cows, and each species is associated with different emerging viruses. Experienced researchers in the veterinary virology field will be needed to understand virus-host interactions and to develop new strategies for vaccine development (Liu *et al.* 2014). Such research will contribute to the understanding of the epidemiology of these viruses and will identify potential animal reservoirs and intermediate hosts for these diseases. Overall, management of animal viruses will directly influence agricultural and economic development, and is therefore not only protecting animals, but human public health and social stability as well.

Table 1.1: Emerging or circulating animal viruses in veterinary industries in China

Family and Genus	Virus	in China since	Infected animals
Asfarviridae, Asfivirus	African Swine Fever Virus	2017	Pigs
Arteriviridae, Arterivirus	Porcine Reproductive Respiratory Syndrome Virus	1996	Pigs
Bunyaviridae, Phlebovirus	Novel Bunya Virus	2013	Human and mammals
Caliciviridae, Lagovirus	Rabbit Hemorrhagic Disease Virus 2	2020	Rabbits
Circoviridae, Circovirus	Porcine Circovirus Type 2	2016	Pigs
Coronaviridae, Alphacoronavirus	Porcine Epidemic Diarrhea Virus	1971	Pigs
Coronaviridae, Alphacoronavirus	Feline Infectious Peritonitis Virus	Not known	Cats, tiger
Flaviviridae, Flavivirus	Japanese Encephalitis Virus	1965	Pigs
Flaviviridae, Flavivirus	West Nile Virus	2013	Human and Culex pipiens
Flaviviridae, Flavivirus	Tembusu Virus	2010	Ducks
Hepeviridae, Orthohepevirus	Hepatitis E virus	2017	Pigs
Orthomyxoviridae,Alpha influenzavirus	Avian Influenza Virus	2004	Chicken
Peribunyaviridae, Orthobunyavirus	Schmallenberg Virus	2018	Sheep, cattle, Ruminants
Paramyxoviridae, Henipavirus	Hendra Virus	2008	Bats
Paramyxoviridae, Morbillivirus	Peste des Petits Ruminants Virus	2007	Sheep, goat, deer
Togaviridae, Alphavirus	Getah Virus	1992	Pigs, horse, cattle, blue fox

Recently, we noticed several emerging RNA virus outbreaks related to farm animals. In my study, I focus on three viruses that had a great impact on the market. Firstly, Peste des Petits Ruminants virus (PPRV), causing "goat plague", a highly contagious disease in small ruminants. PPRV affects both goats and sheep and has been discovered in nearly seventy countries all over the world. PPRV represents one of the economically most important animal diseases for people in rural areas and developing countries that rely on making a living on goat and/or sheep herding. In China, PPRV is known to be present since July 2007 (Wang *et al.* 2009), and has now spread over almost all China and is becoming big burden for farmers (Ma *et al.* 2019).
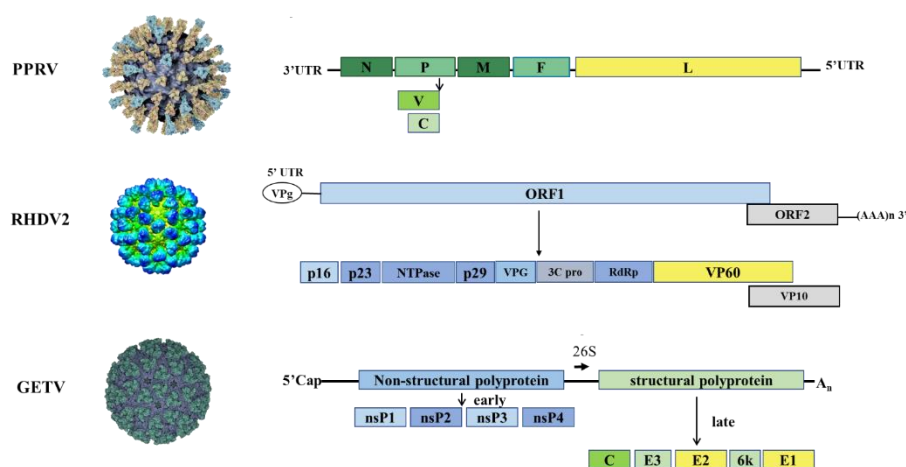
Figure 1.2 Viral particles and genomic organization of viruses studied in the thesis.

Left PPRV(Top), RHDV2(middle) and GETV(bottom). Right, Schematic representation of viral genomes and polyprotein (s).

The second virus included in the study is Rabbit Haemorrhagic Disease Virus (RHDV), which causes "rabbit plague", and was firstly discovered in 1984 in Jiangsu Province (Liu *et al.* 1984), and later spread worldwide (Abrantes *et al.* 2012). RHDV can survive in dead animals for more than 20 days and anything visiting the carcass (predators, flies) can spread the virus easily. The virus can also last for three months in dried feces (Henning *et al.* 2005). Recently, an outbreak of rabbit plague emerged in Europe in young rabbits immunized by vaccination against RHDV. This outbreak was caused by a new serotype, RHDV2/b/G1.2 (Le Pendu *et al.* 2017). In the spring of 2020, an RHDV2 outbreak was reported in rabbit farms in Sichuan province in China

(Hu *et al.* 2021). Now, RHDV2 is replacing traditional RHDV, as the main pathogen for rabbits related haemorrhagic disease (RHD) worldwide (Mahar *et al.* 2018).

Thirdly, an Alphavirus with the name of Getah Virus (GETV) causing abortions in piglets. These abortions were observed in the swine industry since 2016, in pigs that followed the conventional vaccination programme. GETV has mainly been discovered in Asian countries, and uses mosquitoes as vector for transmission (Lu *et al.* 2020). Historically, GETV in China was first reported in 1964 in *Culex* mosquito species in Hainan province (Li *et al.* 1992), however, in recent years, GETV has been detected in various areas in China and is becoming a serious problem.

In this thesis research, I aim to elucidate the molecular mechanisms underlying the pathogenesis of emerging animal viruses with special focus on PPRV and how this virus interacts with innate immunity pathways. I also aim at providing suitable and safe vaccine candidates for RHDV2 and GETV to successfully control these emerging viral diseases. Each virus will be discussed in more detail in the next paragraphs.

1.4.1 Peste des Petits Ruminants virus

Peste des Petits Ruminants virus (PPRV) belongs to the genus *Morbillivirus*, subfamily *Orthoparamyxovirinae*, family *Paramyxoviridae*. In 1942, PPRV was first described in Ivory Coast (Cote d'Ivoire) in West Africa by two French veterinarians (Gargadennec and Lalanne 1942). Peste des Petits ruminants (PPR) is the french name which they gave to the disease, meaning "the plague of small ruminants", but later it was adopted as its scientific name and still used today. In 2016, the official name of the virus species became *Small ruminant morbillivirus* according to the International Committee on Taxonomy of Viruses (ICTV, EC 47, London, UK, July 2015). However, the virus itself is still commonly named PPRV.

PPRV mainly affects goats and sheep with mortality rates are as high as 90% (Parida *et al.* 2015). PPRV infection causes severe symptoms, including pyrexia, oculo-nasal, mucopurulent nasal and ocular discharges, necrotizing and erosive stomatitis, gastroenteritis, bronchopneumonia, cough, pneumonia, and diarrhea, which is similar to Rinderpest Virus (RPV) in cattle (Balamurugan *et al.* 2014). PPRV infections result in significant economic losses worldwide. Until 2020, PPRV has spread to more than 70 countries, mainly in Africa and Asia (**Figure 1.3**) (Baron *et al.* 2016). The disease is currently endemic in both domestic and wild animals in parts of sub-Saharan Africa (Mahapatra *et al.* 2015), the Middle East (Muniraju *et al.* 2014), and Eastern Asia (Banyard *et al.* 2014). Moreover, in central Asia, the virus has been prevalent in many countries, including Kazakhstan (Kock *et al.* 2015), and recently, the

disease has been reported on the edges of European Georgia starting in February 2016 (OIE report) (Donduashvili *et al.* 2018) and in Bulgaria in June 2018 (Altan *et al.* 2019). In Turkey, PPRV infection was also reported earlier in 1996 and 1998, while the first officially reported cases in southern and eastern Anatolia were in 1999 (OIE, 1999). The prevalence, distribution, and host range of PPRV in Turkey have also been investigated in 2002 (Ozkul *et al.* 2002) and the strain information from Marmara region was reported in 2019 (Altan *et al.* 2019). Meanwhile, PPRV is also threatening wild animals in several Asian countries (Marashi *et al.* 2017, Zhou *et al.* 2018).

The *Morbillivirus* genus contains RPV, PPRV, Feline Morbillivirus (FMV), Canine Distemper Virus (CDV), Cetacean morbillivirus (CeMV), Phocine morbillivirus (PDV) and Measles Virus (MV) (Fenner 1976). MV still causes ongoing outbreaks and sometimes pandemic disease in non-vaccinated humans (Fraser-bell 2019). In contrast, due to an efficient eradication campaign, RPV has been eradicated in May 2011 as declared by the United Nations Food and Agriculture Organization (FAO) (Morens *et al.* 2011). Now, the Organization for Animal Health (OIE) also launched the PPR Global Eradication Programme (PPR GEP), to eradicate PPRV by 2030.

Morbilliviruses have enveloped, spherical virons with a diameter ranging from 150 to 300 nm. They are negative-sense single-stranded RNA viruses and the genome encodes eight major proteins in the order of 3'-N-P-M-F-H-L-5', of which P is further processed to give two non-structural proteins, C and V (Figure.1.2). For infection, morbilliviruses need specific cellular receptors, including signalling lymphocyte activation molecule (SLAM, also called CD150) (Tatsuo and Yanagi 2002), which is exclusively expressed in immune cells, or Nectin 4 which has been discovered in epithelial cells and is efficiently involved in morbillivirus entry processes (Birch *et al.* 2013).

For PPRV four lineages have bene identified: I, II, III, and IV, based on the partial nucleotide sequence of N and F genes (Baron *et al.* 2016). The first isolate of PPRV (Cote d'Ivoire) belongs to lineage I, which was circulating in West Africa in the 1970s-1990s and later disappeared, at least for a while. Recent reports indicate that lineage I was circulating in Niger from 1994 until at least 2001, and meanwhile, in south-western Niger, lineages I and II were cocirculating.
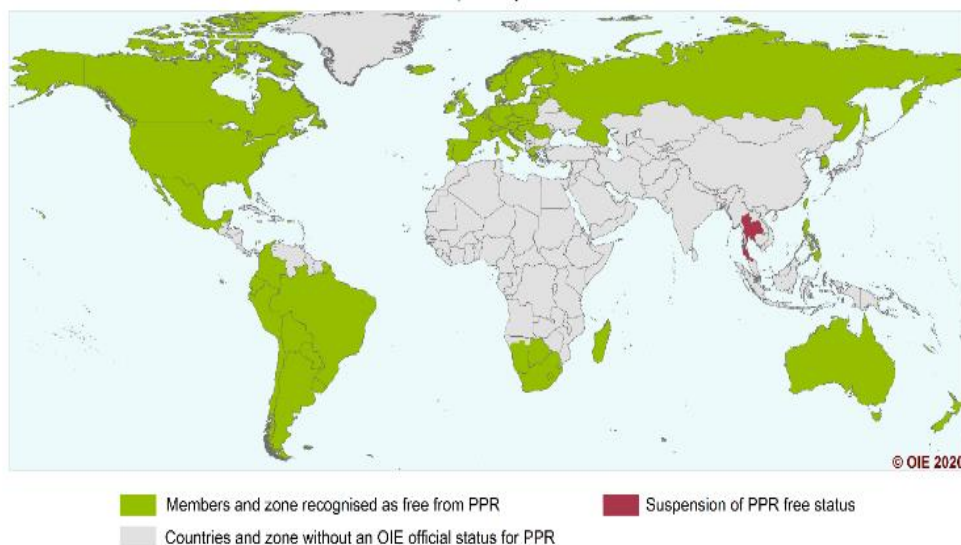
Figure 1.3. Distribution of PPRV worldwide
Pictures were adjusted from www.oie.int.

However, the outbreak in 2013 in West Africa belonged to lineage IV (Tounkara *et al.* 2018). PPRV lineage III now is more prevalent in Middle East and East Africa (Muniraju *et al.* 2014). PPRV IV is found in Israel since 1993 (Clarke *et al.* 2017) and in Ethiopia in 2010 (Muniraju *et al.* 2016), but PPRV lineage IV is not only circulating in Africa but also in Asia (Fine *et al.* 2020). In China, PPRV is being detected since July 2007 and is believed mainly to be of lineage IV (Bao *et al.* 2012). In addition, a PPRV lineage II was discovered in a water deer (*Hydropotes inermis*) in Anhui province, China (Zhou *et al.* 2018).

The distribution of sheep and goat populations is relatively condensed in developing countries, including China, India, and some countries in South Africa. PPRV is now spreading all over China (2013–2014) (FAO, 2013). PPRV is regarded as one of the main animal transboundary diseases that constitute a threat to livestock production and a threaten for 300 million rural families globally, with an estimated economic loss of $2.1 billion per year (FAO). Controlling

this disease may result in improved productivity, food security, income generation, and social stability and empowerment (Dossa *et al.* 2008). Moreover, growing evidence suggested that multiple wildlife species can be infected with PPRV (Dou *et al.* 2020). Recently, an outbreak of PPRV was discovered in several wild animal species such as in endangered saiga antelope (*Saiga tatarica mongolica*) with large-scale mortality (Pruvot *et al.* 2020). PPRV in China caused near-catastrophic herd depletion for endangered wild species, in addition to the severe economic losses for farmers in 2007 (Wang *et al.* 2009). The host range of PPRV has been continuously expanding, resulting in the potential maintenance of PPRV in different susceptible hosts. This is a big threat to wildlife populations and resilience of ecosystems and makes efficient control of PPRV complicated (Fine *et al.* 2020).

PPRV interactions with innate immunity are poorly understood, but are believed to be modulated by both PPRV structural and non-structural viral proteins. The live attenuated vaccine strain PPRV/Nigeria 75/1 is widely used in China, but how the vaccine strain counteracts the innate immunity is hardly known. Furthermore, deep sequencing of the PPRV genome has identified attenuating mutations during the passaging that severely impacted the pathogenicity of PPRV. Nonetheless, the attenuated PPRV strain Nigeria 75/1 can still potentially revert to virulence (Eloiflin *et al.* 2019), which asks for more focused studies on PPRV Nigeria 75/1 vaccine strain to map the mutations required for attenuation. Studies on virus-host interactions and innate immunity were performed by using a novel PPRV strain isolated from *Capra ibex* in China during 2015 (Zhu *et al.* 2016) or by testing the virus on human cell lines, but how the virus interacts might differ between natural hosts. The development of several cell lines have greatly promoted the understanding of PPRV related pathogenesis. An example is the monkey Flp-In-CV-1 cell line (CV1) expressing the sheep–goat SLAM protein and generated for efficient isolation of PPRV (Adombi *et al.* 2011). Other examples are a murine cell line used to distinguish virulent PPRV strains from attenuated PPRV strains (Comerlato *et al.* 2020) and MDBK cell lines stably expressing the PPRV receptor Nectin-4 generated for isolating a clinical PPRV strain. Nevertheless, these are all cell lines that do not originate from PPRV susceptible hosts. A cell line derived from caprine endometrial epithelial cells (EECs) was recently generated and was applied to study the PPRV Nigeria 75/1 internalization and the role of apoptosis and autophagy during infection. Moreover, the gene expression profile of EECs infected with PPRV Nigeria 75/1 was determined with a DNA microarray to investigate the cellular response after viral entry (Yang *et al.* 2018). The new applications of this cell line shed light on PPRV related innate immunity in host cells.

In conclusion, understanding how PPRV vaccine strain Nigeria 75/1 counteracts cellular antiviral responses in host cells, will contribute to the understanding of PPRV pathogenesis and virus-host interactions and may lead towards the development of improved live attenuated PPRV vaccines and/or efficacious antiviral drugs to control this virus in the future.

1.4.2 Rabbit Haemorrhagic Disease Virus

Rabbit Haemorrhagic Disease Virus (RHDV) belongs to the genus *Lagovirus* from the *Caliciviridae* family. In 1984, RHDV was first reported in China. Two years after the initial outbreak, RHDV reached the European continent, and proved to be an acute and highly lethal hemorrhagic disease in European rabbits (*Oryctolagus cuniculus*) (Liu *et al.* 1984). Now RHDV can be found almost all over the world (Abrantes *et al.* 2012). RHDV is severe in domestic and wild European adult rabbits (*Oryctolagus cuniculus*), with symptoms including anorexia, apathy, dullness, groans prostration, nervous signs (convulsion, ataxia, paralysis, opisthotonos, paddling), and respiratory signs (dyspnoea, frothy and bloody nasal discharge), and also cyanosis of mucous membranes. RHDV infection is associated with mortality rates up to around 80~100% (Abrantes *et al.* 2012).

The *Lagovirus* genus contains two proposed genogroups, in which genogroup GI includes all viruses related to RHDV and GII includes viruses related to European brown hare syndrome virus (EBHSV). GI was further subdivided into four phylogenetical variants, including GI.1 (RHDV) and GI.2 (RHDV2/b) that are both highly pathogenic viruses for both rabbits and hares related to rabbit hemorrhagic disease (RHD), whereas, GI.3 and GI.4 are nonpathogenic strains of viruses infecting rabbits (Abrantes *et al.* 2012, Le Pendu *et al.* 2017).

Lagovirus particles are non-enveloped, around 27~40 nm in diameter, with T=3 icosahedral symmetry and composed of 180 capsid proteins. The positive single-stranded RNA genome is around 7.4 kb with two open reading frames (ORFs). The virus genome has a genome-linked protein (VPg) at the 5' end and the 3' end has a poly (A) tail. The ORF1 can be translated into a polyprotein, which is cleaved into seven non-structural proteins including p16, p23, helicase, p29, Vpg, protease, and polymerase (Urakova *et al.* 2015), and one structural capsid protein (VP60), which is also expressed from a subgenomic mRNA. ORF2 is translated into the minor structural protein VP10, which is required for subgenomic RNA formation, and absence of VP10 downregulates the expression of VP60 *in vivo* (Chen *et al.* 2009). Histo-Blood Group Antigens (HBGA) act as attachment factors of RHDV to favor the establishment of infection (Nyström *et al.* 2011). By generating an Arg-Gly-Asp receptor-recognition motif in the RHDV

capsid which enabled the virus to propagate in the rabbit kidney 13 (RK-13) cell line (Zhu *et al.* 2017).

Numerous cases of RHD were reported in European rabbits since 2010 in North-western France (Le Gall-Recule *et al.* 2011). This outbreak affected not only wild rabbit populations but also RHDV-vaccinated rabbits; instead of RHDV which only caused severe symptoms in adults, RHDV2 was characterized by causing disease and high mortality even in young animals from 15~20 days old onwards, and was believed to be a new virus called RHDV2 (Dalton *et al.* 2012). The mortality of RHDV2 in young rabbits and hares is up to 80% which suggests it is less virulent than RHDV. However, the isolation of RHDV2 from RHDV-immunized rabbits indicates the current vaccine is not protective against RHDV2, which underscores the need of new RHDV2 vaccine (**Figure 1.4**).

The differences in pathogenicity between Lagovirus isolates are still poorly understood. Researchers claimed that rabbit calicivirus 1 (RCV-A1) and RHDV(GI.4) cocirculating in Australia with RHDV(G1.1) lead to occasional recombination, and highly virulent strains of RHDV2 might have evolved from nonpathogenic ancestors such as RCV-A1 (Mahar *et al.* 2016). Other studied claimed that the pathogenic strains might be the result of recombination events happening between non-pathogenetic and pathogenic viruses (Esteves *et al.* 2015), such as gene recombination based on RNA dependent RNA polymerase (RdRp) (Hukowska-
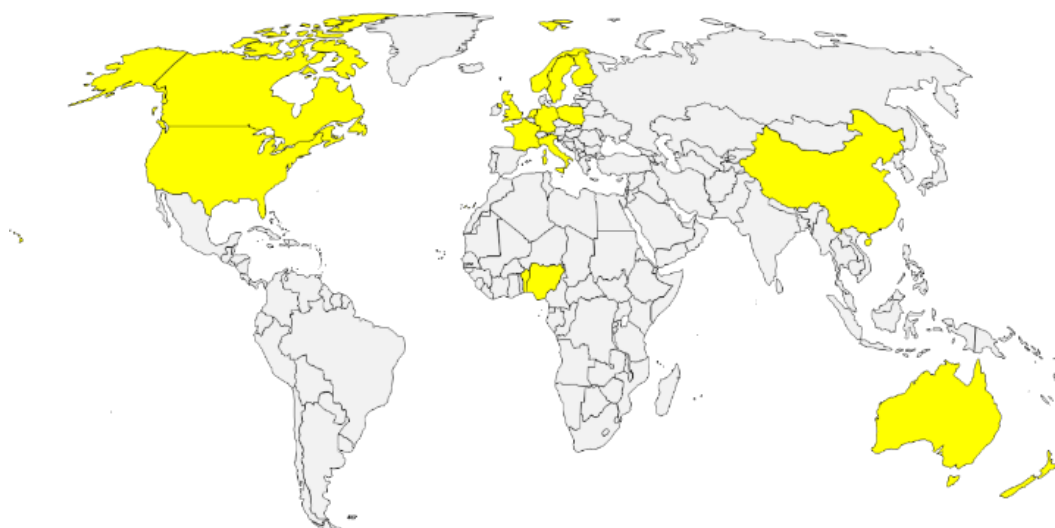

Figure 1.4. Global distribution of RHDV2

Data was summarized by reports from NCBI and OIE until June,2021

Szematowicz 2020), or use GI.3 as the major donor for the non-structural part and GI.2 for the structural part (Abrantes *et al.* 2020). Interestingly, some recent results also showed that non-structural genes may also serve as critical drivers of RHDV2 epidemiological fitness (Mahar *et al.* 2021).

In China, fatality cases related to RHDV2 were reported in early 2020 (Hu *et al.* 2021) , posing a severe risk to the large-scale rabbit meat production industry (Li *et al.* 2018). Moreover, several reports claimed that RHDV2 could be highly infectious for different species in the family *Leporidae*, including *Lepus corsicanus* (Camarda *et al.* 2014) *Lepus timidus* (Neimanis *et al.* 2018) and *Oryctolagus cuniculus* (Camarda *et al.* 2014). China with its various geographic landscapes has a variety of rabbit and hare species including *Lepus sinensis*, *Lepus timidus*, *Lepus hainanus*, *Lepus comus*, *Oryctolagus cuniculus*, and some endangered species such as *Ochotona koslowi*, *Ochotona argentata*, and *Ochotona iliensis* (Li and Smith 2005). As a consequence, the possibility of spill-over of RHDV2 from the rabbit industry to wild rabbits is extremely high, posing a great threat to wild animal conservation. This is an extra reason to generate a safe and effective RHDV2 vaccine, next to the existing inactivated RHDV vaccine.

The conventional method of generating RHDV vaccines has become ethically questionable in recent years, because of the production from liver preparations of infected rabbits. The vaccination also needs several administrations to achieve high levels of protection, requiring massive amounts of propagated virus and a high standard of manufacturing. Besides, potential risks of incomplete inactivation and virus spillover, is a good reason to develop new vaccine candidates using a different method. The property of the RHDV structural capsid protein (VP60) to self-assembly into VLPs, can be exploited to produce immunogenic vaccine candidates.

In this thesis, we developed a new vaccine candidate for an emerging RHDV2 strain from the Netherlands, by using the Baculovirus expression vector system (BEVS), and test the immunogenicity in rabbits. Moreover, in a follow-up study, a novel bivalent virus-like particle bearing VP60 of classic RHDV(GI.1) and RHDV2(GI.2) was also generated to provide a vaccine candidate with dual efficiency to help control both RHDV and RHDV2 related infections with only one shot to be used in industrial reared and pet rabbits.

1.4.3 Getah virus

Getah virus (GETV) belongs to the genus *Alphavirus* within the *Togaviridae* family. In 1955, Getah virus was firstly discovered in Malaysia. The term "getah" means rubber in Malay and refers to its discovery in rubber plantations. The prototype GETV strain MM2021 was isolated

from *Culex gelidus* (Takashima and Hashimoto 1985) mosquitoes. GETV infections were found to be pathogenic in multiple vertebrates. In horses, the symptoms caused by GETV infections are fever, rashes, edema of the hindlegs, and lymph node enlargement, which caused significant economic losses, thus it is regarded as an important pathogen in horses (Fukunaga *et al.* 2000) ; in pigs, GETV infection can cause abortion, fetal death, and reproductive disorders (Yago *et al.* 1987, Yang *et al.* 2018). Furthermore, in blue foxes, GETV infection resulted in fever, depression, neurological symptoms(Shi *et al.* 2019), and even death while in cattle, GETV infection was also detected with fever, anorexia, and depression symptoms (Liu *et al.* 2019). Since the first outbreak of GETV in Japan from 1970 and the 1980s in horses (Kono *et*

Figure 1.5. Global distribution of Getah virus

Countries and areas with GETV, as of April,2021, achieved from ArcGIS(https://efsa.maps.arcgis.com/)

al. 1980, Sentsui and Kono 1980), GETV has spread to most Asian countries and Ethiopia (**Figure 1.5**).

The *Alphavirus* genus currently includes 31 viruses, such as Semliki Forest virus (SFV), Chikungunya virus (CHIKV) and Sindbis virus (SINV) which are known to infect human beings. Alphavirus particles are enveloped, spherical, with a diameter around 60~80 nm (see **Figure1.2**). The capsid has a T=4 icosahedral symmetry made of 240 monomers. The alphavirus envelope contains 80 spikes, each spike made of trimers from E1/E2 heterodimers (Voss *et al.* 2010). E2 is believed to mediate receptor attachment, while E1 is responsible for membrane fusion into host cells during virus infection (Ziemiecki *et al.* 1980). The alphavirus genome is a positive-sense, single-stranded RNA molecule, which is around 12 kb in length. Alphavirus genomes are methylated (7-methylguanosine) at the 5'end and with a poly(A) tail

at the 3'end and with two open reading frames (ORFs). In infected cells, two-thirds of the genome carries ORF1, which is translated into a polyprotein that is cleaved into four non-structural proteins (nsP1-4) by the viral protease nsP2. ORF2 encodes several structural proteins including C (Capsid), E3, E2, 6K, and E1, which are translated form a 26S subgenomic RNA. into a large polyprotein precursor (C/E3/E2/6K/E1) (Strauss and Strauss 1994, Pfeffer *et al.* 1998). Alphaviruses have been demonstrated to attach to cell hosts by receptors, such as Mxra8 (Zhang *et al.* 2018), then start translation of the polyprotein which later forms replication complexes (RC) by four non-structural proteins (nsP1-4). Th exact function of nsp3 in the RC is less known, but it significantly inhibits antiviral stress granules by sequestration of G3BP and several motifs have been recognized that could interact with several host cellular proteins (Nowee *et al.* 2021).

GETV isolates are classified within four lineage groups based on the E2 sequence by phylogenetic analysis (Lu *et al.* 2020). According to the phylogenetic tree, the prototype strain MM2021 represents the oldest group and shows a closer relationship with Sagiyama virus (SAGV), isolated in 1956 in Japan. SAGV initially was considered to belong to an independent virus species, but now it is also believed to be a type of GETV (Shirako and Yamaguchi 2000). The spread of GETV in different vertebrates in China has been reported in provinces including
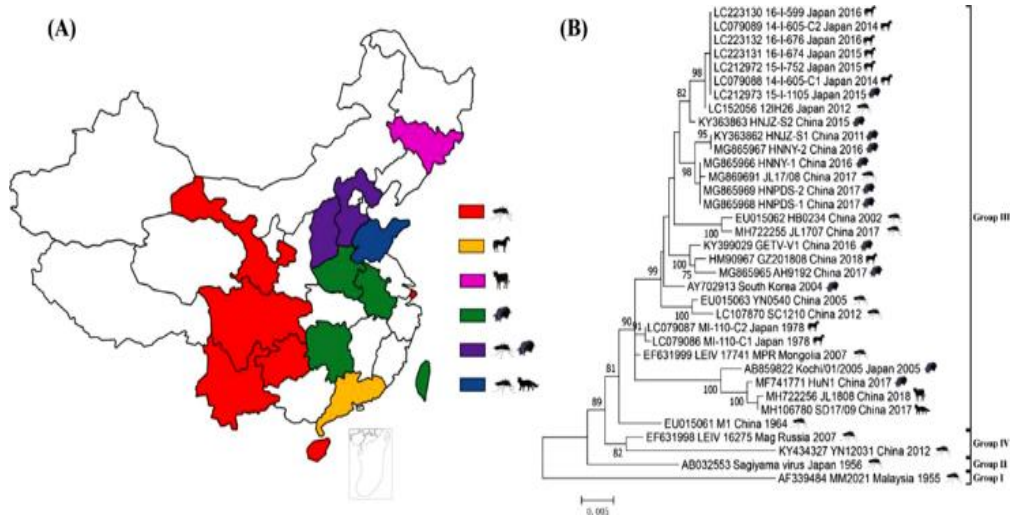

Figure 1.6. Genographic distribution of Getah virus in China and phylogenetic analysis

(A) The distribution of GETV in China's areas (B) The groups I–IV of GETV in different species are classified, respectively. (Pictures took from with permission from Lu *et al*, 2020)

Hunan (Yang *et al.* 2018), Guangzhou, in horses in Guangzhou (Lu *et al.* 2019), in Northeastern China (Liu *et al.* 2019) in cattle and blue foxes in Jilin province (Shi *et al.* 2019). Epidemiological investigations in mosquitoes also discovered GETV in Gansu, Shanghai, Hebei, and Hubei province (Lu *et al.* 2020) (**Figure 1.6A**). Even with the limited reported cases, the wide geographic distribution in China suggests ongoing circulation of GETV in the natural environment. GETV strains discovered in China belong to genotype III, except for LEIV/16275/MAG (isolated in 2010), and YN12031 (isolated in 2012), which are classified into lineage IV (Lu *et al.* 2020) GETV is transmitted between individual hosts by mosquito bites, and infected vertebrates can act as a reservoir for virus amplification. The large host range of GETV suggest there may be a number of potential reservoirs within the environment, which could account for outbreaks and possible co-infections with other mosquito-transmitted diseases (**Figure 1.6**). GETV therefore poses a significant future risk. In general, emerging mosquito-transmitted viruses in the swine industry may have potential risks not only for veterinary but also for public health. For instance, the major cause of human viral encephalitis, Japanese encephalitis virus (JEV), uses pigs as intermediate hosts (Ladreyt *et al.* 2020). JEV infected pigs have been regarded as the main amplifying host for JEV maintenance in nature. Arbovirus co-infections have been discovered worldwide (Tajima *et al.* 2014, Fang *et al.* 2018) and are becoming a serious concern for public health. The emerging infections of GETV in pigs increase the possibility of co-infection with for instance JEV.

For now, the knowledge on GETV is quite limited and there is only one GETV vaccine on the market (Nisseiken, Tokyo, Japan), derived from the Japanese GETVMI-110 horse isolate of 1978 (Nemoto *et al.* 2015). By mixing this strain in inactivated form with the inactivated Japanese encephalitis virus (JEV), it is used to protect racehorses against JEV and GETV-related disease (Bannai *et al.* 2016). However, the traditional approach of making vaccines by inactivating viruses, has an inherent risks of virus spill over during manufacture, and the occurrence of outbreaks of GETV in horses in Japan in recent years (Nemoto *et al.* 2015, Kuwata *et al.* 2018), even after immunization, suggest that these inactivated vaccines might not provide enough protection against new GETV strains in circulation. More recently, researchers in Australia demonstrated that the JEV/GETV vaccine was protective only by immunization with two shots using each time 20 ug of antigen (Rawle *et al.* 2020). For vaccination procedures for horses and pigs, it needs to be determined how much antigen is optimal, but it becomes clear that there is a need for a safe and more efficacious GETV vaccine. Preferably, such a vaccine is produced via a methodology that can easily be adapted to new strains if needed. In this study, we aim to develop a new GETV vaccine candidate that after thorough testing can be furtherly developed to control GETV and reduce the risks of GETV co-infections.

1.5 Scope of the thesis

The research presented in this thesis is focusing on emerging animal viruses, that cause substantial economic losses and raise public concerns during outbreaks in China. Moreover, this study focuses on the molecular mechanisms of innate immune responses against such viruses, which is crucial to understand virus-related pathogenesis and will help in the development of prevention strategies, which may limit the virus spread. Furthermore, applications to develop virus-like particle vaccine candidates are also an important part of this thesis.

Several recently emerging animal viruses from different mammalian species will be focused on in this thesis, which include PPRV, RHDV2 and GETV. The first sections of this thesis will concentrate on understanding molecular mechanisms of virus-host interactions of the emerging animal virus PPRV, which has caused significant economic losses; in **Chapter 2**, to better understand PPRV-induced immune suppression, we will characterize the caprine Mitochondrial antiviral-signaling protein (MAVS) from goat-derived cell lines and characterize its abilities as a RIG-I interacting type I IFN inducer. In **Chapter 3**, to better understand the virus-induced infection and immune response, we will focus on an interferon-stimulated gene (ISG), called BST2, and study its ability to inhibit PPRV budding. Moreover, as the molecular mechanisms which determine PPRV infection are largely unknown, the antiviral ability of BST2 will help to understand PPRV-induced innate immunity. Meanwhile, we characterized the novel Dutch RHDV2 strain, and in **Chapter 4**, by using baculovirus expression system (BEVS), we aim to develop a VLP-based vaccine to further be applied against RHDV2 in the rabbit industry. In **Chapter 5**, a bivalent VLP vaccine bearing both classic RHDV(GI.1) and RHDV2(GI.2) VP60 proteins is made and its immunogenicity in rabbits is analysed. In **Chapter 6**, based on the recent outbreaks of GETV in pigs in China, we will develop a VLP vaccine candidate using the same BEVS technology as for the RHDV2 vaccine.

All the work presented in this thesis is to gain a deeper understanding of virus-host interactions at the molecular level and also to apply the research results into practice by generating effective vaccine candidates for the veterinary industry. As indicated above, this thesis deals with various aspects of emerging animal viruses, from the beginning of virus discovery to deep understanding of the molecular mechanism and how this knowledge can be applied for future veterinary virus control. **Chapter 7** summarizes and integrates the major findings of this thesis research.

Chapter 2

Caprine MAVS Is a RIG-I Interacting Type I Interferon Inducer Downregulated by Peste des Petits Ruminants Virus Infection

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Caprine MAVS Is a RIG-I Interacting Type I Interferon Inducer Downregulated by Peste des Petits Ruminants Virus Infection

Abstract: The mitochondrial antiviral-signaling protein (MAVS, also known as VISA, IPS-1, or CARDIF) plays an essential role in the type I interferon (IFN) response and in retinoic acid-inducible gene I (RIG-I) mediated antiviral innate immunity in mammals. In this study, the caprine MAVS gene (caMAVS, 1566 bp) was identified and cloned. The caMAVS shares the highest amino acid similarity (98.1%) with the predicted sheep MAVS. Confocal microscopy analysis of partial deletion mutants of caMAVS revealed that the transmembrane and the so-called Non-Characterized domains are indispensable for intracellular localization to mitochondria. Overexpression of caMAVS in caprine endometrial epithelial cells up-regulated the mRNA levels of caprine interferon-stimulated genes. We concluded that caprine MAVS mediates the activation of the type I IFN pathway. We further demonstrated that both the CARD-like domain and the transmembrane domain of caMAVS were essential for the activation of the IFN-β promotor. The interaction between caMAVS and caprine RIG-I and the vital role of the CARD and NC domain in this interaction was demonstrated by co-immunoprecipitation. Upon infection with the Peste des Petits Ruminants Virus (PPRV, genus Morbillivirus), the level of MAVS was greatly reduced. This reduction was prevented by the addition of the proteasome inhibitor MG132. Moreover, we found that viral protein V could interact and colocalize with MAVS. Together, we identified caMAVS as a RIG-I interactive protein involved in the activation of type I IFN pathways in caprine cells and as a target for PPRV immune evasion.

2.1 Introduction

The induction of the interferon (IFN) response is vital for vertebrate hosts to counteract invading viruses and microbial pathogens. In general, cytoplasmic pathogen-associated molecular patterns (PAMPs), such as dsRNA molecules generated during viral infection, are sensed by host pattern recognition receptors, which include toll-like receptors (TLRs), and retinoic-acid inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) (Kawai and Akira 2009). Once invading pathogens are recognized, these receptors activate downstream signal transduction pathways.

RLRs constitute a critical group of intracellular viral RNA sensors that share similar structures and functions: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and Laboratory of Genetics and Physiology 2 (LGP2). RLRs contain an ATP-dependent RNA helicase domain that is required for double-stranded RNA binding and ATP hydrolysis (Takeuchi and Akira 2009). RLRs share the ability to detect distinct viral RNA structures as it has been shown for many RNA viruses, including retrovirus, influenza virus and Sendai virus, and for some DNA viruses, such as Epstein–Barr virus, and adenovirus (Wilkins and Gale 2010). RIG-I and MDA5 each possess two N-terminal tandem caspase activation and recruitment domains (CARDs) that mediate the signaling to Mitochondrial Antiviral Signaling protein (MAVS), leading to the activation of IFN-mediated antiviral immunity. LGP2, on the other hand, lacks CARDs and is thought to have a regulatory function (Yoneyama and Fujita 2010).

MAVS functions as the central signaling protein in the RLR-mediated innate immunity pathway and signals to downstream cytokine production, including type I IFN and ultimately IFN-stimulated genes (ISGs). MAVS is also referred to in the literature as IPS-1, VISA and CARDIF (Kawai *et al.* 2005, Meylan *et al.* 2005, Seth *et al.* 2005, Xu *et al.* 2005), and orthologs have been found in several vertebrate species such as mouse (Liu *et al.* 2014), tree shrew (Xu *et al.* 2015), cats (Wu *et al.* 2016), and goose (Sun *et al.* 2019), but also in mollusc (Huang *et al.* 2017). The deletion of MAVS has been shown to significantly reduce the levels of antiviral and other pro-inflammatory cytokines normally induced by virus infection (Deng *et al.* 2015). The central role of MAVs in host antiviral immunity is further illustrated by the fact that double-knockout mice (MAVS-/-) are highly susceptible to multiple RNA virus infections (Sun *et al.* 2006).

Structurally, MAVS contains an N-terminal CARD-like region, a Proline-Rich region (PRR), and a C-terminal transmembrane (TM) domain, of which the CARD-like and TM domains have been demonstrated to be essential for MAVS signalling in human cells (Seth *et al.* 2005).

Initially, MAVS was reported to predominantly localize in mitochondria, where it maintains the stability and function of these organelles (Seth *et al.* 2005). In addition, MAVS was detected in peroxisomes. Upon viral infection, peroxisomal MAVS provides short-term protection by rapidly inducing IFN-independent expression factors, whereas mitochondrial MAVS has been associated with the activation of a more stable antiviral response involving IFN production (Dixit *et al.* 2010). During viral infection, the cellular localization of MAVS may change. It has been shown that, following infection with several RNA viruses, mitochondrial-associated endoplasmic reticulum membrane (MAM) was recruited by RIG-I, which then bound to MAVS, and in that way, coordinated the cellular localization of MAVS to regulate the activation of an effective antiviral response (Horner *et al.* 2011). Functional differences among mitochondria MAVS, peroxisomal MAVS, and MAM localized MAVS suggest that the MAVS protein could recruit distinct cellular components, depending on its cellular localisation and as a consequence may form a powerful network during the innate immune response (Weinberg *et al.* 2015).

Viruses have been shown to apply different strategies to counteract MAVS-mediated signaling, and as a consequence, evade the associated innate immune responses. An example is the hepatitis C virus (HCV) NS3/4A protease, which localizes between the MAM and mitochondria, and particularly targets MAVS that is associated with MAM. Since MAM-localized MAVS has been recognized as being capable of transducing RIG-I signalling, HCV NS3/4A protease is predicted to interrupt RIG-I mediated signalling by cleaving MAVS (Horner *et al.* 2011).

Thus, MAVS is a central protein in the induction of type I IFNs in a number of mammals, but data for goats are missing thus far. Infections with the morbillivirus, Peste des Petits Ruminant Virus (PPRV, family *Paramyxoviridae,* genus *Morbillivirus*) are characterized by conjunctivitis, rhinitis, pneumonia and stomatitis, and enteritis, and reach mortalities as high as 90% in small ruminants. PPRV replication occurs in the respiratory tract, where antigen-presenting cells (APC) (or dendritic cells (DC), macrophages and monocytes) are infected and lymphocytes play a major role during immune responses (Rojas *et al.* 2016). PPRV causes immunosuppression in its natural hosts. The virus counteracts the IFN response and signalling pathways. Even though IFNs and ISGs are important during innate immune response, PPRV controls the induction of type I IFNs (Sanz Bernardo *et al.* 2017). To better understand the pathogenesis of PPRV infection and other caprine-related infectious diseases we aimed to characterize the function of caprine (ca) MAVS as a central protein during the innate immune response in caprine cells and analyse its role in type I IFN induction. Furthermore, we

investigated whether PPRV infection affected caMAVS levels and whether specific viral proteins interacted with caMAVS. We found that the viral protein V could interact and colocalize with MAVS. By expanding our insight in the mode of action of PPRV in the suppression of innate immunity, we aim to contribute to the control of viral diseases of goats, in particular in PPRV and related morbillivirus infections.

2.2 Materials and methods

Cloning and sequence analysis of caprine MAVS

Total RNA from immortalized caprine endometrial epithelial cells (EECs) was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA concentration, as well as the 260/280 and 260/230 ratio, were measured by spectrophotometer (Biotek, Winooski, VT USA). Then cDNA was generated by using M-MLV reverse transcriptase (Promega, USA) with random primers (Invitrogen). Primers (Table.s1) were designed for amplification of caMAVS and caRIG-I based on the predicted sequence in GenBank (accessions XM_018057391.1 and XM_005683566.3). PCR amplification was performed in a reaction mixture of 50 μL containing TransStart® FastPfu Fly DNA polymerase (Transgen Biotech, Beijing, China). The PCR products were purified on spin columns from Sangon Biotech (Shanghai, China) and cloned to pEASY®-BLUNT Zero Vector (Transgene Biotech, China). Inserts of five positive clones were sequenced (Sangon Biotech, Shanghai, China). To analyze the evolution of caprine MAVS, a phylogenetic tree was constructed based on the DNA coding sequence of MAVS proteins from 19 animal species selected as listed in Table.s2. The phylogenetic tree was built with the neighbor-joining method with 1000 bootstrap replications by MEGA5.0. The amino acid sequence of caMAVS and those of MAVS from other species, such as human, rabbit, mouse, and sheep, were aligned using the MEGA 5.0 program and edited by the MegAlign package of Lasergene.

Construction of expression plasmids

Expression plasmids of Myc-tagged caMAVS and caRIG-I were constructed by inserting the full-length open reading frames (ORFs) into the pCMV-MYC vector with MYC fused to the N-terminus (Clontech, Takara Bio, Kusatsu, Shiga, Japan). HA and 3*Flag-tagged caMAVS constructs were cloned with Infusion technology (Vazyme Biotech Co., Ltd., China) into pCMV-HA (N-Terminal) (Clontech, Japan) and p3*FLag-CMV-10 (Sigma-Aldrich, USA) vectors, respectively. Truncated forms of caMAVS, which lacked the CARD domain (residues 10–77), PRR domain (residues 78–199), Transmembrane (TM) domain (residues 495–517), or Non-Characterized (NC) domain (residues 201–494) were constructed by using Infusion

technology or overlapping extension PCR, as described previously (Zhu *et al.* 2018). Flag-tagged human MAVS (huMAVS) plasmid was used as a control and was kindly provided by Dr. Yuzhi Fu from the Wuhan Institute of Virology, Chinese Academy of Sciences. The PRDIII/I-luc and PRDII-Luc plasmids were purchased from Beyotime. pRL-TK (Promega Company, USA) expressing Renilla luciferase was used as endogenous transfection control to allow normalization.

Cell Culture, transfection, and virus infection

Caprine EECs were kindly provided by Prof. Yaping Jin from the Northwest Agricultural University of China and were grown in Dulbecco's Modified Eagle Medium/F-12 (Thermo Fisher, Waltham, MA, USA) with 10% fetal bovine serum (Gibco, Thermo Fisher, Waltham, MA, USA). HEK-293T cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (Gibco, USA). Vero-SLAM cells expressing SLAM receptors were generated at the Shanghai Veterinary Research Institute and grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (Gibco, USA). Vero-SLAM cells support efficient replication of PPRV and the downstream signaling from caMAVS is functional. In a previous study, we demonstrated that nucleolin could inhibit PPRV replication in Vero-SLAMs (Dong *et al.* 2020). All cells were grown with 1% antibiotics of penicillin and streptomycin (P/S) at 37 °C and with a 5% atmospheric CO_2 concentration. For transfection experiments, cells were seeded in 6-well plates (NEST Biotechnology, Jiangsu, China) overnight, and transfected with indicated expression plasmids and 36 h post transfection, cells were harvested and used for further experiments.

The PPRV vaccine strain, Nigeria/75/1 (GenBank: HQ197753), was obtained from the Shanghai Veterinary Research Institute cell culture collection and amplified on Vero-SLAM cells. After experiments were completed, all experimental materials were autoclaved at 120 °C for 30 min to kill PPRV. The laboratory was confirmed to P2 (BSL-2) laboratory requirements and all experiments were completed under P2 laboratory conditions.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to measure the mRNA levels of selected ISGs, including caprine RSAD2 (caRSAD2), caprine OASL (caOASL2), caprine IFITM3 (caIFITM3), caprine MX1 (caMX1). The caprine GAPDH was used as endogenous control and for normalization of the data. Gene-specific primers were designed by using Primer3 software or according to previous reports (Wani *et al.* 2019) and then synthesized by Sangon Biotech, China. Total RNA was extracted by using the TRIzol Reagent (Invitrogen), and cDNA synthesis was performed using

M-MLV reverse transcriptase (Promega, USA) with Oligo dT primer or Random primers (Invitrogen). qRT-PCR was performed using SYBR Premix Ex Taq reagents (Takara, Dalian, China) and StepOne Real-Time PCR Detection System (Thermo Fisher, USA). Fold expression was presented to show the relative abundance of the mRNAs by using the comparative CT (ΔΔCT) method. All experiments were performed in triplicate and all experiments were carried out with at least three repeats.

Reporter plasmids and luciferase assays

HEK-293T cells or EEC Cells (1.25×10^5) were cultured in 24-well plates. Transfections were performed with different amounts of expression plasmid for the full-length caMAVS to compare the activity of stimulating IFN-β-luc, and the same amount of full-length or mutant caMAVs to compare their difference in stimulating IFN-β-luc. Each caMAVS plasmid was co-transfected with the same amount of luciferase reporter plasmid and the pRL-TK plasmid, which is used for data normalization. Transfection with different amounts of expression plasmid for the full length caMAVS (250 ng, 500 ng, 1 μg) was used to compare the induction of IFN-β-luc. The same amount of DNA was used to compare full-length caMAVS with the deletion mutants. The same amount of pRL-TK plasmid (40 ng/well) for data normalization was co-transfected along with 500 ng of firefly luciferase reporter constructs (IFN-β-luc or PRD-III/I-luc and PRD-II-luc). Cells were collected at 24 h post-transfection. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay kit (Promega, USA) according to the manufacturer's protocols. All reporter assays were repeated at least three times. The luciferase activity of each sample was normalized to the Renilla luciferase activity and all data were expressed as mean relative luciferase with standard deviation (SD). Furthermore, all experiments were performed at least three times independently.

Confocal immunofluorescence microscopy

To investigate the subcellular localization of the caprine MAVS protein, Vero-SLAM cells were plated on 15 mm cover glasses (NEST Biotechnology, China) and were transfected with Flag-tagged caMAVS (full-length or mutant versions) or together with the mitochondrial indicator plasmid pDsRed2-Mito (Addgene plasmid # 55838) at 60~70% confluence. Vero-SLAMs were selected for transfection instead of EECs because of the much higher transfection efficiency to achieve sufficient levels of simultaneous expression of two plasmids in one cell. The cells were harvested at 24 h post-transfection and fixed with 4% paraformaldehyde in PBS, and subsequently blocked with 5% Bovine Serum Albumin (BSA) in PBS at 37 °C for one hour. Immunofluorescence analysis was performed with primary antibodies (1:200, see next paragraph) directed against the tags and Alexa Fluor-488-conjugated goat anti-mouse antibody

(Invitrogen, USA) or Alexa Fluor-633-conjugated goat anti-rabbit antibody (Invitrogen, USA) (1:1000) as secondary antibodies, respectively. The cell nucleus was stained with DAPI (Beyotime, China) for 5 min and then washed four times with PBS-Tween. The images were taken with a Zeiss LSM880 confocal microscope and analysed by Zen Blue software (Zeiss, Germany).

Immunofluorescence and western blot analysis

Anti-PPRV-N monoclonal antibody directed against the nucleocapsid protein (N) of PPRV was generated by GenScript (Nanjing, China). Flag-tag primary antibodies were purchased from Sigma (USA). Myc-tag primary antibodies were purchased from Santa Cruz (USA), HA-tag primary antibodies were purchased from Abcam (UK). GAPDH and β-actin antibody were purchased from CoWin Biosciences (China). At 24–36 h after transfection with expression plasmids (caMAVS, huMAVS, or truncated caMAVS), cells were washed thoroughly with cooled PBS and lysed in cell lysis buffer containing a protease inhibitor cocktail (Merck Millipore, Darmstadt, Germany). The lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Whatman, Maidstone, UK). The membranes were then blocked at 37 °C with 5% skimmed milk for an hour and incubated with specific primary antibodies (1:1000) overnight at 4 °C and then subjected to horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research Laboratories) (1:10000) for 1 h at room temperature. The bound secondary antibodies were visualized with enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Pittsburgh, PA, USA). For the virus infection experiment, cell lysates were harvested with indicated time intervals after infection and generated by adding 5×loading buffer to the collected cells and separated by SDS-PAGE and subjected to Western blot analysis. By using indicated primary antibodies which recognized different cellular proteins downstream of the PPRV-induced RIG-I pathway, including MAVS (Cell Signaling Technology, Danvers, MA, USA), TBK1 (CST, USA), P-TBK1(CST, USA), the different protein expression levels were analyzed in the presence or absence of a PPRV infection. Fis-1(CST, USA), which is a component of a mitochondrial complex, was used as indication for mitochondria.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed to confirm the interaction between caRIG-I and caMAVS, and to determine whether PPRV protein V (PPRV-V) interacts with caMAVS. HEK-293T cells were seeded on 100 mm dishes and transfected with 5 μg of the Myc-tagged caRIG-I encoding plasmid together with 5 μg of HA-tagged wild-type (WT) or truncated caMAVS (caMAVS-ΔCARD, caMAVS-ΔPRR, caMAVS-ΔNC or caMAVS-ΔTM) encoding

plasmids. Alternatively, Flag-V was co-transfected together with Myc-caMAVS. Then, 24 h post-transfection, cells were lysed with cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% NP-40) containing protease inhibitors (Merck-Millipore). Lysates were centrifuged at $12,000{\times}g$ for 10 min to remove the cell debris. Precipitation was performed overnight at 4 °C with anti-Myc mAb conjugated to Resin Agarose beads (Thermo Fisher Scientific). The beads were washed four times with lysis buffer and the bound proteins were eluted with SDS loading buffer by heating for 10 min. Fifty microliter volumes of cell lysate were eluted and then subjected to Western blot analysis using specific antibodies.

Statistical analysis

All statistical analysis was performed using GraphPad Prism Version 5. Data were expressed as means ± standard deviations. A one-way ANOVA test was used to compare multiple groups (>2). p values < 0.05 were considered to indicate significant differences.

2.3 Results

Cloning and sequence analysis of caprine MAVS Gene

A 1566 bp-long caMAVS cDNA sequence was amplified by conventional RT-PCR. The nucleotide sequence of *Capra hircus* MAVS (caMAVS) has been submitted to GenBank (accession number: MT501722). The predicted 522 amino acid caMAVS protein shares the highest (98.1%) amino acid similarity with the predicted sheep MAVS (XM_015099722.2). A neighbor-joining phylogenetic tree was constructed based on selected cDNA sequences of MAVS from several species (**Figure 1A**). The phylogenetic tree contains two major branches of mammalian MAVS, which are separated from avian and fish MAVS. In the MAVS amino acid sequence alignment, it was shown that caMAVS has the same structural domains as MAVS found in other mammalian species (**Figure 2.1B**).

Schematic representation and identification of caprine MAVS and its Mutants

Online structural analysis software (https://www.expasy.org/, 2020) predicts that caMAVS is a membrane-bound protein with a single C-terminal transmembrane (TM) domain. Based on the comparison with human MAVS and the predicted domains therein, in-frame deletion mutants of caprine MAVS were constructed to analyze the role of the CARD-domain, as well as the PRR, NC, and TM domains (**Figure 2.2A**). Western blotting was used to confirm the expression of caMAVS and its mutants before Luciferase and immunofluorescence assay (IFA) were conducted (**Figure 2.2B**).
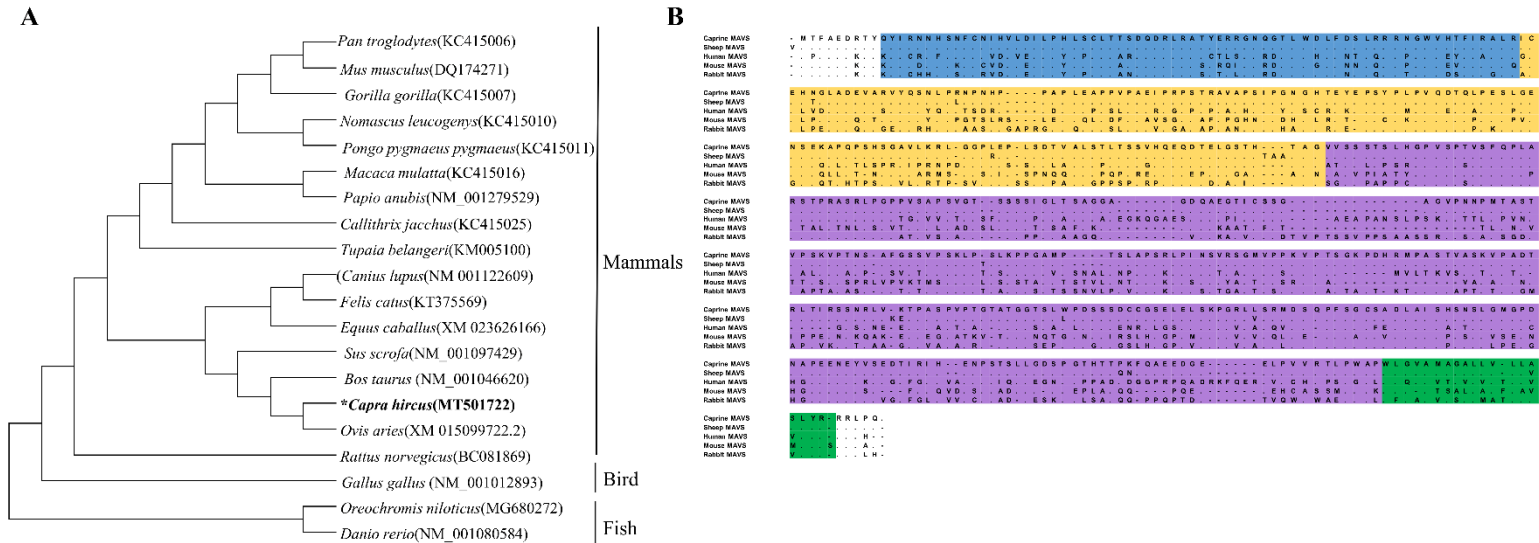
Figure 2.1. Identification of caprine MAVS.

(A) Multiple sequences of MAVS from different species were selected to construct the phylogenetic tree. The tree was constructed by using the neighbour-joining method by MEGA5.0 and the scale bar is 2. The amino acid sequences of MAVS used in this program were listed in Table s2. The asterisk indicates caprince MAVS (B) Amino acid alignments of caprine, sheep, human, mouse and rabbit MAVS. Sequence alignments were performed and edited with the Lasergene MegAlign program. Structural caspase activation and recruitment domain (CARD), proline-rich region (PRR), non-characterized (NC) and C-terminal transmembrane (TM) domains are indicated in blue, yellow, purple, and green, respectively.
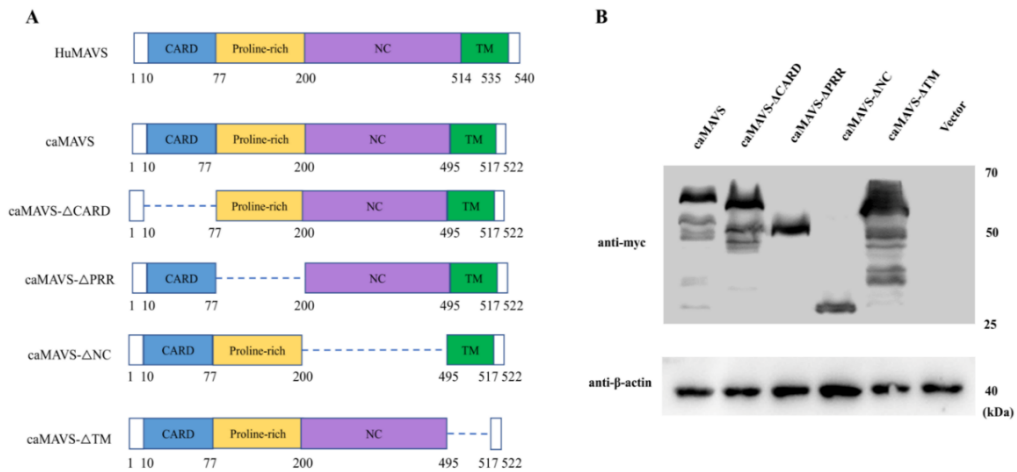
Subcellular localization of caprine MAVS

Figure 2.2. Schematic diagram of caprine MAVS mutants and their expression.

(**A**) Schematic representation of caprine MAVS was presented based on the conserved points according to human MAVS. The CARD, PRR, NC and TM domains are represented in blue, yellow, purple, and green, respectively. The schematic representation is also used for constructing truncated mutants. The indicated numbers represent amino acid positions. (**B**) Western blot analysis of the expression of caMAVS and its mutants by transfection with caMAVS and mutant plasmids in HEK-293T cells.

Vero-SLAM cells were transfected with expression plasmids encoding caMAVS and mutants and subjected to IFA to determine the effect of the deletions on the subcellular location of caMAVS. As shown in **Figure 2.3A**, full-length caMAVS was abundantly present in the cytoplasm and had a punctate localization. The mutants caMAVS-△CARD and cAMAVS-△PPR also had a punctate localization like the full-length caMAVS. However, when the TM or the NC domains were deleted, the mutant proteins lost their punctate localization and were dispersed over both the cytoplasm and the nucleus.

As the distribution of caMAVS to mitochondria is vital for its role in signaling, the subcellular location of caMAVS and the various mutants was compared with that of a co-transfected pDsRed2-Mito marker designed for fluorescently labeling mitochondria. As shown in **Figure 2.3B**, full-length MAVS co-localized with the mitochondrial marker, as well as caMAVS-△CARD and caMAVS-△PRR. For caMAVS-△TM the mitochondrial localization was disrupted, suggesting that the mitochondrial colocalization was dependent on the TM domain. The caMAVS-△NC mutant, from which a large (295 aa) central part of caMAVS was deleted, did also not colocalize with the mitochondrial marker.
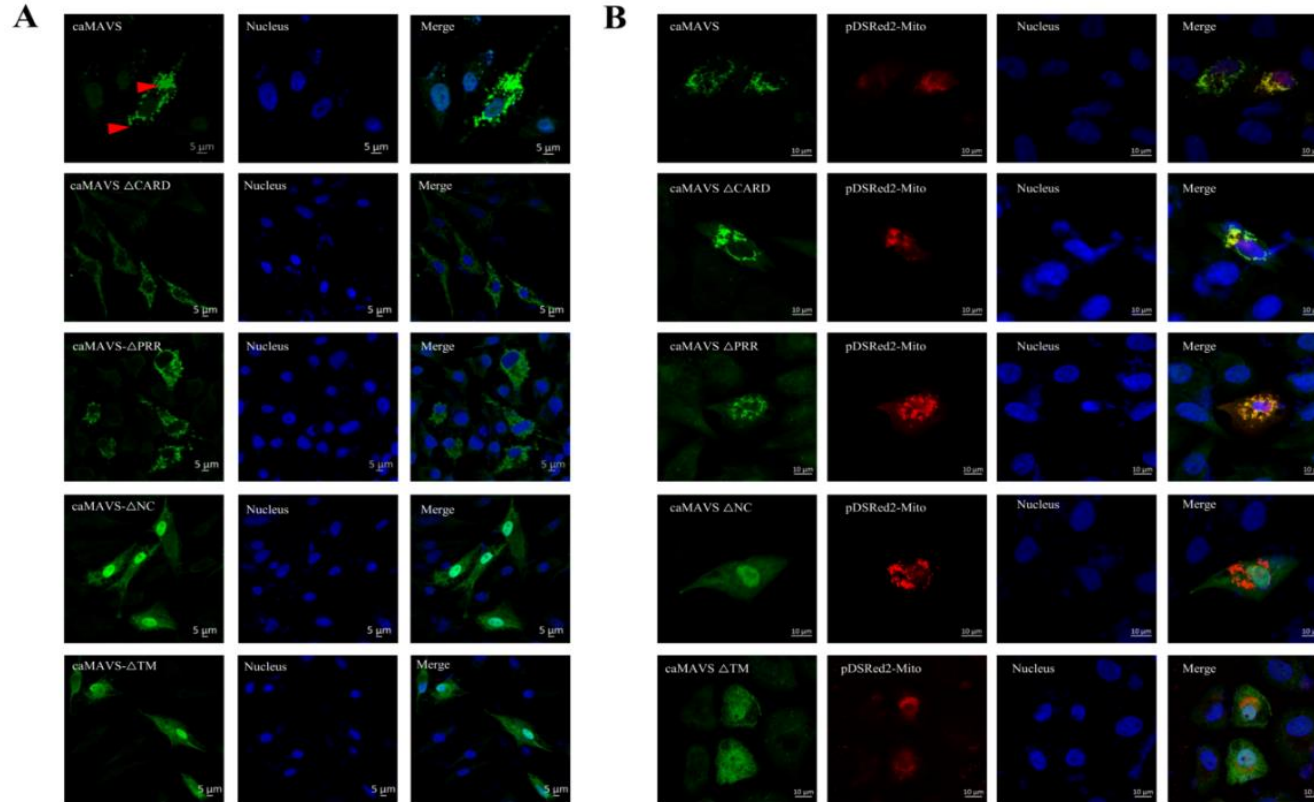
Figure 2.3. Localization study of caMAVS and its mutants.
 (**A**) Expression plasmids encoding caMAVS and its different deletion mutants were transfected into Vero-SLAM cells. At 36 h post transfection, cells were fixed and stained with Myc primary antibodies and secondary Alexa-488 labeled antibodies. (**B**) To verify the colocalization of caMAVS (green) and Mitochondria (red), expression plasmids encoding Myc tagged caMAVS and its mutants were transfected together with pDsRed2-Mit. At 36 hpt, cells were fixed and stained as in panel A.

Overexpression of caMAVS-Induced IFN-β via the NF-κB and IRF-3-Mediated Pathways
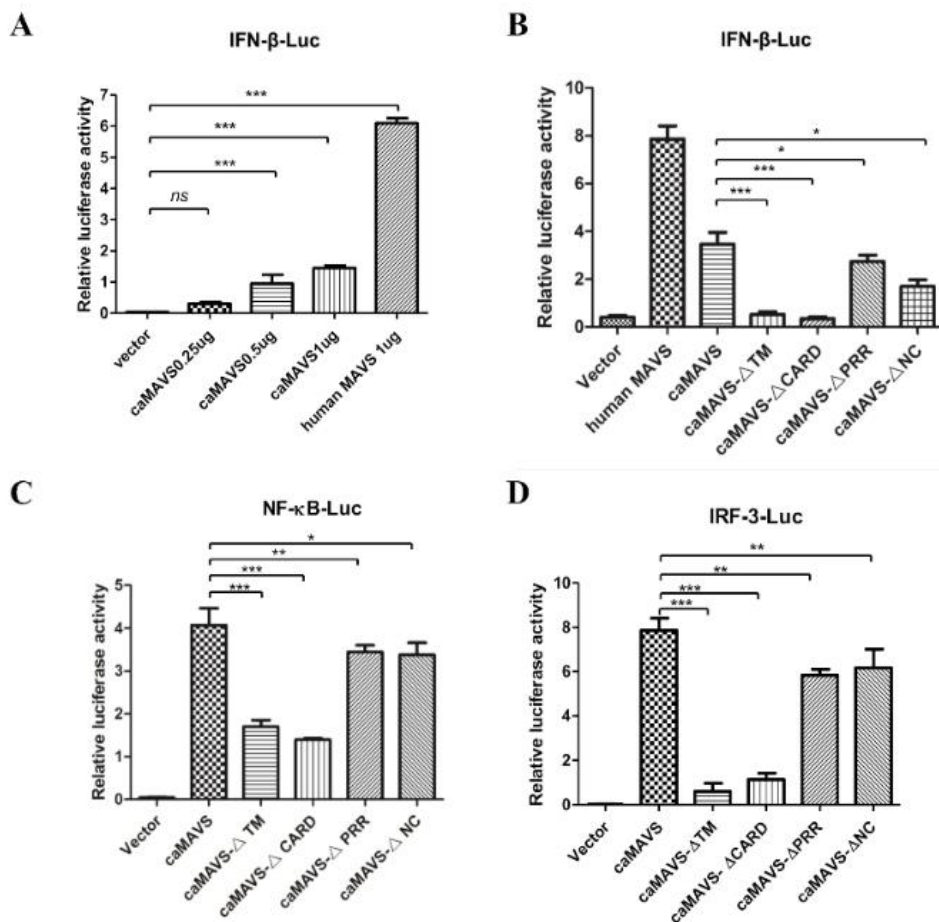

Figure 2.4. Overexpression of caMAVS induced IFN-β via the NF-κB and IRF-3-mediated pathways.

(**A**) HEK-293T cells were transfected with increasing amount of caMAVS, IFN-β-Luc together with endogenous control pRL-TK Plasmid (40 ng/well). (**B**–**D**) HEK-293T cells were transfected with caMAVS or its mutants (500 ng) along with PRDII-Luc (NK-κB-luc), PRDI/III-luc (IRF3-luc) or IFNβ-Luc together endogenous control pRL-TK Plasmid (40 ng/well). Human MAVS was used as a positive control. At 36 hpt, the HEK-293T cells were lysed, and Rluc and Fluc activities were evaluated using the Promega Dual-Luciferase Reporter Assay System. Furthermore, all the experiments were performed at least three times to ensure the results consistency (The data represent the mean ± SD of three independent experiments. One-way ANOVA was used for statistical analysis; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

As one of the most important adaptor molecules, MAVS is hypothesized to be critical for virus-induced type I IFN expression in goats, as a link between RLRs and downstream NF-κB and IRF3 activation (Seth *et al.* 2005). To confirm that caMAVS is functional in the type I IFN pathway, HEK293T cells were co-transfected with plasmids expressing caMAVS and human IFN-β promoter-driven luciferase. All cells were also co-transfected with pRL-TK encoding Renilla luciferase to allow for data normalization. As expected, overexpression of caMAVS activated the IFN-β promoter in a dose-dependent manner (**Figure 2.4A**). However, the observed expression of luciferase was lower than with human MAVS, which acted as a positive control in this study.

The next experiment illustrated that caMAVS mutants lacking either the CARD or the TM domains were unable to activate the IFN-β promotor (**Figure 2.4B**), in line with previous analysis of mutants of feline MAVS (Wu *et al.* 2016). In the next experiment, we used a set of
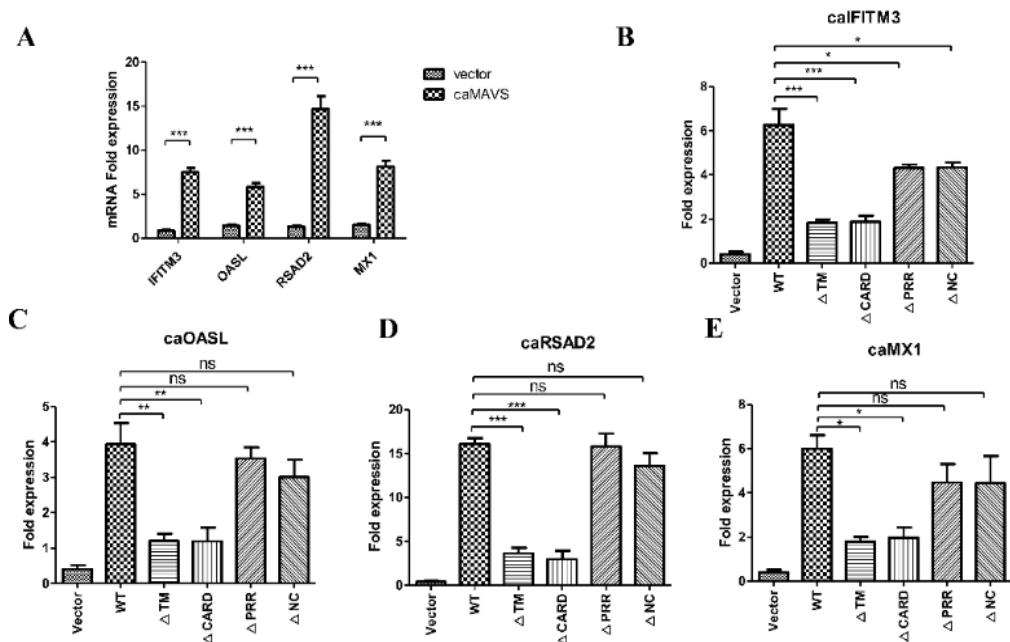

Figure 2.5. Evaluation of ISGs by overexpression of caMAVS and its mutants in EECs.

(**A**) EECs were transfected with either empty vector or caMAVS. The relative mRNA levels of selected caprine ISGs (IFITM3, OASL, RASD2, MX1) were analyzed by qRT-PCR. (**B**–**E**) EECs were transfected with either empty vector or caMAVS and its mutants. The relative mRNA levels of selected ISGs were analyzed by qRT-PCR. Data presented were from at least three independent experiments. Significance was analyzed by GraphPad prism 5.0 software with a one-way ANOVA test (One-way ANOVA was used for statistical analysis; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

luciferase report plasmids containing IRF-3 and NF-κB-binding motifs (PRDIII/I-Luc, referred to as IRF-3-Luc and PRDII-Luc, referred to as NF-κB-Luc) to further increase our understanding about caMAVS as an adaptor protein. HEK 293T cells were transfected with caMAVS, or mutant versions thereof, together with one of the luciferase reporter plasmids. The results demonstrated that overexpression of caMAVS, as well as the mutants caMAVS-△PRR and caMAVS-△NC, could activate NF-κB and IRF-3 pathways, while caMAVS-△TM and caMAVS-△CARD had lost this ability (**Figure 2.4C-D**). Together, the data demonstrated that caMAVS could activate the IFN-β promoter through both NF-κB and IRF-3 signaling.

caMAVS overexpression upregulates the mRNA level of caprine ISGs

IFN production results in the expression of IFN-stimulated genes (ISGs), which have an important role in morbillivirus antiviral innate immunity (Wani *et al.* 2019). To further establish the role of caMAVS in ISGs induction, the effect of overexpression of caMAVS or its mutants on the transcription of a number of ISGs (caIFITM3, caOASL, caRSAD2, caMX1) was explored by qRT-PCR. Transfection of caMAVS in EECs resulted in the upregulation of the mRNA expression levels of all tested ISGs (**Figure 2.5A**). Furthermore, the deletion of either CARD or TM domains significantly limited the induction level of these ISGs (**Figure 2.5B–E**). All findings demonstrated that the CARD and TM domains are both essential for the MAVS-medicated IFN production.

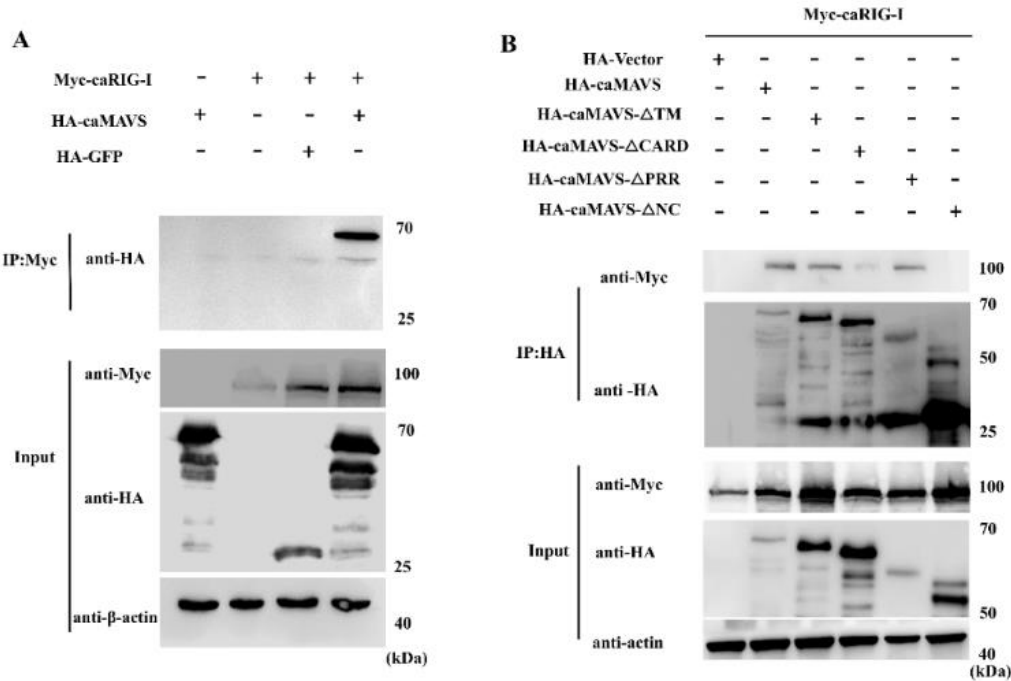**caMAVS interacts with caRIG-I through its CARD and NC Domain**

Figure 2.6. Identification of the interaction between caMAVS and caRIG-I by Co-IP.

(**A**) HEK-293T cells seeded in 10 cm dishes and were transfected with HA-tagged MAVS or HA-GFP together with (without) Myc-RIG-I when cells were at around 60% confluency, respectively. Cells were harvested after 24 h post transfection. Cell lysates were precipitated with anti-Myc mAb resin overnight at 4 °C and precipitated proteins detected with anti-HA and anti-Myc mAbs. β-actin was used as protein loading control. (**B**) HEK-293T cells were seeded in 10 cm dishes and were transfected with HA-tagged MAVS and its mutants together with Myc-RIG-I when cells were around 60% confluency. Cells were harvested after 24 hpt and cell lysates were precipitated with anti-HA mAb resin overnight at 4 °C and precipitated proteins were detected with anti-HA and anti-Myc mAb. β-actin was used as protein loading control.

To verify that caMAVS functions as the adaptor of RIG-I, the caprine RIG-I gene was amplified and subcloned into pCMV-Myc (Clontech, USA) and tested in co-immunoprecipitation assays. HEK293T cells were transfected with HA-caMAVS (or its mutants), together with Myc-caRIG-I, and cell lysates were analyzed at 36 h post transfection. Myc-caRIG-I was precipitated with anti-Myc conjugated resin beads and Myc-caRIG-I and HA-caMAVS were detected using anti-bodies against the Myc and HA-tags, respectively. As shown in **Figure 2.6A**, the protein samples were incubated with Myc antibody and then the elution was subjected for HA antibody check. The results confirmed the interaction between caRIG-I and full-length caMAVS.

Moreover, as shown in **Figure 2.6B**, the mutants with PPR or TM domains deleted still interacted with caRIG-I. On the other hand, the CARD domain deletion mutant displays a strongly reduced interaction with caRIG-I, indicating that the CARD domain of MAVS plays a key role in this interaction. Interestingly, also the NC domain mutant, which did not colocalize with mitochondria in the fluorescence studies also lost the ability to interact with caRIG-I.

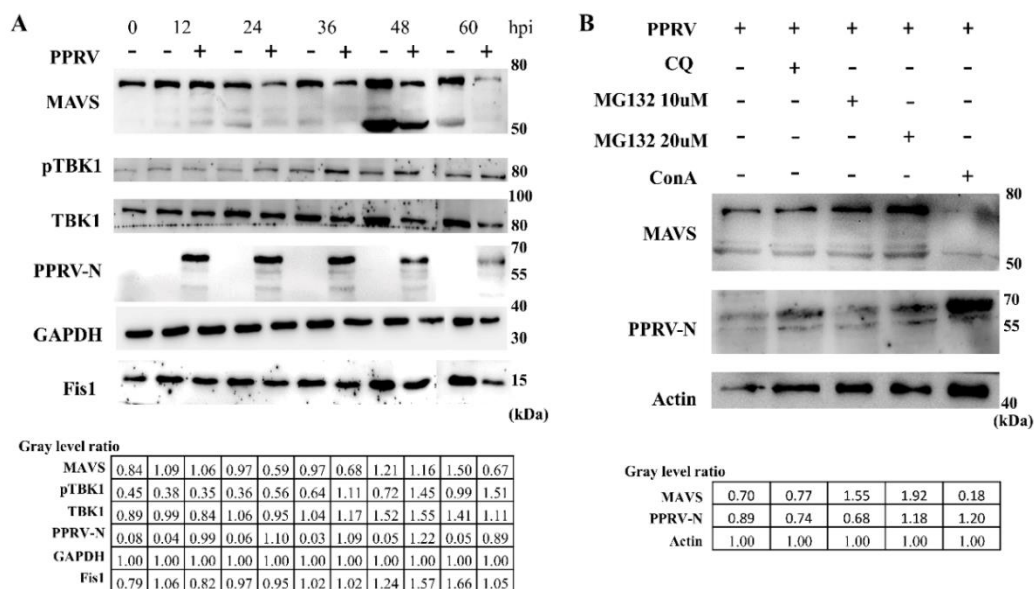**caMAVS response to PPRV infection in EEC Cells**

Figure 2.7. caMAVS is degraded through the proteasome pathway during PPRV infection.

(**A**) EECs were infected with the PPRV vaccine strain, Nigeria/75/1, at MOI 10. The relative protein level of several important molecules during viral infection was checked by WB. (**B**) Several inhibitors were used after infection of PPRV on EECs to demonstrate which pathway is involved in MAVS degradation. The relative level of individual proteins was quantified with respect to β-actin.

PPRV has been responsible for severe infectious disease in small ruminants, including goats and sheep. The virus caused significant economic losses in the goat and sheep industry and also threatens wildlife conservation (Baron *et al.* 2016, Dou *et al.* 2020). To determine where caMAVS functions as a key factor in the antiviral innate immune response to PPRV, EEC cells were infected with PPRV. Through screening for several proteins known to be important for the induction of innate immunity, we found that caMAVS was degraded after virus infection, as shown in **Figure 2.7A.** The endogenous protein expression level of the 70 kDa MAVS was

significantly decreased following infection with a high dose of PPRV. The 70 kDa MAVS was downregulated, while the expression level of a smaller (45–50 kDa) band reactive with caMAVS antibodies was upregulated at 48 h with/without virus infection for reasons unknown. A truncated MAVS isoform was also identified in other species but was unable to trigger IFN production (Brubaker *et al.* 2014). Furthermore, we observed that caMAVS mRNA levels remained relatively stable after PPRV infection (data not shown), which is consistent with other studies. In infections with other viruses, MAVS cleavage was a result of apoptosis-activated degradation (Scott and Norris 2008), autophagic degradation (Jin *et al.* 2017) or ubiquitin-proteasome pathways (Nakhaei *et al.* 2009). Several inhibitors have been widely used to elucidate which pathway is involved in MAVS cleavage. MG132 is a commonly used proteasome inhibitor, chloroquine (CQ) is a classic inhibitor of autophagy, and Concanavalin A (ConA) induces autophagy. In our study, we found that the proteasome inhibitor MG132 was able to rescue caMAVS expression to mock-infection levels, in contrast to the lysosome inhibitor chloroquine (CQ). ConA treatment markedly reduced MAVS expression levels. Overall, this suggests that caMAVS degradation during PPRV infection is dependent on the ubiquitin-proteasome pathway (**Figure 2.7B**).

PPRV V Protein interacts with caMAVS

To identify a putative viral protein responsible for caMAVS degradation in PPRV-infected cells, HEK-293T cells were co-transfected with viral protein-encoding plasmids V and Myc-caMAVS because Newcastle Disease Virus (NDV) V could target MAVS degradation to inhibit host type I interferon production (Sun *et al.* 2019). Clearly, caMAVS expression level was greatly reduced during co-transfection with PPRV-V (**Figure 2.8A**). Co-IP assays between with the flagged tagged V protein of PPRV and Myc-caMAVS demonstrated that PPRV-V and caMAVS co-immunoprecipitated, irrespective of which of the two proteins was targeted for pull-down, indicative of a (direct) interaction between these two proteins (**Figure 2.8B**). Furthermore, Flag-V co-localized with Myc-caMAVS and with the mitochondrial marker pDsRed2-Mito (**Figure 2.8C**) when overexpressed in Vero-SLAM cells, indicating that PPRV-V most likely acts on mitochondrial MAVS.

2.4 Discussion

PPRV is the causative agent of a highly contagious disease that affects both domestic and wild small ruminants (Baron *et al.* 2016). Except for farm animals, studies also reported the infection of PPRV lineage II in the Chinese water deer, *Hydropotes inermis* (Zhou *et al.* 2018) and recent outbreaks of PPRV have also been reported to occur in other wild animals in different countries

(Shahriari *et al.* 2019, Pruvot *et al.* 2020). PPRV appears to induce immune suppression during the acute phase of the disease (Rojas *et al.* 2014), which may favor the establishment of secondary infections with other pathogens. Recently, it was demonstrated that PPRV utilizes the viral Nucleoprotein (N) and Phosphoprotein (P) to inhibit interferon signalling by blocking the JAK-STAT Pathway (Li *et al.* 2019). Another study showed that the N protein inhibits IFN-
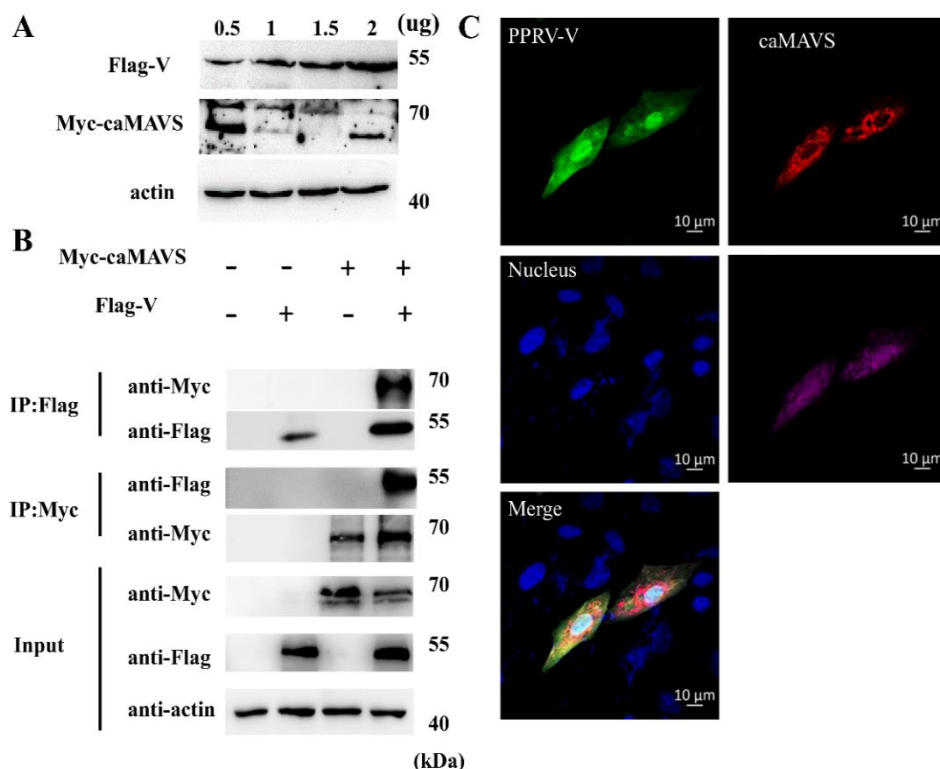

Figure 2.8. caMAVS interacts and colocalizes with PPRV viral protein V.

(**A**) Increasing amounts of plasmids encoding Flag-tagged PPEV protein V (Flag-V) (0.5 ug, 1 ug, 1.5 ug, 2 ug) were co-transfected with plasmid-encoding Myc-tagged caMAVS (2 ug) in 6-well plates. PPRV V and caMAVS were detected by Western blot using β-actin as loading control. (**B**) Co-IP was performed to identify interaction between PPRV V and caMAVS. Flag-V and Myc-caMAVS plasmids were transfected into HEK-293T cells independently or together. Cell lysates were subjected to immunoprecipitation with anti-Myc or anti-Flag mAb overnight at 4 °C. The expression of the transfected proteins was determined by Western blotting by anti-Flag and anti-Myc mAbs, respectively. (**C**) The colocalization study was performed by transfection of Myc-caMAVS (red), Flag-V (green) together with pDsRed2-Mito (pink) and subjected for IFA with anti-Flag and anti-Myc mAbs as primary antibodies.

β production by interacting with IRF3 (Zhu *et al.* 2019); however, up to now, studies on PPRV were mostly performed in the human cell line 293T, while humans are no natural PPRV host.

We hypothesized that PPRV may also inhibit other immune signaling pathways to effectively evade innate immune responses. Interference with the RIG-I-like receptor (RLR) mediated pathways, for instance, might lead to a reduction in the production of ISGs. The activity of this pathway during a PPRV infection was studied by analysing the MAVS adaptor protein in caprine cells. MAVS is crucial in the RIG-I/MDA-5 sensing of various RNA viruses and has been characterized in several species as a key factor in RLR pathways. In our study, we successfully cloned the MAVS homolog from caprine endometrial epithelial cells (EECs), a host cell line for PPRV. By sequence comparison, we found that caMAVS shares conserved structural domains (CARD-PRR-NC-TM) with MAVS of other mammalian species and that caMAVS shares the highest identity with sheep MAVS (98.1%). Further analysis using transiently expressed MAVS mutants mapped individual domains of caMAVS responsible for its distribution in Vero-SLAM cells, which have been described as a useful cell model to study PPRV infection (Dong *et al.* 2020). The full-length caMAVS protein was localized in mitochondria, while caMAVS lacking the TM domain lost the mitochondrial localization and was found in both cytoplasm and nucleus. Further, by using a luciferase-based assay, caMAVS lacking the deleted CARD domain did not induce IFN-β reporter activity and did not activate downstream ISGs. Interestingly, caMAVS carrying a deletion of the NC domain, which has not been studied by others before, also lost mitochondrial localization, but could still induce IFN-β-Luc, NF-κB-Luc, and IRF-3-Luc, albeit less efficiently than the full-length caMAVS. A potential limitation of the luciferase-based assay is the use of HEK 293T cells instead of a natural host cell line (e.g., EECs), but the very low transfection efficiency in such cells precluded us from generating meaningful results. Given that PPRV can replicate in primate cells, the transfection efficiency or reporter plasmids in HEK293T cells is much higher, and the fact that caMAVS overexpression induces IFN-β transcription, we considered this a suitable alternative cell model. Because the induction of downstream signalling by overexpressed caMAVS is independent from interaction with RIG-I, this may explain why deletion of the NC domain abrogates interaction with RIG-I (**Figure 2.6B**) but can still induce downstream signalling (**Figures 2.4 and 2.5**). Similarly, in a mouse model of hantaan orthohantavirus (HTNV, genus *Orthohantavirus*, family *Hantaviridae*), it has been observed that IFN production is MAVS independent (Kell *et al.* 2020).

By activation of innate immunity, host cells aim to restrict the spread of invading viruses and other pathogens. Once the infection is cleared, cells have their own system to downregulate

such responses to return to a normal physiological state. The cellular proteins PCBP2 (You *et al.* 2009) and PCBP1 (Zhou *et al.* 2012) can mediate the degradation of the adaptor protein MAVS. NLRX1 (a nucleotide binding oligomerization domain (NOD)-like receptor X1) recruits PCBP2 to induce MAVS degradation through the proteasomal pathway (Qin *et al.* 2017). During HCV infection, the cellular Golgi protein 73 (GP73) acts as a negative regulator of innate immunity by interacting with MAVS/TRAF6 to promote degradation of this complex (Zhang *et al.* 2017). Alternatively, viruses may also encode proteins to specifically downregulate MAVS. For example, MAVS is degraded by the Rotavirus (RV) RNA methyl- and guanylyl-transferase (VP3) in a host-range-restricted manner (Ding *et al.* 2018). Likewise, MAVS is cleaved followed by infection with the Avian infectious bronchitis virus (Yu *et al.* 2017). In our study, we observed that PPRV infection induced MAVS degradation in host cells. Moreover, by testing a series of inhibitors, we found that PPRV-induced caMAVS degradation in EECs was medicated by the proteasome. When EECs were infected with a high MOI, the degradation of caMAVS was significant within 36 h post-infection. At the same time, the expression level of a smaller (45–50 kDa) polypeptide that also reacted with the caMAVS antibodies increased from 36 h post infection (**Figure 2.7A**), suggesting that caMAVS variants might exist, that may possess diverse biological functions in innate immune regulation. Surprisingly, we also found that the truncated variant is also significantly visible at the 48 h point without virus infection; however, it also decreased later at the 60 h point, which remains unclear. Truncated variants of MAVS (mini MAVS), which is expressed from a bicistronic mRNA of MAVS have been demonstrated to possess the ability to antagonize the signalling function of MAVS and thereby downregulate type I IFN expression by regulation of cell death as described previously (Brubaker *et al.* 2014).

Finally, we showed that overexpression of the PPRV V protein, in absence of a virus infection, also led to the downregulation of caMAVS. Although we did observe co-localization of PPRV-V with caMAVS in transfected cells, further studies will be required to fully understand the degradative interplay between PPRV-V and caMAVS. Interestingly, PPRV-C protein has recently been demonstrated to inhibit IFN-β induction by interacting with RIG-I and MAVS (Li *et al.* 2021), which might indicate that both PPRV-V and PPRV-C are critical for counteracting the innate immune response. Since the results described in this study are based on the PPRV vaccine strain from lineage II, it would be interesting to follow up on this research to investigate commonalities and potential differences between the four circulating lineages (Libeau *et al.* 2014) in their ability to counteract caMAVS.

In summary, our study characterized a MAVS homolog from immortalized goat cells (EECs) and investigated the functions of caMAVS during type I IFN signalling. We identified caMAVS as a RIG-I interacting type I interferon inducer and showed that caMAVS degraded during PPRV infection, possibly due to an interaction of PPRV-V and caMAVS.

Supplementary Materials: Table 2.S1: Primers designed and used in this study; Table 2.S2: The sequence of MAVS selected and included for polygenetic tree construction

Table 2.s1. Primers designed and used in this study

Primer name	Primer sequence (5'-3')
caMAVS-F	GAGAATTCATGACGTTTGCCGAGGACAGA
caMAVS-R	TCGATATCTCACTGGGGTAGGCGCCGCCG
caRIG-I-F	AGCAGAGTCGCCGG CATGACGGCC
caRIG-I-R	AATTTCTTCATTCAAGGCCATTCATTGCC
Caprine-qIFITM3-F	GGGCTATGAGGTGCTCAAGG
Caprine-qIFITM3-R	TGAACAGGGACCACACGATG
Caprine-qMX1-F	ACTCCCGACTGTTTACCAAAG
Caprine- qMX1-R	ACAAACCCTGGCAACTCTC
Caprine-qRSAD2-F	GCCCGAGTCTAACCAGAAG
Caprine-qRSAD2-R	CTACACCCACGTCCAAGATG
Caprine-qOASL-F	GCTGACCCCACCTACAATG
Caprine-qOASL-R	AGGACTCTTTCAGGCAATGG
Caprine-qGAPDH-F	GATGGTGAAGGTCGGAGTGAAC
Caprine-qGAPDH-R	GTCATTGATGGCGACGATGT
caMAVS-△TM-inF	ATGGAGGCCCGAATTCGGATGACGTTTGCCGAGGACAGA
caMAVS-△TM-inR	GTACCTCGAGAGATCTTCACAAGGTCCTGACCACAGGCAA
caMAVS-△CARD-inF	ATGGAGGCCCGAATTCGGATGATCTGTGAGCATACTGGT
caMAVS-dTM-inR	GTACCTCGAGAGATCTTCACTGGGGTAGGCGCCGCCGGTA
caMAVS-△PRR-F	ATCAGGGCACTGAGGCTGGGCAGTACCCACACAGCAGGCGG
caMAVS-△PRR-R	CCTCAGTGCCCTGATGAAGGT
caMAVS-△NC-F	CAGGAGCAAGACACAGAATTGCCCTGGGCTCCGTGGCTC
caMAVS-△NC-R	TTCTGTGTCTTGCTCCTGATG

Table 2.s2. The sequence of MAVS selected and included for polygenetic tree construction

Species	GenBank accession number
Ovis aries	XM_015099722.2
Tupaia belangeri	KM005100
Oreochromis niloticus	MG680272
Equus caballus	XM_023626166
Gallus gallus	NM_001012893
Danio rerio	NM_001080584
Rattus norvegicus	BC081869
Callithrix jacchus	KC415025
Papio anubis	NM_001279529
Pan troglodytes	KC415006
Pongo pygmaeus pygmaeus	KC415011
Nomascus leucogenys	KC415010
Gorilla gorilla	KC415007
Mus musculus	DQ174271
chimpanzee	KC415006
Sus scrofa	NM_001097429
Bos taurus	NM_001046620
Felis catus	KT375569
Canis lupus familiaris	NM_001122609

Chapter 3

BST2/tetherin restricts interactions with Peste des Petits Ruminants virus production and interferes with PPRV-V mediated interferon suppression

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BST2/tetherin restricts interactions with Peste des Petits Ruminants virus production and interferes with PPRV-V mediated interferon suppression

Abstract: BST2/Tetherin is an interferon-inducible transmembrane protein that inhibits a variety of enveloped viruses by interfering with the budding of progeny virions from the plasma membrane of infected cells. Here, we report two goat BST2 (GBST2) paralogs and test their antiviral activity against Peste des Petits Ruminants virus (PPRV), an emerging morbillivirus (family Paramyxoviridae) that causes severe disease in goats and sheep. Ectopic expression of either of the two goat BST2 isoforms blocked the release of PPRV from Vero cells overexpressing the Signalling lymphocytic activation molecule (SLAM) that serves as receptor for PPRV. Furthermore, it was found that GBST2 interacted and colocalized with the PPRV protein V. By generating a series of mutants of both GBST2 and V, we mapped the domains crucial for that interaction. Moreover, we found that GBST2 interfered with the ability of PPRV-V to inhibit IFN-β production. Overall, these results expand our understanding of the antiviral activity of BST2 paralogs against non-human morbilliviruses.

3.1 Introduction

When viruses invade host cells, the expression of host restriction factors (HRFs) helps the host to fight against viral infections. Many HRFs have been identified, especially in primates, like tripartite motif 5 (TRIM5) (Luban 2007), apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3 (APOBEC3) (Cullen 2006), CCCH-type Zinc-Finger Antiviral protein (ZAP) (Gao *et al.* 2002), and BST2/tetherin (Neil *et al.* 2008). BST2 is also known as bone marrow stromal antigen (BST2) or CD317/HM1.24. Initially, BST2 was identified in humans as an HRF that blocks the release of human immunodeficiency virus type-1(HIV-1) from infected cells (Neil *et al.* 2008). BST2 is a glycosylated, type II transmembrane protein with a mass of approximately 20 kDa. BST2 is constitutively expressed in for instance B cells and cultured Hela cells, whereas in various other cell lines its expression is upregulated by treatment with type I Interferons (IFNs) (Kuhl *et al.* 2011). Structurally, BST2 has an N-terminal cytoplasmic tail(CT domain), followed by a transmembrane domain(TM domain), an extracellular coiled-coil domain (CC domain), and a C-terminal glycosylphosphatidylinositol (GPI) anchor (Neil 2013). For a variety of species BST2 has been identified as a potential antiviral factor (Dietrich *et al.* 2011, Takeda *et al.* 2012, Yin *et al.* 2014, Murphy *et al.* 2015, Kim *et al.* 2016). Whether BST2 also functions as HRF in ruminants and if so via which inhibitory mechanism(s) is not known. BST2 paralogs have not been characterized, so far, for goat cells and putative interactions between goat BST2 and proteins of goat-infecting viruses have not been analysed.

Peste des Petits Ruminants virus (PPRV) causes an acute and highly contagious disease in goats, sheep, and related wild artiodactyl animals, with mortality rates as high as 90% (Abubakar *et al.* 2009). The disease is also known as goat plague and is characterized by conjunctivitis, rhinitis, stomatitis pneumonia and enteritis, and abortion. The disease was first described in 1942 in Côte d'Ivoire and is currently endemic in domestic and wild animals in parts of sub-Saharan Africa (Mahapatra *et al.* 2015), the Middle East (Muniraju *et al.* 2014), and Eastern Asia (Banyard *et al.* 2014). The virus is re-emerging on a regular basis in other countries (Marashi *et al.* 2017). In 2007, the disease was reported to be introduced into the Ngari region of Western Tibet (Wang *et al.* 2009, Zhou *et al.* 2018), causing near-catastrophic herd depletion for affected species, associated with huge economic losses. Now, PPRV has spread to more than 70 countries world-wide (Baron *et al.* 2016) and four distinct lineages of PPRV are recognised today (Baron *et al.* 2016). Even though PPRV outbreaks mostly happen in goats and sheep, the known host range of PPRV has been continuously expanding over the last decades, which brings a potential challenge to effectively control virus reservoirs and

eradicate PPRV (Dou *et al.* 2020). In summary, PPRV has become a global problem due to the severe symptoms, the significant economic losses and the risks it poses to both reared and wild animals.

PPRV (family *Paramyxoviridae*, subfamily *Orthoparamyxovirinae*, genus *Morbillivirus*) is a negative-sense, single-stranded RNA virus, which encodes a number of conserved, essential proteins (N, M, F, H, P, and L). The viral genome also encodes two accessory proteins, C and V. Protein C is expressed from the unedited mRNA for P, whereas V is expressed from an edited mRNA for P (Mahapatra *et al.* 2003). The PPRV V protein has been found to interact through its C-terminal region with several host proteins, such as MDA-5, LGP2, and RIG-I (Sanz Bernardo *et al.* 2017). Expression of the PPRV V protein can also block the induction of IFN-β (Sanz Bernardo *et al.* 2017). For some other paramyxovirus V proteins interaction with TRIM25 has been demonstrated, and this interaction prevents the TRIM25-mediated ubiquitination of RIG-I and thereby disrupts the downstream RIG-I signalling to the mitochondrial antiviral signalling protein (MAVS) (Sanchez-Aparicio *et al.* 2018).This interaction is also mediated by the conserved C-terminal domain of the respective V proteins of measles virus, Sendai virus, and parainfluenza virus (Sanchez-Aparicio *et al.* 2018). Hence, promoting viral growth by counteracting the host's innate immune response appears to be one of the main functions of the morbillivirus V proteins.

In the present study, we characterized the goat BST2 in goat-derived endometrial epithelial cells (EECs) and confirmed that goat BST2 (GBST2) has three N-glycosylation sites. We analysed the cellular localization of this protein by ectopic overexpression and also studied its antiviral action during PPRV virion production. We also studied the interactions of GBST2 with viral proteins, by using co-immunoprecipitation, and we mapped the domain crucial for the interaction of GBST2 with the PPRV V protein. To further understand the antiviral activity of GBST2, we tested the ability of GBST2 to counteract V-induced IFN-β inhibition.

3.2 Materials and methods

Cells and viruses

Goat-derived Endometrial Epithelial Cells (EECs) immortalized by transfection with human telomerase reverse transcriptase (Wang *et al.* 2015), were kindly provided by Prof. Yaping Jin (Northwest A&F University, Yangling, Shanxi, China), and we confirmed that their secretory functions were consistent with that of primary EECs. EECs were cultured in Dulbecco's Minimal Essential Medium/Nutrient Mixture F-12 Ham's (DMEM/F12) medium supplemented with 10% fetal bovine serum (FBS, Gibco, United States), 100 IU/ml of

penicillin, and 10 µg/ml of streptomycin at 37°C with 5% CO_2. Vero-SLAM cells, generated in the Shanghai Veterinary Research Institute and over-expressing the goat signalling lymphocytic activation molecule (SLAM), which is required as PPRV receptor, were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FBS. The PPRV vaccine strain, Nigeria/75/1 (GenBank Accession: HQ197753), was obtained from our laboratory cell culture collection at the Shanghai Veterinary Research Institute and titrated on the Vero-SLAM cells. Sendai virus (SeV) was proliferated in Specific pathogen-free (SPF) chicken eggs (Melia, Beijing) and titrated with erythrocyte agglutination tests. All experimental materials containing viruses were autoclaved at 120°C for 30 min after experiments were completed. Our laboratory confirms to meet the P2 (BSL-2) laboratory requirements, and all experiments were completed under P2 laboratory conditions.

Cloning and plasmids constructs

The immortalized EEC cells were treated with 1000 units/ml IFN-α for 24 h (Sigma, USA) before the total cellular RNA was isolated using Trizol (Invitrogen, USA). The isolated total RNA was subjected to reverse transcription with random primers and M-MLV reverse transcriptase (Promega, USA). Based on the predicted goat genome data, the GBST2 and GBST2-like cDNA sequences were amplified with conventional PCR, with primer sets designed specifically for the GBST2 gene (XM_018051379) (5'-ACGCGTCGACCATGATGGACAAATTGGAAGGA-3' and 5'-CGGGGTACCTCATTTCTTCAGACACTTGCGG-3'), and the GBST2-like gene (XM_018051380.1) (5'-ACGCGTCGACCATGGAGACCGAGATGGACGTGA-3' and 5'-CGGGGTACCTCATGTCAGCGGAGCGTTGAG -3'). After the PCR products were obtained, they were cloned into the pEASY-Blunt vector (Transgen, China). After confirmation by sequence analysis obtained from Sangon Biotech (Shanghai, China), the inserts were subcloned into the pCMV-HA(N-terminal) plasmid to attach an N-terminal HA tag. Based on online N-glycosylation prediction software (http://www.cbs.dtu.dk/services/NetNGlyc/) and the predicted structure of the proteins (http://ps2.life.nctu.edu.tw/), several mutants were generated with the Fast MultiSite Mutagenesis System (Transgen, Beijing, China) or by using Infusion technology (Vazyme, China) following previously described methods (Zhu *et al.* 2018). PPRV- Nigeria 75/1 gene fragments were all obtained from RT-PCR and then cloned into the p3xFLAG-CMV-10 (Sigma, USA) vector as described previously (Dong *et al.* 2020). Flag-GFP was generated with conventional PCR methods by inserting the GFP coding sequence into p3xFLAG-CMV-10 (Sigma, USA).

Antibodies, chemicals, and reagents

The anti-PPRV-N monoclonal antibody was generated by GenScript (Nanjing, China). Primary antibodies were used at a 1:1000 dilution and included antibodies directed against the Flag tag (Sigma, USA), the Myc tag (Santa Cruze, USA), and the HA-tag (Abcam, UK), as well as anti-β-actin antibodies (CWbiotech, China). HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch (USA). Alexa Fluor™ secondary antibodies for immunofluorescence assays were purchased from Thermo Fisher Technologies (Invitrogen, USA). RIPA lysis buffer and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Beyotime (Shanghai, China). PNGase F was purchased New England Biolabs (UK) and the Pierce™ Co-immunoprecipitation kit was obtained from Thermo Fisher (USA). Flasks, cell-culture plates, and plates for IFA were purchased from Nest Biotechnology (China).

Cell transfection, and Co-immunoprecipitation assay

Vero-SLAM cells were transfected with HA-tagged GBST2 and GBST2-like constructs at a confluency around 60% with Lipofectamine 2000 (Thermo Fisher, USA). Five hours after transfection, cells were washed three times with ice-cold PBS and the medium was exchanged for culture medium containing 2% FBS. The transfected Vero-SLAM cells were infected or mock-infected with PPRV at 12 hours post transfection.

HEK293T cells were seeded in 10 cm cell culture petri dishes (NEST biotech, China) co-transfected with each 5 ug of different tagged two protein expression plasmids, respectively serve as bait or prey plasmids for protein interaction studies. Forty-eight hours after transfection, total protein was isolated using IP lysis buffer (Thermo Fisher, USA). Co-immunoprecipitation (Co-IP) assay was performed using Pierce™ Co-immunoprecipitation kit (Thermo Fisher, USA) according to the manufacturer's instructions. At 36 hours post transfection, cells were washed with ice-cold PBS, and harvested with lysis buffer from the co-immunoprecipitation kit (Thermofisher, USA). The protein mixture was incubated overnight at 4°C and later subjected for WB analysis. AminoLink Plus Coupling Resin was incubated with anti-Flag monoclonal antibody (MAb) (Sigma, USA) or anti-HA Mab (Abcam, UK), and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblot analysis of the proteins was subsequently conducted using anti-HA or anti-Myc, or anti-Flag MAb independently.

Western blot analysis

Cells were washed with ice-cooled PBS (Hyclone, GE, USA) at the indicated time points and then harvested by adding RIPA lysis buffer (Beyotime, China) to the cells and a further

incubation on ice for 30 min. After centrifugation at 2000 rpm for 10 min, supernatants were collected. Then protein samples were separated in 12% SDS-PAGE gels and then transferred onto 0.22 µm Nitrocellulose membranes (GE healthcare, USA). Membranes were blocked with 5% skimmed milk diluted in TBST at 37℃ for an hour and subsequently washed with TBST and then incubated at 4℃ overnight with primary antibodies. After washing with TBST for four times (10 min each), the membranes were incubated with HRP-conjugated secondary antibodies for half an hour. The membrane was developed with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher, USA) and analysed on a Tanon 6600 Luminescent Imaging Workstation (ShanghaiTanon, China).

Reporter plasmids and luciferase Assays

HEK293T cells were cultured in 24-well plates and co-transfected with 40 ng of the Renilla luciferase-expressing plasmid pRL-TK (Promega, USA) along with 0, 50, 100 or 200 ng of each GBST2-expression plasmid, together with 100 ng encoding PPRV-V plasmid and 200 ng of a firefly luciferase reporter construct under control of the human IFN- β promoter (IFN-luc). The cells were infected at 12 hours post transfection with SeV as a positive control and at 36 hours post-transfection cells were harvested. The pRL-TK plasmid (Promega, USA) was used as an internal control for the normalization of gene transfection efficiency. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay kit (Promega, USA) according to the manufacturer's protocol, and all reporter assays were repeated three times. The luciferase activity of each sample was normalized to the Renilla luciferase activity and all data were expressed as mean relative luciferase activity with standard deviation (SD). Furthermore, all transfection experiments were independently performed three times.

Immunofluorescence Assay (IFA) and Confocal microscopy

Vero-SLAM cells were seeded onto glass coverslips (NEST biotech, China) to a confluency of ~60% and transfected with 2 µg of the constructs expressing either Flag-V or HA-GBST2 individually or combined, or with the Flag-V constructs together. At 36 hours post-transfection, cells were washed with cold PBS and fixed in 4% paraformaldehyde. Cells were then washed four times with PBS and treated with 0.1% Triton X-100 for 15 min, and incubated with 1% bovine serum albumin (BSA) with primary antibodies (1:500) for 1 h at 37°C or overnight at 4°C. Then, cells were washed with PBST for four times and then further incubated with FITC- or TRITC-conjugated secondary antibodies. Finally, cells were treated with 1 µg/ml of DAPI solution (Beyotime, China) for 5 min and washed with PBST for four times, later the samples were analysed by confocal microscopy Zeiss LSM880 and images were analysed by confocal microscopy Zen Blue software (Zeiss, Germany).

Virus titrations

Culture supernatants were collected and centrifuged to remove cell debris. Virus titers were determined by end point dilution assay. Briefly, Vero-SLAMs were cultured in 96-well plates and inoculated with virus dilutions (100 μL/well) prepared by 10-fold serial dilutions. The cells were incubated at 37℃ with 5% CO_2 for about 120 h, and cytopathic effect (CPE) was recorded. Virus titres were calculated using the Reed-Muench method and recorded as the $TCID_{50}$/ml. Each test was performed in triplicate and all experiments were done at least three times to make sure they were consistent.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, United States). The two-tailed, unpaired Student T-test was used to determine the statistical significance of the data. The difference between more than two groups was accessed by One-way ANOVA, it was considered to significant different if the $P < 0.05$ (*), whereas $P < 0.01$ was considered as highly different (**), whereas $P < 0.001$ was considered as significantly different (***). All data were analysed based on at least three individual independent experiments.
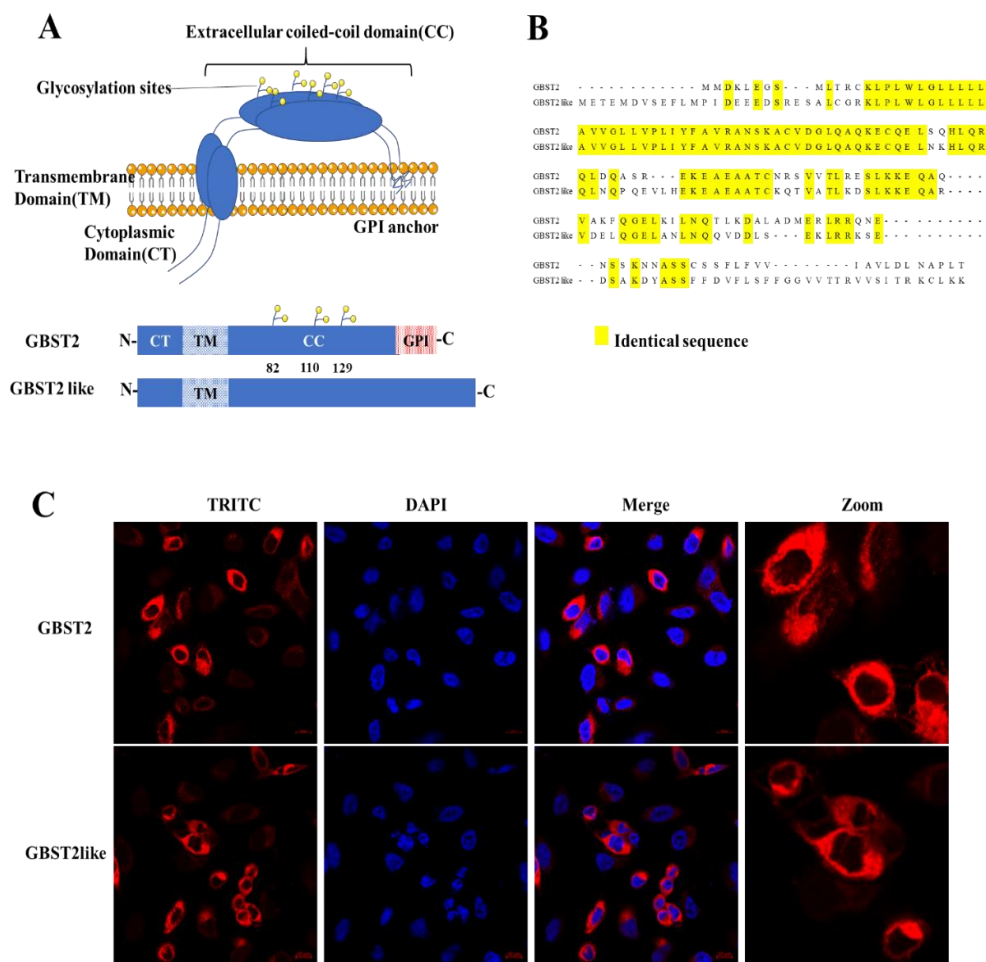
3.3 Results

Figure 3.1. Schematic structure of Goat BST2 and BST2 paralogs

(A) Schematic diagram of caprine BST2 and its structure base, (B) Alignment of caprine BST2 paralogs, (C) To explore the cellular distribution of GBST2 and GBST2like, Vero-SLAM cells were transfected with GBST2, GBST2 like and a number of mutants of GBST2 using Lipofectamine 2000. 36 hours later, samples of cell culture were harvested and analysed by IFA with specific antibodies.

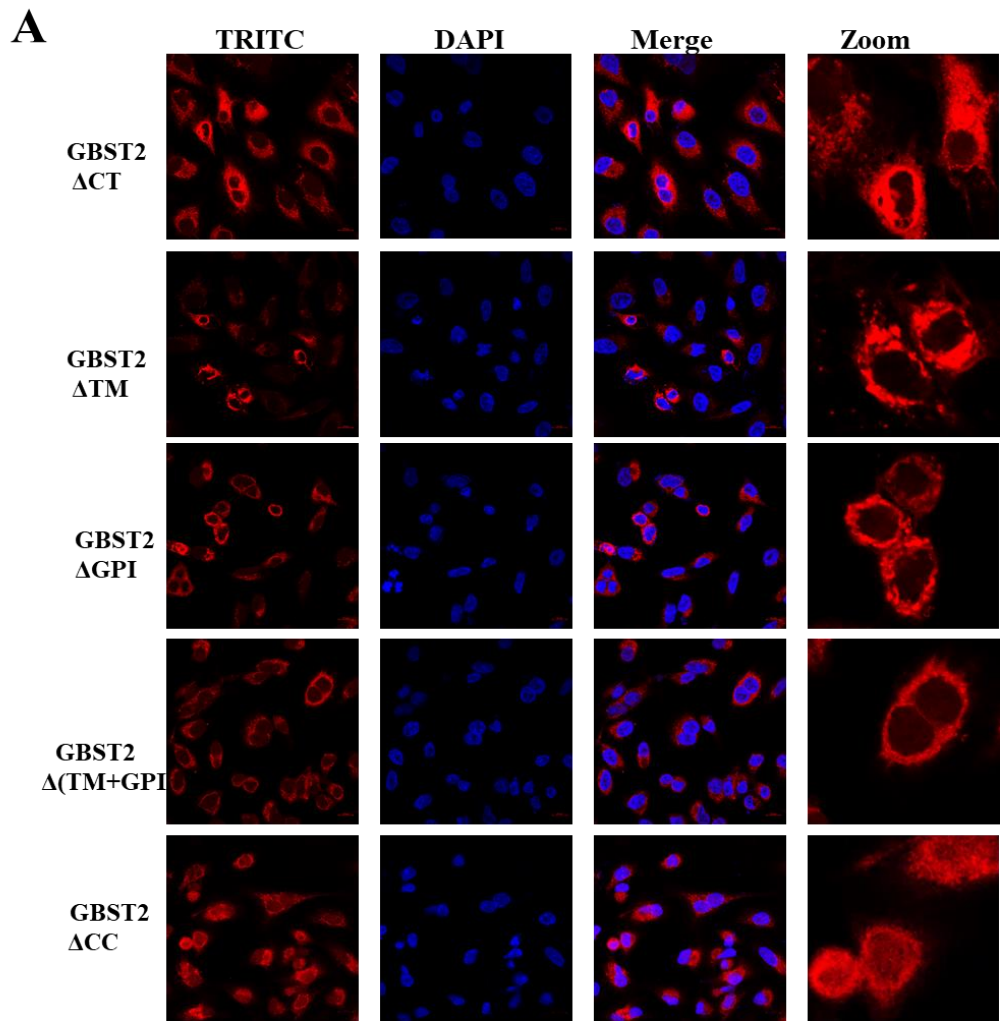**Goat BST2 paralogs and cellular distribution**

Figure 3.2 Cellular localization of the caprine BST2 mutants.

To explore the cellular distribution of GBST2 mutants, Vero-SLAM cells were transfected with a number of mutants of GBST2 respectively using Lipofectamine 2000. 36 hours later, samples of cell culture were harvested and analysed by IFA with specific antibodies.

Two paralogs of goat BST2 (GBST2 and GBST2like) were identified based on genome sequences and cloned as cDNA. Based on an amino acid alignment with the BST2s from other mammalian species, an N-terminal transmembrane (TM) domain, and a C-terminal hydrophobic domain (CT), a predicted GPI anchor, and an extracellular coiled-coil (CC) domain can be annotated (**Figure 3.1A**). Identical sequences of GBST2 and GBST2-like are coloured yellow. GBST2like shares 77% similarity with GBST2 (**Figure 3.1B**), but it lacks the predicted N-glycosylation sites, of which three are present in GBST2.

Immunofluorescence assays (IFA) followed by confocal microscopy showed that HA-tagged full length GBST2 and GBST2like proteins located similarly in both the cell membrane and in the cytoplasm (**Figure 3.1C**). But GBST2like showed condensed protein display in the cytoplasm. Based on the predicted structural domains for GBST2, several mutated constructs were generated, and these constructs were named GBST2-ΔCT, GBST2-ΔTM, GBST2-ΔGPI, GBST2-ΔCC, and a GBST2-Δ(TM+GPI), in which both the TM and the GPI anchor were deleted. The hypothesis is that the TM domain and GPI anchor would be essential for GBST2 localization and that deleting these domains would influence the intracellular localization of GBST2. On the contrary, full length GBST2 and its mutants showed a similar distribution, except for GBST2-ΔCC which was more abundantly found in the cytoplasm (**Figure 3.2A** lowest panel). Further analysis demonstrated that the TM domain of GBST2 was important for the presence of BST2 in the cytoplasm and that the deletion of the GPI domain did not affect its cellular distribution (**Figure 3.2A**).

Both goat BST2 paralogs are able to inhibit PPRV budding.

Vero-SLAMs cells were transfected with increasing amounts of ectopic HA-tagged GBST2 and GBST2-like constructs to analyse the effect of BST2 on virus amplification. Twelve hours post-transfection, cells were infected with PPRV-Nigeria/75/1. At 120 hours post infection, the culture medium was analysed for the presence of viral proteins by western blotting and by virus titration assays. We found that the expression of increasing amounts of goat BST2 lead to lower
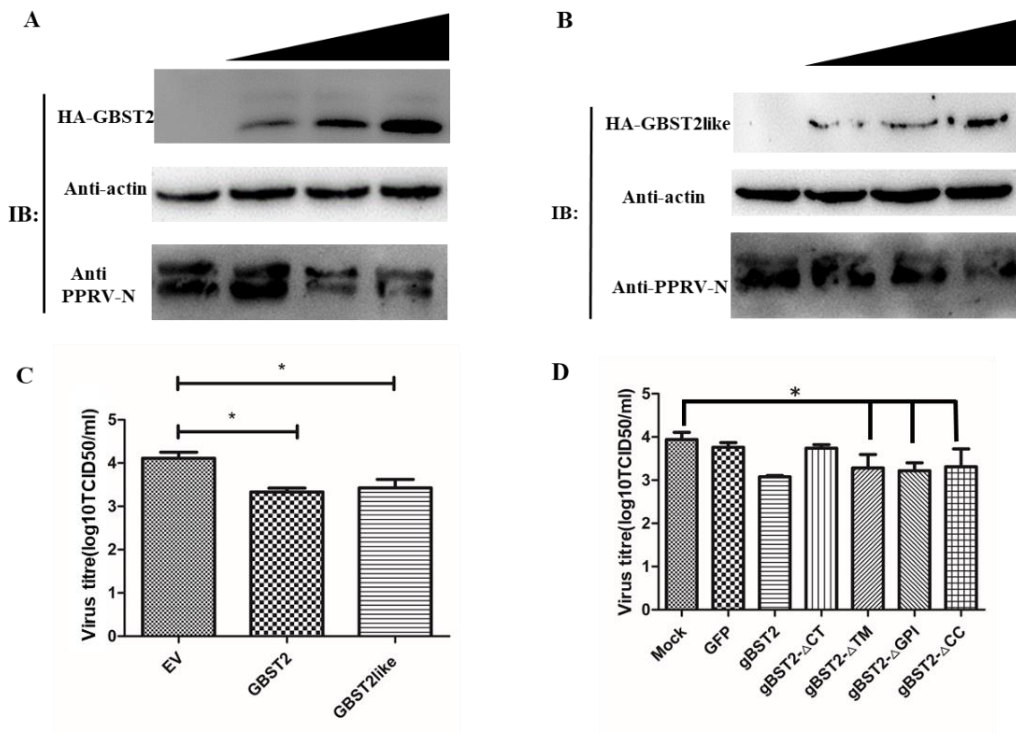

Figure 3.3. Caprine Tetherin blocks PPRV release from infected cells.

(A.B) Vero-SLAMs cells were transfected with increasing amounts (2 ug, 4 ug, 8 ug) of GBST2 and GBST2like 12h hours post-transfection, cells were were washed with fresh DMEM containing 1% FBS after infection. Supernatants was harvested after 120 hours post-infection and subjected to ultracentrifugation for WB analysis or virus titration. Western blot and virus titration were performed to check the influence of increasing amount of caprine BST2 on PPRV production. (C) Equal amount of GBST2 and GBST2like and empty vector were transfected to Vero-SLAMs, later cells were changed with fresh medium after infection and supernatants were harvest for titration 5days later. (D) Equal amount of GBST2 and its mutants without domain deletion were transfected to Vero-SLAMs, and 12 hours later, cells were changed with fresh medium after infection and 5days later, supernatants were harvest for titration.

levels of PPRV in the supernatants (**Figure.3A**). Moreover, the GBST2like paralog also inhibited virus production, however on the western blot (Figure.3B) this affect appeared less obvious than for GBST2. Furthermore, the infectious virus titres in the culture medium for the GBST2 and GBST2like overexpression did not differ significantly from each other (**Figure 3.3C**). More in-depth analysis was carried out for mutant GBST2 constructs lacked the TM, GPI, or CC domain, and tested these in a similar way in infected Vero SLAM cells. From the titration results (**Figure 3.3D**), it became clear that the GPI and the TM domain are crucial for the restriction activity of GBST2.

GBST2 interacts and co-localizes with PPRV-V

Measles virus V protein was previously reported to block IFNα/β signalling (Takeuchi *et al.* 2003), but for PPRV it is not fully understood how the V protein affects innate immunity by interfering with IFN production and induction of IFN-stimulated genes (ISGs). Co-IP experiments were performed to test the interactions of BST2 with a set of virus protein, and found that HA-tagged GBST2 interacted with a Flag tagged PPRV protein V. Flag-GFP was used as negative control. In these Co-IP experiments in protein extracts of co-transfected cells we demonstrated that GBTS2 could interact with Flag-PPRV-V (**Figure 3.4A**). Furthermore, a co-localization study was conducted in Vero-SLAM cells. GBST2 localized to the cytoplasm
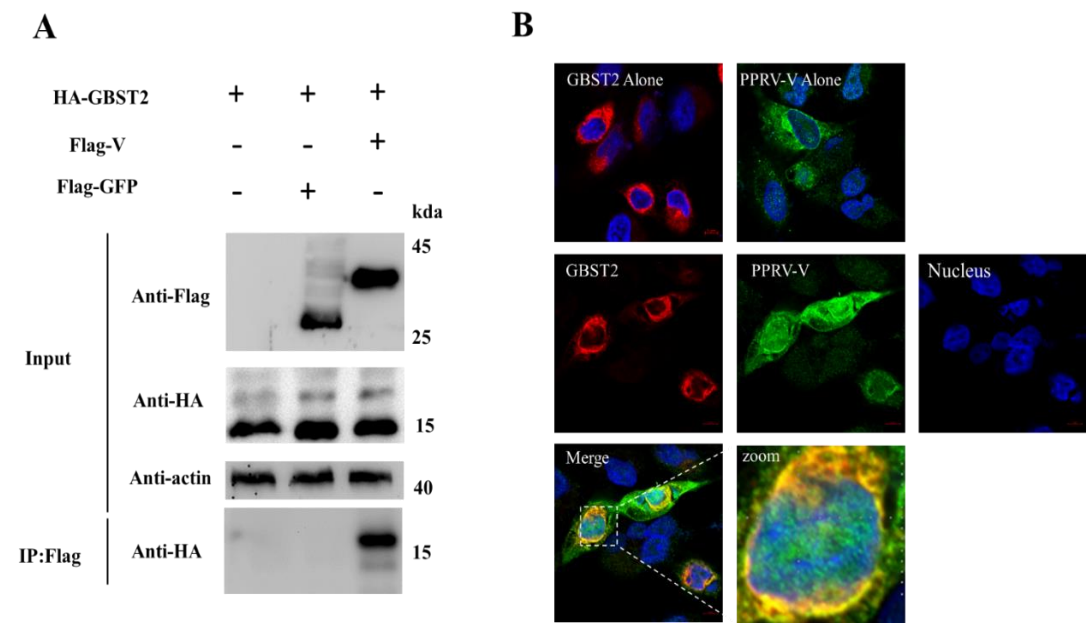

Figure 3.4. The interaction between GBST2 and PPRV-V

To further validate the interactions between GBST2 and PPRV-V, HEK293T cells were transfected with equal amounts of HA-GBST2 and Flag-V or Flag-GFP (5 ug each). Cells were harvested at 48 h post transfection and subjected for Co-IP procedures. (B,C) Vero-SLAM cells and EECs were transfected with GBST2 and PPRV individually or combined for (co-) localization studies, twenty four hours later, cells were harvested and stained with specific fluorescently-labeled antibodies for immunofluorescence studies, DAPI was applied for staining the nucleus, immunofluorescence was analyzed using a Zeiss LSM880 confocal microscope and analysed by Zen Blue software (Zeiss, Germany) , and GBST2(red) was found to co-localized with PPRV-V (green) around nucleus(blue).

and to the cell membrane when expressed alone, while the PPRV-V protein was distributed over both the cytoplasm and the nucleus. GBST2 co-localized with the PPRV-V protein around the nucleolus (**Figure 3.4B**).

N-glycosylation affect but not abolish the interactions between GBST2 and PPRV-V

Furthermore, Co-IP experiments were performed to find out whether the N-glycosylation sites of GBST2 are needed for the interactions between GBST2 and PPRV-V. HA-tagged constructs of GBST2 with/without N-glycosylation sites (**Figure 3.5A**) were co-transfected with Flag tagged PPRV-V to HEK293T cells . The results indicated that removal of N-glycosylation sites in GBST2 did not abolish its interaction with PPRV-V totally (**Figure 3.5B**), but do influence their levels between compared with each other, our results proved that the glycosylation modification which happened after transcription is not required for their interaction, which is consistent with some previous findings (Wang *et al.* 2015).
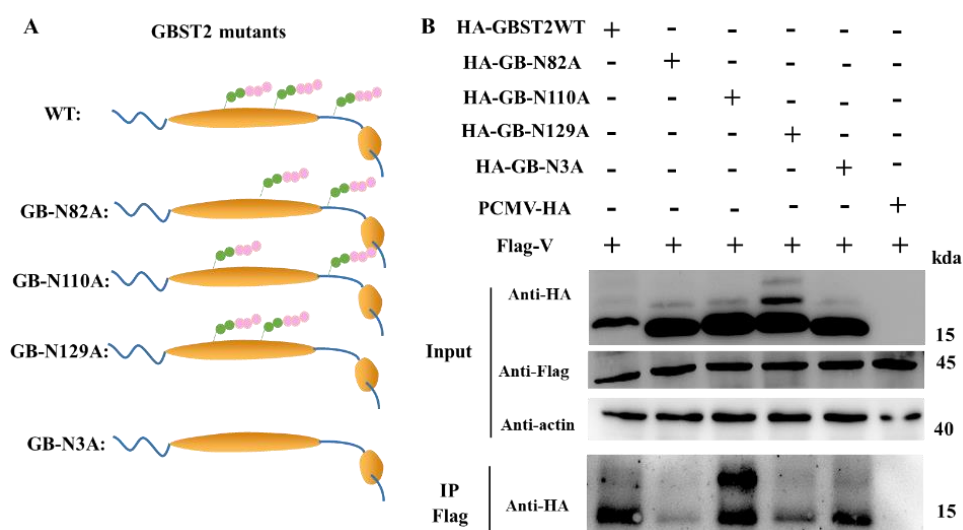
Figure 3.5 The N-Glycosylation modifications of GBST2 does influence the interaction between GBST2 and PPRV-V

(A) Diagram presenting the N-Glycosylation sites of GBST2. (B) Equal amounts of HEK 293T cells were seeded into 6-well plates. Cells were transfected with Flag-V together with several HA-tagged mutants of GBST2, in which the individual or all three N-glycosylation sites were mutated, thereby converting the N into an A residue. Cells were harvested with lysis buffer and subjected for Co-IP experiment overnight. The eluted protein was subjected for SDS-PAGE and WB using specific antibodies.

Mapping PPRV-V and GBST2 interaction domains

To further map the domain(s) required for interaction between PPRV-V and GBST2, several mutants were constructed based-on the schematic diagram shown in **Figure 3.6A**. PPRV-V N-Terminal (V-NTD) and C-terminal domain (V-CTD) mutants of PPRV-V were generated with the N-terminal Flag tag and transfected with HA-tagged GBST2 into HEK293T cells. All HA-GBST2 based mutants that lacked the TM, GPI, or CC domains were generated and transfected together with Flag-V also into HEK293T cells. Thirty-six hours post transfection, cells were harvested and subjected for Co-IP by following the procedures. From the co-IP experimental results, we clearly showed that for PPRV-V protein, both domains are required for interacting with GBST2 (Figure. 6A). However, for GBST2, only deletion of the Central domain (CC) domain abolished the interaction of GBST2 with GBST2 with PPRV-V (**Figure 3.6B**).
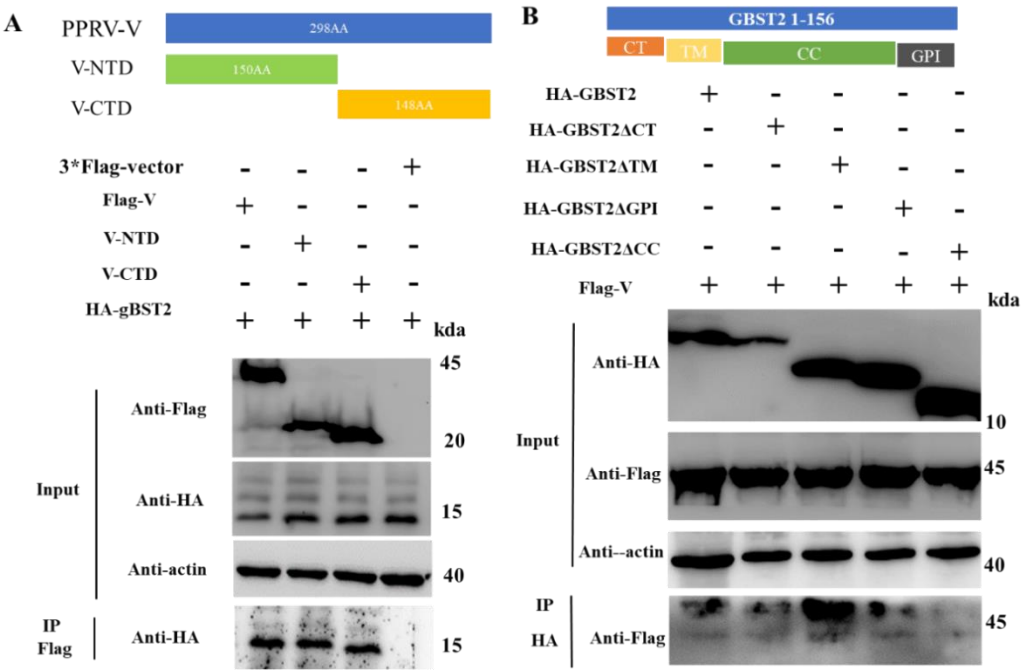
Figure 3.6. Mapping the crucial domain of both GBST2 and PPRV for their interaction.

HEK293T cells were transfected with mutants and wide type (WT) constructs for further domain verification. (A) An equal amount of WT Flag-V, V-NTD, or V-CTD was used for transfection together with HA-GBST2 (5 ug). (B) Equal amounts of WT HA-GBST2 or HA-tagged GBST2 mutants (5 ug) were used for transfection together with WT Flag-V (5 ug). Cells were harvested and subjected to further Co-IP experiments. The eluted protein was subjected to SDS-PAGE and Western blot analysis using specific antibodies.

GBST2 interfered with PPRV-V induced IFN inhibition

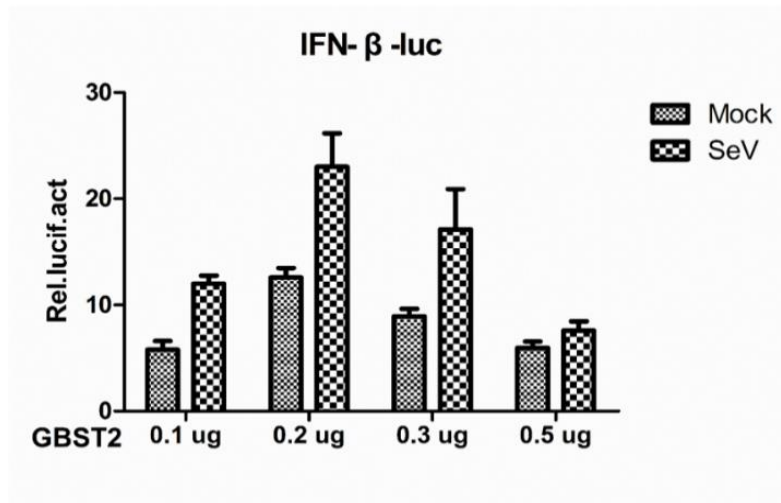**Figure 3.7. GBST2 influence the IFN-β inhibition ability of PPRV-V significantly**

PPRV-V induced IFN-β inhibition is counteracted by GBST2 overexpression. HEK293T cells were transfected with increasing amounts of a GBST2 expressing plasmid (ranging from 100 to 500 ng) ,together with a standard amount of PPRV-V plasmid (100 ng). , Twenty four hours hours post transfection, SeV was added to duplicate cells for full activation of the signaling pathway leading to IFN- β expression.

Type I IFNs are a key component of the innate immune response and form the first line of defense against viral infections. Previously PPRV-V was demonstrated to inhibit IFN-β induction (Sanz Bernardo *et al.* 2017), and here we investigated whether the interaction between GBST2 and PPRV-V played a role in preventing IFN-β induction. In our study, a reporter plasmid was used that expressed luciferase under control of the IFN-β promoters to analyze whether GBST2 affects the production of IFN-β. HEK293T cells overexpressing PPRV-V were co-transfected with increasing amounts of GBST2 and IFN-β transcription was induced by SeV infection. As shown in **Figure 3.7**, PPRV-V could induce IFN-β inhibition in HEK293T cells, and the IFN-β inhibition was affected by GBST2 in a dose-dependent manner. Similar experiments were performed with SeV as a positive control.

3.4 Discussion

BST2 or BST2 was first reported to be a potential antiviral host factor in 2008 (Neil *et al.* 2008). At that time, it was demonstrated that BST2 can inhibit the release of HIV-1 particles from host cells, but only when the HIV-1 viral protein Vpu was absent (Neil *et al.* 2008, Van Damme *et al.* 2008), indicating that Vpu counteracts BST2. Meanwhile, BST2 has been found on cell membranes inhibiting the budding of a variety of enveloped viruses such as alphavirus (Jones *et al.* 2013, Ooi *et al.* 2015), retrovirus (Jia *et al.* 2009, Mangeat *et al.* 2012, Yin *et al.* 2014), filovirus (Vande Burgt *et al.* 2015), poxvirus (Sliva *et al.* 2012), paramyxovirus (Kong *et al.* 2012), herpesviruses (Zenner *et al.* 2013), rhabdoviruses (Weidner *et al.* 2010) and arenavirus (Radoshitzky *et al.* 2010). In support of an antiviral role for BST2, knockout cells of BST2 increased the release of progeny virus (Sauter 2014).
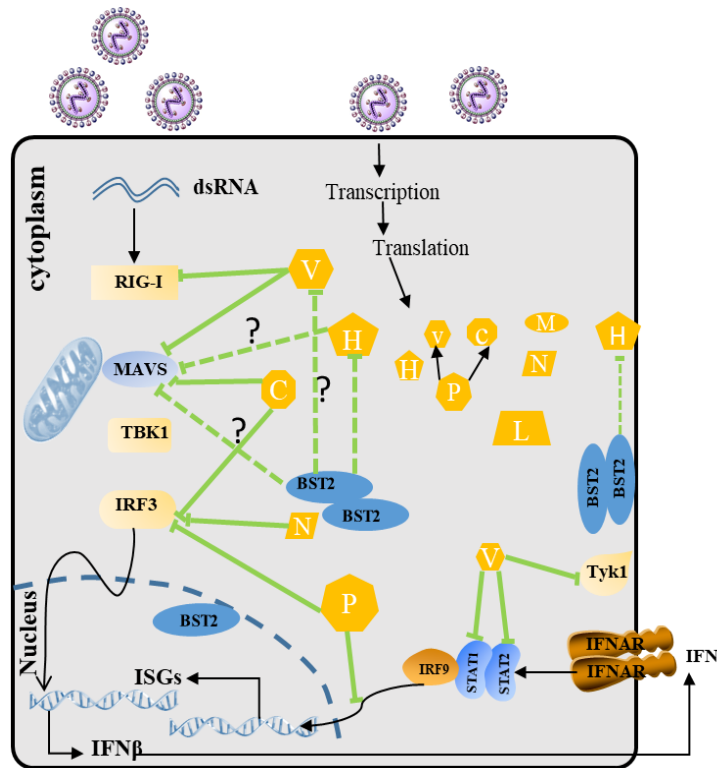

Figure 3.8 Complex interactions modulated by PPRV viral proteins

BST2 has been proved interacted with PPRV-V by Co-IP and IFA , and the detailed mechanism remains unclear. PPRV-V mediated MAVS degradation have been studied in other chapters in the thesis, the other antagonism related to PPRV viral proteins and host cellular proteins were reviewed in José M Rojas, 2016

BST2 had been characterized in mammals, however, recently, BST2 was also reported to be present in other vertebrates, including fish, reptiles and birds (Heusinger *et al.* 2015). An interesting discovery is that for ruminants there is more than one BST2 gene, such as the three bovine paralogs (Takeda *et al.* 2012), and the two paralogs in sheep (Arnaud *et al.* 2010). In this study, we identified two goat BST2 paralogs, which we named GBST2 and GBST2like. For GBST2, by prediction, the cytoplasmic domains are similar in size and sequence with human BST2. First, we confirmed that goat BST2 (GBST2) is an ISG by isolating the sequence through treatment of EECs with IFN-α. Furthermore, we demonstrated the antiviral activity of GBST2 against PPRV. Ectopic overexpression of the GBST2 paralogs could significantly reduce the virus load in cell culture supernatants, which maybe directly by GBST2 the progeny viruses on the cell surfaces (**Figure 3.1A-B**), but unluckily we did not have date show the qualification of intracellular Virus RNA which limited our conclusion. Over all, based on domain analysis, we observed that GBST2 shares major structural properties with its homologues in other mammals. The observed effects of overexpressed GBST2 mutants in Vero-SLAM cells on progeny virus titres, suggested that the TM and GPI domain are important for its function in restricting PPRV. The limitation of the current study is that we the experiments did not discriminate between effect of GBST2 PPRV replication in general, or specifically the budding process.

N-glycosylation is one of the most common posttranslational modifications in the endoplasmic reticulum (ER) and the Golgi apparatus during protein synthesis and is important for the stability of numerous glycoproteins. Both human and feline BST2s have N-glycosylation at conserved asparagine residues in their extracellular domains, but there are conflicting reports about the effect of the N-glycosylation status on virus restriction ability (Han *et al.* 2016, Bai *et al.* 2020). In our study, by introducing asparagine(N) to alanine(A) mutations in GBST2, we demonstrated that some BST2 N-glycosylation sites decreased but not abolish the interaction with PPRV-V.

Given the ability of IFNs to fight against viral infection, many viruses have evolved to counteract or evade the antiviral IFN response, such as Nef protein of SIV, HIV-1 Vpu (Jia *et al.* 2009), HIV-2 Env (Chen *et al.* 2016). In our study, we demonstrated that GBST2 could interact with PPRV V directly after overexpression of tagged proteins. However, for another paralog of goat BST2 (GBST2like), we did not observe any interactions during the Co-IP, but the interaction of GBST2 with PPRV-H is also immunoprecipitated (Supplementary **Figure 3.s1**) which is similar as the researchers demonstrated BST2/BST2 could interact with MeV H

protein to modulates morbillivirus glycoprotein production to inhibit cell-cell fusion (Kelly *et al.* 2019).

We continued focusing our study on the interaction between GBST2 and PPRV-V by carrying out a intracellular localization study. We found that PPRV V expression alone was widely distributed in the both cytoplasm and also the nucleus, but when overexpressed together with the expression of GBST2, PPRV-V redistributed together with GBST2 around the nucleus. PPRV-V protein has been discovered to inhibit IFN production by blocking its activation and downstream signalling of ISRE promoter (Ma *et al.* 2015). However, in the future, additional studies are needed to fully address the relative importance of goat BST2's activity and its relevance for innate immune interference *in vivo*, and together how PPRV antagonizes BST2's antiviral ability by viral V or other viral protein (**Figure 3.8** working model for BST2 interactions during signalling of innate immunity). Except for its inhibitory effect on virus release, BST2 was shown in other mammals to affect cell signaling by induction of NF-κB-dependent pro-inflammatory responses, and the YxY motif located in the cytoplasmic domain of BST2 was shown to be critical for this signaling function (Galao *et al.* 2012, Sauter *et al.* 2013, Tokarev *et al.* 2013). Inspection of the cytoplasmic domain of predicted a similar YxY motif in goat BST2. Moreover, further studies need to demonstrate whether GBST2 could activate the NF-κB pathway and to see if it is consistent with other mammalian BST2 studies (Rizk *et al.* 2017).

In summary, we characterized two paralogs of goat BST2 and demonstrated their ability on PPRV budding by exogenous overexpression in Vero-SLAM cells. Moreover, we discovered that the antiviral ability of GBST2 relies on the presence of specific structural domains on virus production,. We performed more in depth studies with GBST2 and showed that the PPRV V protein could interact and co-localize with GBST2., and mapped the crucial domain for their interaction. But how the PPRV viral protein V antagonizes GBST2 and the interactions on innate immunity signaling pathway process remains to be explored.

Acknowledgments

Supplementary Materials: Figure.3.s1: GBST2 interacted with PPRV-V and PPRV-H by Co-IP

	3*Flag	+	-	-	-	-
	Flag-V	-	+	-	-	-
	Flag-P	-	-	+	-	-
	Flag-H	-	-	-	+	-
	Flag-N	-	-	-	-	+
	HA-GBST2	+	+	+	+	+

Figure. 3.s1 GBST2 interacted with PPRV-V and H

HEK293T cells were transfected with equal amount of HA-GBST2 together with PPRV viral protein V, P, H, N respectively, for CO-IP assays. The results indicated the direct interaction of GBST2 with PPRV-V, and PPRV-H, but not with PPRV-P and PPRV-N

Chapter 4

Immunogenicity in Rabbits of Virus-Like Particles from a Contemporary Rabbit Haemorrhagic Disease Virus Type 2 (GI.2/RHDV2/b) Isolated in The Netherlands

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Immunogenicity in Rabbits of Virus-Like Particles from a Contemporary Rabbit Haemorrhagic Disease Virus Type 2 (GI.2/RHDV2/b) Isolated in The Netherlands

Abstract: Rabbit haemorrhagic disease virus (RHDV) type 2 (GI.2/RHDV2/b) is an emerging pathogen in wild rabbits and in domestic rabbits vaccinated against RHDV (GI.1). Here we report the genome sequence of a contemporary RHDV2 isolate from the Netherlands and investigate the immunogenicity of virus-like particles (VLPs) produced in insect cells. RHDV2 RNA was isolated from the liver of a naturally infected wild rabbit and the complete viral genome sequence was assembled from sequenced RT-PCR products. Phylogenetic analysis based on the VP60 capsid gene demonstrated that the RHDV2 NL2016 isolate clustered with other contemporary RHDV2 strains. The VP60 gene was cloned in a baculovirus expression vector to produce VLPs in Sf9 insect cells. Density-gradient purified RHDV2 VLPs were visualized by transmission electron microscopy as spherical particles of around 30 nm in diameter with a morphology resembling authentic RHDV. Immunization of rabbits with RHDV2 VLPs resulted in high production of serum antibodies against VP60, and the production of cytokines (IFN-γ and IL-4) was significantly elevated in the immunized rabbits compared to the control group. The results demonstrate that the recombinant RHDV2 VLPs are highly immunogenic and may find applications in serological detection assays and might be further developed as a vaccine candidate to protect domestic rabbits against RHDV2 infection.

4.1 Introduction

Rabbit haemorrhagic disease virus (RHDV, family *Caliciviridae*, genus *Lagovirus*) was first reported in 1984 in China as the causal agent of Rabbit Haemorrhagic Disease (RHD), an acute and highly lethal disease in wild and domestic rabbits (*Oryctolagus cuniculus*). Two years after the initial outbreak, RHDV reached the European continent. RHDV is highly contagious and can have a mortality rate of around 80-100% in adult rabbits (Abrantes *et al.* 2012). RHD is considered the most economically important disease in rabbits around the world (Ohlinger *et al.* 1990). Several inactivated and live myxoma vector vaccines are commercially available that confer protection against circulating RHDV strains (Abrantes *et al.* 2012). Even after large scale vaccination numerous cases of RHD remained to be reported, e.g. in 2010 in North-western France(Le Gall-Recule *et al.* 2011). This disease affected not only wild populations, but also RHDV-vaccinated rabbits, suggesting the emergence of a novel RHDV serotype. Indeed, a newly identified lagovirus named RHDV type 2 (RHDV2) was identified on an industrial farm in the province of Udine in North-eastern Italy (Epizooties 2011). Later, a similar virus isolated in Spain was named RHDVb (Dalton *et al.* 2012). In 2017, a new RHDV nomenclature was proposed(Le Pendu *et al.* 2017), and RHDV2 and RHDVb were designated GI.2. RHDV2 (RHDVb/GI.2) has been reported in France (Le Gall-Recule *et al.* 2011), Italy (Le Gall-Recule *et al.* 2013), the Iberian Peninsula (Abrantes *et al.* 2013, Dalton *et al.* 2014), Sardinia (Puggioni *et al.* 2013), the United Kingdom (Westcott *et al.* 2014), Madeira (Carvalho *et al.* 2017), the Canary Islands, the Azores (Duarte *et al.* 2015), Australia(Hall *et al.* 2015), Scandinavia (Neimanis *et al.* 2018), Africa (Lopes *et al.* 2019) and America (Carlos Rouco *et al.* 2019). In Sweden, Australia and on the Iberian Peninsula, all recent cases of RHD in wild and domestic rabbits were caused by RHDV2, which suggests that RHDV2 replaces RHDV as the main cause of RHD (Dalton *et al.* 2014, Lopes *et al.* 2014, Peacock *et al.* 2017, Mahar *et al.* 2018). These outbreaks clearly indicated that RHDV2 is antigenically different from RHDV and has the capacity to kill rabbits vaccinated against RHDV (Capucci *et al.* 2017, Peacock *et al.* 2017).

In the period December 2015 to August 2016, the Dutch Wildlife Health Centre (DWHC) in the Netherlands examined numerous dead rabbits that were found to be RHDV2-positive. In January 2017, the first case of RHDV2 in the Netherlands was confirmed as the cause of death in a wild hare. The dead hare presented with severe acute inflammation in liver and spleen, and RHDV2 infection was confirmed by PCR analysis.

At present, the emergence of RHDV2 raises concerns regarding the impact of the disease among domestic and wild rabbit populations. RHDV2 is replacing existing strains of RHDV

and is expected to cause severe problems to the rabbit industry. Thus, there is a clear need for a safe and effective RHDV2 vaccine. The traditional approach is an inactivated vaccine, typically produced from the liver collected from artificially infected rabbits. Because inactivated RHDV2 vaccines may not always confer full protection against RHD, alternative vaccines including those based on a Myxoma virus vector are in development. Another promising approach to develop safe and effective vaccines is the application of virus-like particles (VLPs). VLPs have been shown to induce a potent B-cell mediated immune response, and can induce highly effective CD4+ T cell proliferative responses and a cytotoxic T lymphocyte (CTL) response (Wagner *et al.* 1994, Schirmbeck *et al.* 1996, Peng *et al.* 1998, Win *et al.* 2011, Kushnir *et al.* 2012). VLP-based vaccines have been recognized as safe and effective and are commercially available to protect humans against Hepatitis B virus and human papillomavirus (Monie *et al.* 2008, Ji *et al.* 2018) and swine against porcine circovirus (Fort *et al.* 2009). Numerous prototype VLP vaccines against novel emerging diseases, such as chikungunya and Zika viruses have been generated and are in different stages of (pre-)clinical trials (Akahata *et al.* 2010, Boigard *et al.* 2017). RHDV VLPs can be generated by overexpression and self-assembly of VP60, the major viral structural protein, using different expression systems including bacteria, yeast and insect cells (Nagesha *et al.* 1995, Peacey *et al.* 2008, Fernandez *et al.* 2013, Gao *et al.* 2013, Chen *et al.* 2014, Guo *et al.* 2016, Song *et al.* 2017). The baculovirus expression vector system (BEVS) is a versatile expression platform ideally suited for the large-scale production of complex (glyco)proteins and VLPs in insect cells (Metz and Pijlman 2011, van Oers *et al.* 2015). Commercial VLP-based vaccines for human (Cervarix, GlaxoSmithKline) and veterinary (Porcilis PCV, MSD Animal Health) applications utilize BEVS technology.

In the present study, we identified the complete genome sequence of a contemporary RHDV2 strain from the Netherlands (NL2016) and expressed the VP60 capsid protein of RHDV2-NL2016 to generate VLPs. Subsequently, we have evaluated the immunogenicity of the RHDV2 VLPs in rabbits.

4.2 Materials and methods

Ethics statement

All animal experiments involved in these studies were conducted following the guide for care and use of Laboratory Animals of the Ministry of Science and Technology of people's republic of China. All experiments were performed in a secondary biosecurity laboratory. All animal procedures were approved by the Institutional Animal Care and Use committee of Shanghai

Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permit number:20180511). Every effort was made to minimize suffering.

RHDV2 virus sequence analysis and phylogenetic analysis

RHDV2 was discovered by the Dutch Wildlife Health Centre in the Netherlands. The liver of the infected rabbit (*Oryctolagus cuniculus*, no. 3151221028) was ground with liquid nitrogen in a mortar and total RNA was isolated with Trizol (Invitrogen, USA). Primers (**Table 4.1**) were designed based on conserved regions of RHDV2 sequences available in GenBank (**Table 4.2**).

Reverse transcription of total RNA with M-MLV reverse transcriptase and random primers was used to generate cDNA. The cDNA was used as template for PCR amplifications using primers (Table 1) to obtain a collection of DNA fragments spanning the entire RHDV2 genome. For these PCR amplifications the proof-reading Phusion polymerase (Thermo Fisher, USA) was used. Each PCR product was cloned into pJET1.2 (Thermo Fisher, USA) and 3 clones per fragment were sent for Sanger sequencing (Macrogen). The sequences were assembled into a consensus complete genome sequence named *Lagovirus europaeus*/GI.2/*O cun*/NL/2016/3151221028 or in short RHDV2-NL2016 (Genbank accession no. MN061492). The phylogenetic tree was constructed by Mega5.1 using a selection of VP60 sequences (**Table 4.2**).

Table4.1. Primers designed for amplification of the complete RHDV2-NL2016 genome.

Primer name	Primer sequence
Gate-VP60F(*NcoI*)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGGACCATGGAGGGCAA AGCCCG
Gate-VP60R(*NsiI*)	GGGGACCACTTTGTACAAGAAAGCTGGGTAATGCATTCAGACATAAGAAAG CCATTG
RHDV2(1-405)-F	GTGAAAGTTATGGCGGCTATG
RHDV2(1-405)-R	TCGGTAAGCACAGGGGATGAC
RHDV2(80-1355)-F	TCCTGGACCTCAGGGACAAGA
RHDV2(80-1355)-R	GCCATTTTCACAACTGTCAT
RHDV2(1332-3101)-F	GGTTATGACAGTTGTGAAAATGGC
RHDV2(1332-3101)-R	GTCATGTCATGTGCGTTGACA
RHDV2(3083-5319)-F	TCAACGCACATGACATGACTG
RHDV2(3083-5319)-R	GGCTTTGCCCTCCATAACATT
RHDV2(6964-7378)-F	CGCCCTGTGGGACCCAGA
RHDV2(6964-7378)-R	TCAAGCACTGGACTCGCCAGT
RHDV2(5295-7047)-F	TGTGAATGTTATGGAGGGCAAAGC
RHDV2- 3'dTNNN	GACTGACTGCCATGGCCGGCGCTAGCTTTTTTTTTTTTTTTTTTTTTTTTT

RHDV2 VP60 cloning and expression vector construction

Primers with attB1/2 recombination sites and *Nco*I and *Nsi*I unique restriction sites were designed to subclone the RHDV2 VP60 gene in the Gateway donor vector pDONR207 (Invitrogen, USA). Next, the VP60 gene was transferred to the Gateway destination vector pDEST10 (Invitrogen, USA), which drives expression of N-terminal his-tagged transgenes from the strong baculovirus polyhedrin promoter. The resulting pDEST10-VP60 plasmid was sequenced and used for the construction of a recombinant baculovirus.

Cells and recombinant baculovirus expressing RHDV2 VLPs

Spodoptera frugiperda (Sf9) insect cells were maintained in Sf-900 Ⅱ SFM medium (Gibco, USA) at 28 ℃. The plasmid pDEST10-VP60 was transformed into DH10Bac *E.coli* competent cells, to generate recombinant bacmid DNA (Bac-to-bac expression system, Invitrogen, USA). The recombinant bacmid DNA was used to transfect Sf9 insect cells to generate the corresponding recombinant baculovirus, Bac-RHDV2-VP60, using Cellfectin II transfection reagent (Invitrogen, USA). Viral titres were determined by an end-point dilution assay (EPDA) using Sf9 cells. Subsequent infections were performed by adding virus to cells at a multiplicity of infection (MOI) of 10 $TCID_{50}$ units per cell. Cells were incubated for 2h and then medium was changed by fresh medium. Plaque purification was performed to isolate a pure viral stock. Briefly, the Sf9 cell monolayers were incubated with 10-fold serial dilutions of baculovirus Bac-RHDV2-VP60. After infection for 2h, the medium containing virus was replaced by fresh medium, and the cells were overlaid with 1% low melting-point agarose (Sangon, ShangHai) in Sf-900™ Medium (1.3X) medium (Gibco, USA) and incubated at 28℃. After 7 days, several plaques were randomly picked from serial dilutions. Later the recombinant baculoviruses from individual plaques were amplified, titrated and then used to inoculate Sf9 cells.

Immunofluorescence assay and western blot analysis

Sf9 cells were infected with Bac-RHDV2-VP60 at a multiplicity of infection (MOI) of 10 followed by incubation at 28℃ for three days. Indirect immunofluorescence assay (IFA) was used to check the expression of VP60 in Sf9 cells. Sf9 cells were fixed with 4% paraformaldehyde, then anti-His mouse monoclonal antibody was used for first antibody and FITC-conjugated rabbit-anti-mouse antibody was used for detection. To check the expression by western blot analysis, supernatants and cell lysates were collected. Cells were lysed in RIPA buffer (Beyotime, Shanghai) and anti His-tag antibodies were used for VP60 detection. Furthermore, Sf9 cells were infected with Bac-RHDV2-VP60 at a MOI of 10 to test the VP60 expression level at different timepoints post infection. Culture fluids and cells were collected from six-well plates, in volumes of 2 ml, and lysed by sonication, with a 2 mm Ultrasonic

Amplitude Transformer giving 5 s pulses at 300W with intervals of 9 s for a total of 10 min. Next, the lysates were clarified by centrifugation at 8000 rpm for 15 min to remove the cell debris. Then, the lysates were subjected to ultracentrifugation at 40,000 rpm for 3 h to pellet the VLPs. Finally, the pellets were resuspended in 80 µl PBS and analysed by SDS-PAGE and western blot analysis.

Transmission electron microscopy

To test the ability of RHDV2 VP60 to self-assemble into VLPs, the supernatants of lysed Bac-RHDV2-VP60 infected Sf9 cells were clarified and VLPs were pelleted as above and then subjected to caesium chloride density gradient centrifugation. The VP60 band was isolated with a needle, diluted in PBS and centrifuged again to remove the CsCl. Finally, the pellet was resuspended in PBS. The caesium chloride purified material was checked by TEM via negative staining with 0.5% aqueous uranyl acetate. The grids were observed with a transmission electron microscope (H-7500, Hitachi, Japan) operating at 80kV.

Preparation of RHDV2 VLPs and control antigen

To prepare RHDV2 VLPs for immunization trials, Sf9 cells were infected at a MOI of 1 for 72h with plaque-purified Bac-RHDV2-VP60. Cells were lysed ultrasonically in the growth medium, and then clarified by centrifugation at 8,000 rpm for 15 min. The supernatants were collected and the total protein concentration was measured by a BCA assay and then used for immunization experiments. To formulate the experimental vaccines, MONTANIDE ISA 201VG adjuvant (SEPPIC, Shanghai) was mixed at a ratio of 1:1 with VLPs by Emulsifying Equipment (FLUKO, Shanghai) for injection. Sf9 cell lysates from uninfected cells were subjected to the same treatments to generate the negative control antigen.

Vaccination of rabbits with RHDV2 VLPs and ELISA assays

Fifteen 8-week old New Zealand White Rabbits which lacked anti-RHDV antibodies were purchased from the Laboratory Animal Centre of Shanghai Veterinary Research Institute and raised in pathogen-free, isolated cages. All animals used in the immunization trials were approved in compliance with the guidelines of Animal Research Ethics Board of Shanghai veterinary institute, CAAS. Rabbits were immunized with 2 mg VLPs, 5 mg VLPs or with control antigen via subcutaneous injection. Serum samples were collected from each immunized rabbit at 0, 7, 14 and 21 days after immunization. RHDV2-specific antibodies in immunized rabbits were detected by indirect ELISA assays as described (Guo *et al.* 2016). Briefly, 96-well microplates were coated overnight at 4℃, with GST-tagged RHDV2 VP60 protein produced in *E. coli* using a PGEX-4T-1 expression vector, followed by blocking the

plates with 5% skimmed milk diluted in PBS-Tween (PBST) at 37℃ for 1 h. After washing with PBST, the plates were incubated for 1 h with 100 μl of serum samples (1:500 diluted in PBS), and then washed with PBST. Next, 100 μl of HRP-conjugated goat anti-rabbit IgG (Santa Cruz, USA) was added at a dilution of 1:10000 and again incubated for 1 hour. The plates were washed three times and then 100 μl TMB substrate (Beyotime, China) was added and the plate was incubated in the dark for 15 min. Finally, 50 μl 2M sulfuric acid was added to stop the colour development, after which the absorbance was measured at 450 nm by using a microplate reader (Biotek, USA). All samples obtained were measured in quadruplicate.

Cytokine assays

The level of cytokines at weeks 0,1,2,3 post-immunization was examined to investigate the efficiency of the cellular immune response induced by VLPs. Therefore, the serum collected above was used to determine the levels of the Th1-Type I related cytokine IFN-γ, while IL-4 levels were measured to check for Th2-type 2 cytokines. Commercial Elisa Kits (R&D Minneapolis, MN, USA) were used for these assays.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism Version 5. The data including antibody response and cytokine production were compared by one-way ANOVA among different groups. $P<0.05$ was considered significantly different.

Table 4.2. Selected RHDV sequences to construct a phylogenetic tree.

Genbank Accession number	Genotype	Strain name	Year of isolation
KX844830	RHDVa	SCH04	2016
KY171748	RHDVa	Sch07	2017
AB300693	RHDVa	Hokkaido/2002/JPN	2009
EU003581	RHDVa	NY-01	2007
KY622129	RHDVa	P175	2017
MF598301	RHDVa	K5_08Q712_BatchRelease1/2008	2017
DQ205345	RHDVa	JX/CHA/97	1997
JF412629	RHDVa	HYD	2011
HM623309	RHDVa	NJ-2009	2009
KF677011	RHDVa	STR2012	2012
KY679905	RHDVa	STR2014	2014
AF258618	RHDVa	Iowa2000	2000
EF558583	RHDVa	Triptis	2008
EU003582	RHDVa	UT-01	2001
EF558586	RHDVa	Hartmannsdorf	2007
Z29514	RHDV	SD	2005
KU882095	RHDV	ZD0	2000
EF558576	RHDV	Jena	2007
KY622127	RHDV	P158	1998
DQ189078	RHDV	Saudi Arabia	2005
KX357670	RHDV	AUS/ACT/MtPt-2/2010	2010

KT006735	RHDV	AUS/WA/Bunbury/2000	2000
KT006733	RHDV	AUS/WA/Gnowangerup/1999	1999
U54983	RHDV	Czech strain V351	1997
EF558579	RHDV	NZ54	2007
KT006725	RHDV	NZL/Otago/Queensberry/74/2013	2013
GQ166866	RCV	MRCV	2001
KP129397	RHDV2(RHDVb)	Tar06-12	2015
MF421684	RHDV2(RHDVb)	AUS/VIC/AC-1/2016	2016
KF442961	RHDV2(RHDVb)	Algarve1	2013
KM115680	RHDV2(RHDVb)	CBEstoi13-7	2013
MF598302	RHDV2(RHDVb)	AUS/NSW/CAR-3/2016	2016
KM979445	RHDV2(RHDVb)	CBVal16	2012
KP129395	RHDV2(RHDVb)	Rij06-12	2014
KP090976	RHDV2(RHDVb)	CBAnd1	2012
KM878681	RHDV2(RHDVb)	N11	2011
KP129398	RHDV2(RHDVb)	Zar11-11	2010

4.3 Results

Pathology of RHDV2 infection in a European rabbit

A specimen of the European rabbit (*Oryctolagus cuniculus*) was found dead on 22 December 2015 in a garden in Nederweert, in the province of Limburg, The Netherlands. Post-mortem examination showed it was a young adult male, in good condition (based on muscle development and degree of fat storage). The trachea mucosa was hyperemic, and the anterior lung lobes were dark red and consolidated. The liver was light brown and soft.
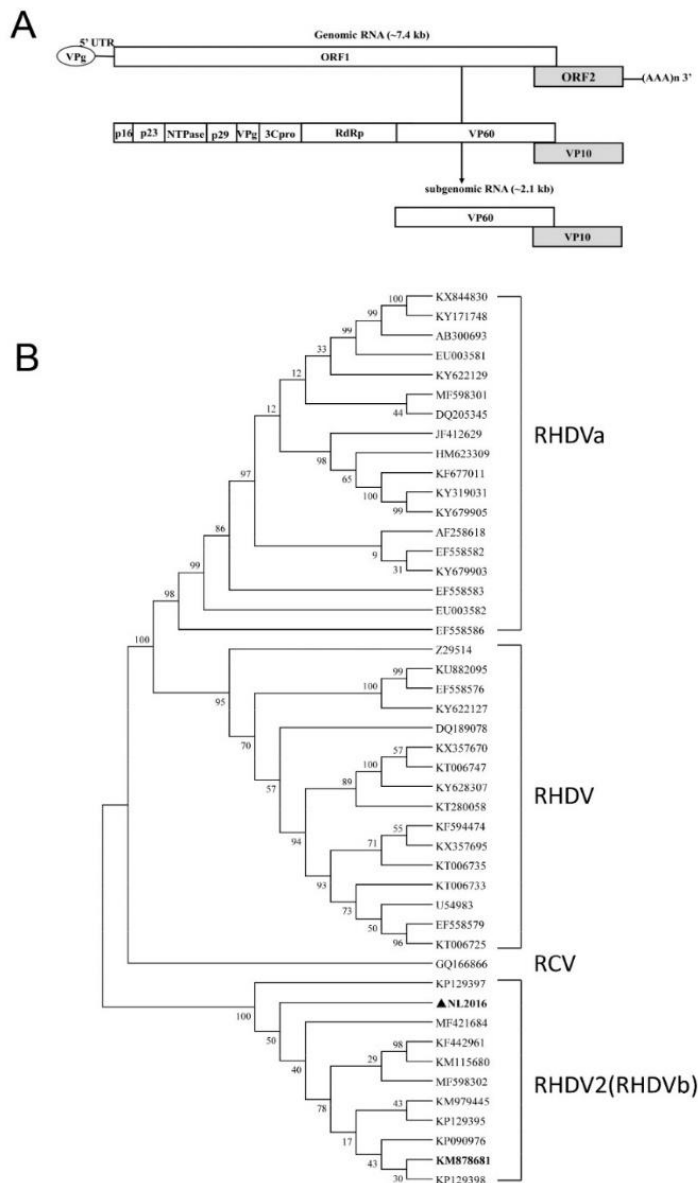
Figure 4.1. Genome organization of strain RHDV2-NL2016 and phylogenetic analysis.

A) Genome organization of RHDV2. The RHDV2 genome is a 3'polyadenylated single-stranded positive-sense RNA of ~7.4 kb encoding 2 open reading frames (ORFs). A subgenomic mRNA of ~2.1 kb formed during viral RNA replication. A viral genome-linked protein (VPg) is covalently attached to the 5' untranslated region (5'UTR). ORF1 encodes a polyprotein that is proteolytically cleaved to form the nonstructural proteins p16, p23, NTPase, p29, VPg, the viral protease 3Cpro, the RNA-dependent RNA polymerase (RdRp) and the major capsid protein VP60. ORF2 encodes the minor structural protein VP10. B) Phylogenetic neighbor-joining tree constructed based on the VP60 gene of RHDV2-NL2016 and multiple reference sequences (Table 4.2).

The gastrointestinal tract was filled with feed remains, and a few coccidia and cestode and nematode parasites were observed (incidental findings). Main histopathologic findings were diffuse swollen and hyper-eosinophilic hepatocytes, diffuse hyperemia and necrosis of lymphoid cells in the spleen, and pulmonary hyperemia with moderate to severe intra-alveolar edema and focal hemorrhage. The observed per acute hepatic necrosis, splenic necrosis and acute pneumonia are fatal lesions consistent with RHDV2 infection, which was confirmed by PCR analysis.

Complete genome sequence and phylogeny of RHDV2 isolate NL2016

The liver of the dead rabbit was collected and used for isolation of total RNA. As the 5'UTR of RHDV2 is conserved among different strains, primers for RT-PCR were designed based on the conserved regions of RHDV2, while for the 3'UTR we designed a primer ending with oligo-dT (**Table 4.1**). RT-PCR was performed and the amplified PCR products were cloned into pJET1.2 and then processed for Sanger sequencing. The sequences were assembled into a consensus complete RHDV2 genome (named NL2016 isolate) with a length of 7473 nucleotides (nt) (Genbank accession no. MN061492).

The RHDV2-2016 genome has two opening reading frames (ORFs) (**Figure 4.1A**), of which the first ORF is processed in a number of nonstructural proteins and the structural protein VP60. By selecting RHDV and RHDV2 GenBank sequences (**Table 4.2**) we constructed a phylogenetic tree based on the VP60 nucleotide sequences. The NL2016 isolate clustered with other RHDV2 (RHDVb) isolates including the type strain from Spain (RHDV2-N11, GenBank: KM878681.1). This indicates that NL2016 is a typical RHDV2 strain (**Figure 4.1B**).
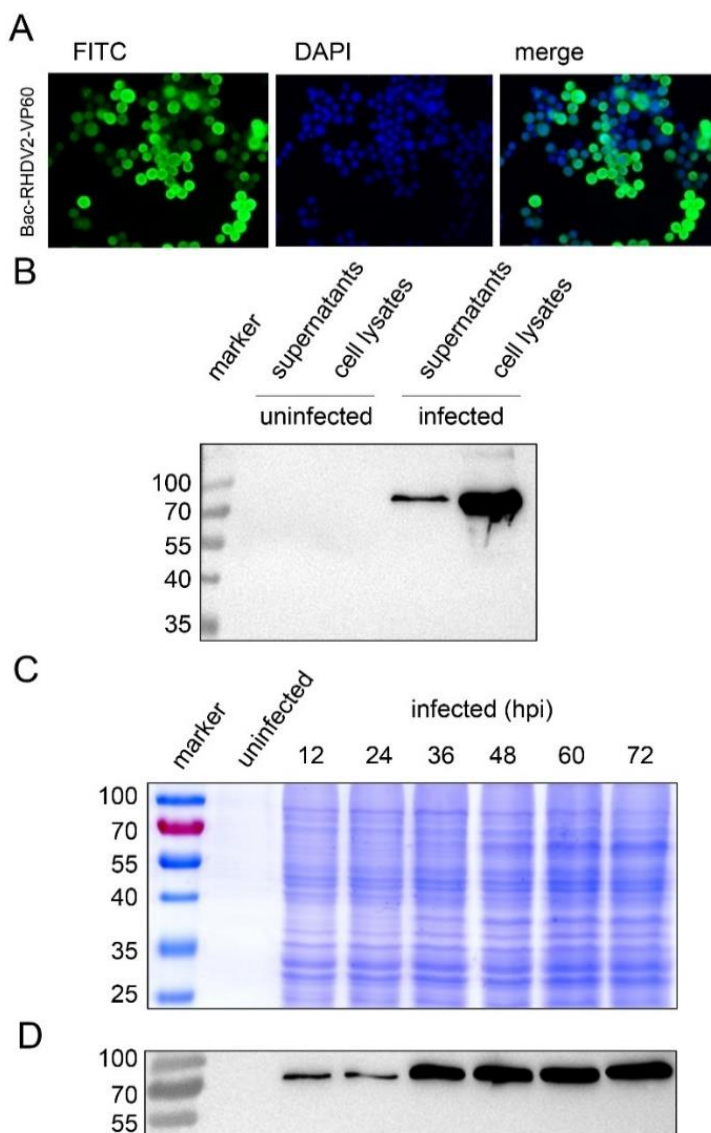
Figure 4.2. VP60 synthesis by infection of Sf9 cells with a recombinant baculovirus.

A) Indirect immunofluorescent assay to show the expression of His-VP60 in transfected Sf9 cells. Anti-His mAb was used as the primary antibody, FITC-conjugated rabbit-anti-mouse antibody was used for detection. DAPI was used to stain cell nuclei. B) Western blot (WB) analysis of Bac-His-VP60 infected Sf9 cells. Cells were infected with the P5 baculovirus stock at MOI 10, collected at 3 days post infection (d.p.i) and checked by WB. Anti-His mAb was used as the primary antibody, HRP-conjugated rabbit-anti-mouse antibody was used for detection. C) SDS-PAGE and D) WB were used to check the expression level of His-VP60 in Sf9 cells at different times point infection. Anti-His mAb was used as the primary antibody, HRP-conjugated rabbit-anti-mouse antibody was used for detection.

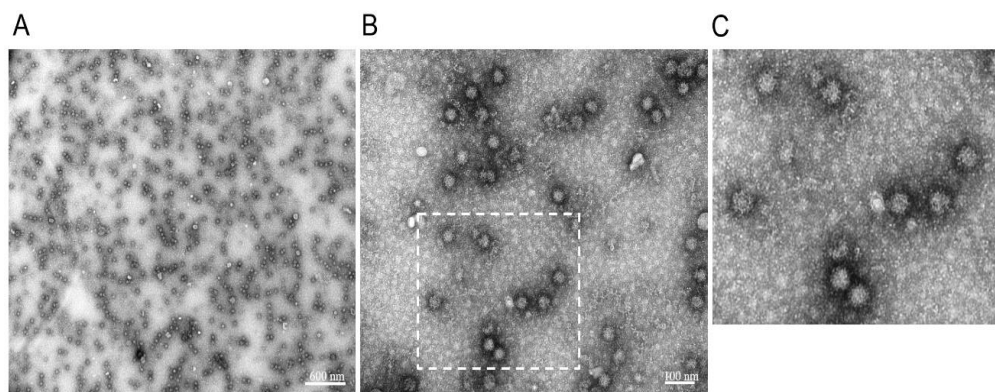**Baculovirus expression of RHDV2 VP60 in Sf9 insect cells**

Figure 4.3. TEM pictures of RHDV2-VLPs at different magnifications.

Transmission electron microscopy of CsCl density gradient purified RHDV2 VLPs. Samples were negatively stained with 0.5% aqueous uranyl acetate. Bars represent 600 nm (A) and 100 nm (B, inset enlarged in C).

In order to produce RHDV2 VLPs in insect cells, the VP60 ORF was PCR amplified and cloned downstream of a His-tag into the expression plasmid pDEST10 under control of the very strong polyhedrin promoter. The pDEST10-VP60 plasmid was used to construct a recombinant baculovirus bacmid, which was then transfected into Sf9 insect cells to generate the recombinant baculovirus Bac-RHDV2-VP60.

Indirect immunofluorescence assay (IFA) using anti-his antibodies showed that VP60 accumulated to high levels upon transfection of bacmid DNA (**Figure 4.2A**). Supernatants and lysates of infected cells were separately collected after 3 days post infection (dpi) with plaque purified, passage 5 (P5) virus stocks. Next, VP60 expression was confirmed by western blot analysis using His-tag specific antibodies. The expected molecular mass of recombinant His-VP60 is approximately 70 kDa (**Figure 4.2B**). Both the supernatant and cell lysates were positive, with more protein present in the cell lysate. Next, Sf9 cells infected with Bac-RHDV2-VP60 at a MOI of 10 and both the culture fluid and cells were collected, lysed by sonication and clarified by low-speed centrifugation to remove cell debris. After ultracentrifugation, the pellet was resuspended in PBS and the expression level of RHDV2-VLPs was checked by SDS-PAGE (**Figure4. 2C**) and western blot (**Figure4. 2D**). RHDV2-VLPs could be detected on western blots as early as 12 hpi (**Figure4. 2D**), but the highest expression level was observed at 60 hpi (**Figure4. 2C**).

Characterization of RHDV2 VLPs by electron microscopy

To determine whether the overexpressed RHDV2 VP60 protein self-assembled into VLPs, CsCl density gradient purified material was visualized by electron microscopy analysis. TEM showed a high concentration of spherical particles **(Figure 4.3A)** with diameters ranging from 30-35 nm (Figs. 4.3BC). This demonstrates the successful assembly of RHDV2 VLPs in Sf9 insect cells.

Immunogenicity of RHDV2 VLPs in rabbits
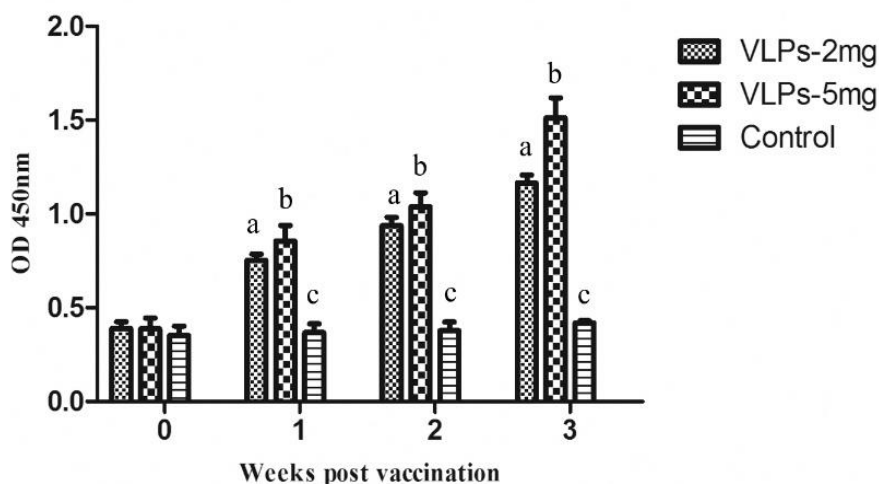
Figure 4.4. Serum antibody responses in rabbits after immunization with RHDV2 VLPs.

Rabbit sera from different groups of immunized animals were collected at 0, 1, 2 and 3 weeks post immunization and then analyzed by indirect ELISA. All rabbit sera were diluted 1:500 before testing.

The RHDV2 VLPs were then used to immunize rabbits. Groups of 5 rabbits were immunized subcutaneously with different dosages of VLPs or with control antigen. Serum was collected every week and RHDV2-specific antibodies in immunized rabbits were detected by indirect ELISA assay. The antibody titers of rabbits immunized with VLPs were shown to increase with time and are statistically different from the control group. As expected, the antibody titers in rabbits immunized with 5 mg VLPs are a little higher than those immunized with 2 mg VLPs **(Figure4. 4)**.

To investigate the cellular immune response to RHDV2 VLP immunization, IFN-γ and IL-4 production levels were measured in sera from immunized rabbits. The levels of IFN-γ (**Figure 4.5A**) and IL-4 (**Figure 4.5B**) in both VLP groups were increasing over time and were significantly different from the control group, indicating that both Th1 and Th2 responses were induced by the VLPs.
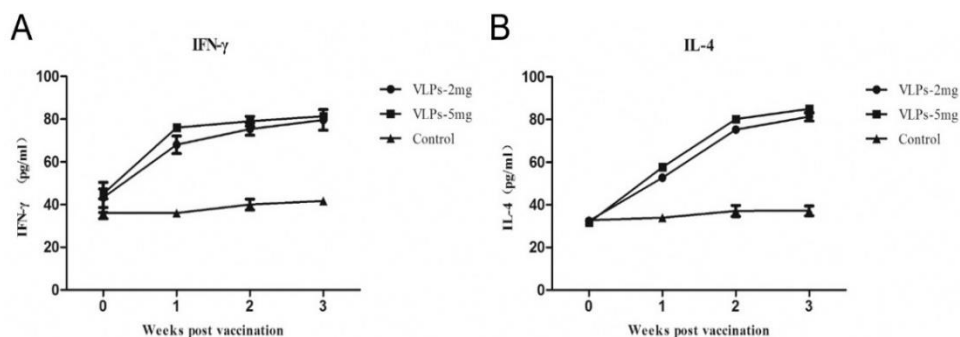

Figure 4.5. Production of cytokines IFN-γ and IL-4 in rabbits after immunization.

The rabbit sera collected were used to analyze the serum levels of IFN-γ and IL-4 by ELISA. The IFN-γ and IL-4 level of rabbits immunized subcutaneously with 2 mg or 5 mg VLPs were both significantly higher as compared to the control group, but not different from each other.

4.4 Discussion

RHD is a highly contagious and lethal viral disease of both wild and domestic rabbits and associated with large economic losses. RHD may be caused by related but different lagoviruses. Since the first discovery of RHDV2 in 2010 in RHDV-vaccinated rabbits, RHDV2 has spread through wild rabbit populations and has replaced RHDV in several countries. RHDV2 is genetically and antigenically distinct from RHDV and thus can overcome immunity to classical RHDV. The case-fatality in mature rabbits infected with RHDV2 is lower compared to classic RHDV infection, however young rabbits are more susceptible to RHDV2 (Capucci *et al.* 2017). Before GI.2 emerged, only *Lepus granatensis* (Lopes *et al.* 2014)and *Oryctolagus cuniculus* had been infected with RHDV. With the emerge of GI.2, more species have been observed to become infected, e.g. *Lepus corsicanus* (Camarda *et al.* 2014), *Lepus europaeus* (Velarde *et al.* 2016, Hall *et al.* 2017), *Lepus capensis mediterraneus* (Puggioni *et al.* 2013) and *Lepus timidus* (Neimanis *et al.* 2018).RHDV2(G1.2) Thus, it is urgent to control and prevent transmission of RHDV2.

Traditional RHD inactivated vaccines are effective, but production of the viral antigens can be cumbersome given the lack of cell lines supporting RHDV and RHDV2 infection. VLPs are a product of modern biotechnology and can be produced at large scale, at low cost, and generally at the lowest biosafety level. VLPs structurally resemble the authentic virus as they are the result of self-assembly of solely the viral structural protein(s). Due to the lack of viral genetic material inside the capsid, VLPs are non-infectious and have a similar safety profile as subunit vaccines. However, in comparison to subunits, VLPs display their antigenic epitopes in a structured, repetitive fashion that may serves as pathogen-associated molecular patterns (PAMPs). In this way, co-stimulatory signals are available for the activation of T-lymphocytes by antigen presenting cells. This is one of the reasons that VLPs in general tend to generate a more potent humoral and cellular immune response (Metz *et al.* 2013).

RHDV VLPs have been produced in multiple expression systems, including the BEVS. The advantage of baculovirus expression in insect cells is the ease of use and robustness of the system, leading to high expression of almost any pro- or eukaryotic protein. Another advantage of the BEVS is the efficiency of post-translation modifications, such as phosphorylation or glycosylation, which is important for the functionality of complex viral (glyco)proteins (van Oers *et al.* 2015). Indeed, it has been shown that RHDV VP60 capsid protein expression in insect cells leads to the formation of VLPs that can easily be purified for downstream applications (Peacey *et al.* 2007). VLPs from new variant RHDV2(G1.2) have been generated to demonstrate the different antigenic properties exhibited by RHDV and RHDV2(G1.2) (Barcena *et al.* 2015), to generate monoclonal antibodies against RHDV2(G1.2) capsid and to evaluate the sensitivity and specificity of an ELISA for the detection of RHDV2(G1.2) in liver extracts (Dalton *et al.* 2018) and to test their immunogenicity and protection potential against lethal virus challenge(Muller *et al.* 2019).

In our study, we obtained the complete RHDV2 genome sequence from a diseased rabbit, which was found in the Netherlands in 2016. The contemporary RHDV2-NL2016 isolate appeared to be closely related to other European RHDV2 genotypes for which the VP60 sequence is available, suggesting that the virus quickly spread over the continent after its initial introduction. It is known that RHDV2(G1.2) strains have evolved by recombination between the non-structural proteins and minor structural proteins, VP60 and VP10. Recombinants may e.g. include the structural proteins of GI.2, with non-structural proteins derived from non-pathogenic lagoviruses (GI.3 and GI.4) or from pathogenic GI.1 strains (Lopes *et al.* 2015, Silverio *et al.* 2018). However, according to the phylogenetic tree we generated based on the amino acid sequence of the NL2016 non-structural proteins, we could only see that NL2016

shares a close relationship with other RHDV2(RHDVb/ GI.2) sequences (KM979445, KP129397) and not with GI.1 strains.

We cloned the RHDV2-NL2016 VP60 gene in a recombinant baculovirus expression vector using Gateway and Bac-to-bac technology and demonstrated the successful expression of RHDV2 VLPs in Sf9 insect cells (Figs. 2-3). The icosahedral VLPs had a regular, spherical appearance. The VLPs could easily be purified and were indistinguishable from infectious virus when visualized by electron microscopy. Immunization of rabbits with RHDV2 VLPs induced high levels of VP60-specific antibodies. Furthermore, the production of the Th1-Type I cytokine IFN-γ, and the Th2-type 2 cytokine IL-4 in the immunized rabbits was significantly higher than in the negative control group.

Together, our results suggest that RHDV2 VLPs produced in insect cells with recombinant baculovirus technology are highly immunogenic in rabbits. The VLPs may be further developed as promising, novel vaccine to protect rabbits against RHDV2. An attractive possibility would be to generate a bivalent RHD vaccine by combining the RHDV and RHDV2 VLPs in a single formulation. Finally, the VLPs may find application as potent antigen in diagnostic serological assays to discriminate RHDV from RHDV2 infections.

Chapter 5

Construction and immunogenicity of novel bivalent virus-like particles bearing VP60 genes of classic RHDV(GI.1) and RHDV2(GI.2).

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Construction and immunogenicity of novel bivalent virus-like particles bearing VP60 genes of classic RHDV(GI.1) and RHDV2(GI.2).

Abstract: Rabbit hemorrhagic disease (RHD) is an acute, inflammatory, septic, and devastating infectious disease caused by Rabbit hemorrhagic disease virus (RHDV), which poses a serious threat to the rabbit industry. RHDV2 (GI.2/RHDVb), a recently reported new variant could cause RHD in wild populations, but also RHDV-vaccinated rabbits. For now, both RHDV and RHDV2 are the main causes of RHD. To develop a new subunit vaccine that could protect rabbits against both classic RHDV and RHDV2 infections, we constructed a recombinant baculovirus (Bac-classic RHDV VP60-RHDV2 VP60) containing the VP60 genes of classic RHDV and RHDV2. Both VP60 genes were well expressed simultaneously in Spodoptera frugiperda cells (Sf9) after infection with the recombinant baculovirus. Transmission electron microscopy showed that the recombinant VP60 self-assembled into virus-like particles (VLPs). The antigenicity and immunogenicity of the bivalent VLPs vaccine were examined with animal experiments. Our results demonstrated that both the humoral and cellular immune responses were efficiently induced in rabbits by a subunit vaccine based on the recombinant baculovirus. In addition, all rabbits immunized with the bivalent VLPs vaccine survived after challenged with classic RHDV, and showed no clinical signs of RHD, whereas all the rabbits in the negative control group died from classic RHDV infection and showed typical clinical signs of RHD. In summary, our results indicated that the recombinant baculovirus carrying two VP60 genes is a candidate construct from which to develop a bivalent VLPs vaccine against both classic RHDV and RHDV2 infections.

5.1 Introduction

Rabbit hemorrhagic disease (RHD) is an acute, inflammatory, septic, and devastating infectious disease. It was first reported in China and spread around the world within a few years, causing important economic losses in the rabbit industry (Xu 1991, Mitro and Krauss 1993, Delibes-Mateos *et al.* 2007). *Rabbit hemorrhagic disease virus* (RHDV) belongs to the family *Caliciviridae* and the genus *Lagovirus*. The genus *Lagovirus* also contains *European brown hare syndrome virus* (EBHSV), which only infects hare species (*Lepus europaeus* and *L. timidus*) (Nauwynck *et al.* 1993). Although RHDV and EBHSV have many similarities in their clinical signs, however, the pathological and histopathological changes they induce, their mortality rates, virion morphology, and antigenicity differs, they are distinct agents and infect different species (Capucci *et al.* 1991, Marcato *et al.* 1991, Chasey *et al.* 1992, Fuchs and Weissenbock 1992, Wirblich *et al.* 1994, Lavazza *et al.* 1996).

Since 2010, there have been numerous cases of RHD in RHDV-vaccinated and wild rabbits in north-western France (Le Gall-Recule *et al.* 2011). A new RHDV variant, named RHDV2/b/GI.2, was identified for the first time on an industrial farm in Udine Province of Italy in 2011(reference). Compared with classic RHDV, RHDV2 affects not only the European rabbit (*Oryctolagus cuniculus*) (Camarda *et al.* 2014) but also the Sardinian cape hare (*L. capensis*) (Puggioni *et al.* 2013), the Corsican hare (*L. corsicanus*) (Camarda *et al.* 2014), and the European hare (*L. europaeus*) (Neimanis *et al.* 2018). The incubation time of RHD caused by RHDV2 is much longer than that caused by classic RHDV, and its morbidity and mortality rates vary. RHDV2 can cause the death of young rabbits aged 2-3 weeks, which suggests that RHDV2 may use different receptors (Le Gall-Recule *et al.* 2011). RHDV2 has now been reported in many countries in Europe, Australia, Africa, and North America (Le Gall-Recule *et al.* 2011, Dalton *et al.* 2012, Abrantes *et al.* 2013, Camarda *et al.* 2014, Dalton *et al.* 2014, Westcott *et al.* 2014, Duarte *et al.* 2015, Hall *et al.* 2015, Martin-Alonso *et al.* 2016, Neimanis *et al.* 2018, Carlos Rouco *et al.* 2019), and in many areas, RHDV2 has replaced classic RHDV as the major cause of RHD (Lopes *et al.* 2014, Peacock *et al.* 2017, Mahar *et al.* 2018).Most strikingly, RHDV2 can cause the death of classic RHDV-vaccinated rabbits (Peacock *et al.* 2017), which suggests that RHDV2 differs antigenically from classic RHDV. Therefore, an RHDV2 vaccine is urgently required to limit the spread of RHDV2 and reduce the risk of outbreaks of RHDV2 infection in other countries. VP60 , a major viral structural protein and immunogenic protein of RHDV (Mikschofsky *et al.* 2009), it self-assembles into virus-like particles (VLPs) in different expression systems, including bacterial systems (Guo *et al.* 2016), insect systems (Perez-Filgueira *et al.* 2007), plant systems (Fernandez-Fernandez *et al.* 2001), and yeast systems (Farnos *et al.* 2005). Whereas, compared with other systems, the baculovirus

expression vector system (BEVS) have the advantages of easy culture, ready protein expression, and the compatible post-translational modifications of proteins, so they are widely used in laboratories or factories to produce VLPs (Roldao *et al.* 2010).

In this study, we used BEVS to simultaneously express the VP60 capsid genes of both classic RHDV and RHDV2 to generate VLPs, then we tested their immunogenicity in rabbits.

5.2 Materials and methods

Construction of recombinant vector and expression of recombinant baculovirus

A recombinant vector containing the RHDV2 VP60 gene was supplied by Prof. Gorben Pijlman of Wageningen University (Wageningen, Dutch). Another recombinant plasmid containing the complete classic RHDV genome (GenBank accession number: DQ205345) is maintained in our laboratory. Two pairs of VP60 primers containing homologous arms and a histidine (His) or hemagglutinin (HA) tag (Table 1) were designed to amplify VP60 gene with *FastPfu* Fly DNA Polymerase (Transgen Biotech, China) from the two recombinant plasmids mentioned above. The PCR products were purified with SanPrep Column DNA Gel Extraction Kit (Sangon biotech, China) and subcloned together into the pFastBac Dual Expression Vector (Invitrogen, Massachusetts, USA) with ClonExpress II One Step Cloning Kit (Vazyme, China). The expression of the classic RHDV VP60 was controlled by the polyhedrin (PH) promoter and the expression of the RHDV2 VP60 gene was under the p10 promoter. The recombinant plasmid pFastBac Dual-classic RHDV VP60-RHDV2 VP60 was confirmed with DNA sequencing (Sangon Biotech, China).

The recombinant bacmid was generated by transforming the recombinant plasmid into DH10Bac competent *Escherichia coli* cells (Invitrogen, USA). Subsequently, the recombinant bacmid DNA was used to transfect Sf9 insect cells to generate the corresponding recombinant baculovirus, Bac-classic RHDV VP60-RHDV2 VP60, using Cellfectin II transfection reagent (Invitrogen, USA) ,and the one-step growth curve was determined according to the titer of the recombinant baculovirus which calculated by an end-point dilution assay (EPDA) using Sf9 cells.

Immunofluorescence assay (IFA) and western blotting analysis

The VP60 expression of the classic RHDV and RHDV2 was confirmed with western blotting analysis and IFA. For the western blotting analysis, Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 1 at 28 °C for 72-96 h. The infected Sf9 cells were then analyzed with western blotting with mouse anti-His monoclonal primary antibody (Sigma, USA) or mouse anti-HA monoclonal primary antibody (Abcam, UK)

and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Jackson Immuno Research, USA). For IFA, Sf9 cells were infected with the recombinant baculovirus at a MOI of 1 at 28 °C for 24-32 h. The infected Sf9 cells were fixed with 4% paraformaldehyde for 10 min and washed with phosphate-buffered saline (PBS)-Tween buffer (pH 7.2), and furtherly blocking with 5% Bull Serum Albumin (BSA). The anti-VP60 monoclonal antibody (Laboratory stock) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Invitrogen, USA) was used as the primary and secondary antibodies, respectively. To detect the expression levels of classic RHDV VP60 and RHDV2 VP60 at different time points after infection. Sf9 cells were infected with the recombinant baculovirus at a MOI of 1 at 28 °C. The infected cells were then collected at 6, 12, 24, 48, 72, or 96 hours post infection (hpi) and lysed with ultrasonication (5 s pulses at 300 W, with intervals of 9 s, for a total of 5 min) using an Ultrasonic Amplitude Transformer (Jingxin, China). The lysates were centrifuged at $10,000 \times g$ for 30 min, and the supernatants were collected and analyzed with SDS-PAGE and western blotting.

Transmission electron microscopy (TEM) detection

To detect if the ability of VP60 to self-assemble into VLPs, the supernatants of infected Sf9 cells were clarified with cesium chloride density gradient centrifugation and washed with PBS to remove the CsCl. The caesium chloride purified material was negatively stained with 1% uranyl acetate (pH 4.5) and analyzed with TEM (H-7500, Hitachi, Japan) operating at 80 kV.

Bivalent VLPs vaccine preparation and Vaccination of rabbits

Sf9 cells were infected with the recombinant baculovirus at a MOI of 1 at 28 °C for 72-96 h. The cells were harvested lysed and centrifuged at $10,000 \times g$ for 30 min to remove the cellular debris. The supernatants were then dissolved in 0.1% formaldehyde at 4 °C for 24 h to inactivate the recombinant baculovirus. Inactivation was confirmed by infecting Sf9 cells. The samples were then emulsified with Montanide™ ISA 201 VG adjuvant to produce the bivalent VLPs vaccine.

Twelve 8-week-old, specific-pathogen-free New Zealand white rabbits that lacked anti-RHDV antibodies were purchased from the Laboratory Animal Centre of Shanghai Veterinary Research Institute (Shanghai, China) and raised in isolated pathogen-free cages. All the immunization trials involving rabbits were approved and performed in compliance with the guidelines of the Animal Research Ethics Board of Shanghai Veterinary Institute (Shanghai, China), CAAS (no. SHVRI-ZD-2019-012). The New Zealand white rabbits were randomly divided into three groups (two rabbits of each sex, n=4). The rabbits were immunized with 0.5 mg of VLPs/rabbit (L-VLPs), 2 mg of VLPs/rabbit (H-VLPs), or the control (2 ml of Sf-900 II

SFM medium/rabbit). Serum samples from each immunized rabbit were collected at 0, 7, 14, and 21 days post-immunization. At 21 days after immunization, all the rabbits were infected with 200 LD$_{50}$ of classic RHDV (JX/CHA/97) (Cheng *et al.* 2013), and observed continuously for 7 days for clinical signs (Table 2). All the surviving rabbits in each group were euthanized with an intravenous injection of sodium pentobarbital. Pathology tests and immunohistochemical (IHC) analysis were used to identify the lesions in various tissues and reverse transcription (RT)-PCR was used to detect the presence of classic RHDV.

Enzyme-linked immunosorbent assays (ELISA)

The levels of specific antibodies against RHDV in the immunized rabbits of each group were measured with the indirect ELISA. In detail, a 96-well microtiter plate (NEST biotechnology, Wuxi, CHINA) was coated with RHDV VP60 protein (5 µg/ml) overnight at 4 °C and then blocked with 5% skimmed milk diluted in PBS at 37 °C for 2 h. After the plates were washed with PBS-Tween, the diluted serum samples (100 µL; diluted 1:1500 in PBS) were added and incubated for 1 h. After washing for three times the plates were incubated with 100 µL of HRP-conjugated goat anti-rabbit IgG antibody (diluted 1:10,000; Santa Cruz Biotechnology, USA) for 1 h. Subsequently, the plates were washed three times with PBS-Tween, and TMB substrate (100 µL; Beyotime, China) was added to them in the dark for 15 min, and the color development was stopped with 2 M sulfuric acid. The optical density of each serum sample in the plates was measured at a wave length of 450 nm with a microplate reader (Biotek, USA). Each serum sample was measured three times.

Hemagglutination inhibition (HI) assay

HI assay was performed to determine the ability of each serum sample to neutralize the RHDV virus with 1% human erythrocytes of blood group O. Serially two-fold diluted serum samples (50 µL) and an equal volume of standardized antigen containing 8 hemagglutination units was added to the wells of microtiter plates. After incubation at 25 °C for 1 h, 50 µL/well 1% human type O erythrocytes were added. The HI point titers were determined after incubation at 4 °C for 2 h.

Cytokine assays

To investigate the levels of the cellular immune responses induced by the bivalent VLPs vaccine, the Th1-related cytokine interferon γ (IFN-γ) and Th2-related cytokine interleukin 4 (IL-4) were measured in each serum sample with commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the instructions.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from each tissue of the rabbits with TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. Each RNA sample was reverse transcribed to cDNA with M-MLV Reverse Transcriptase (Promega, USA). To detect the presence of classic RHDV, the classic RHDV VP60 gene was amplified with the classic RHDV-F/R primers (Table 1), under the following reaction conditions: pre-denaturation at 95 °C for 3 min; 35 cycles denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 2 min; and a final extension step at 72 °C for 10 min. The products were resolved in 1% agarose and detected with the ChemiDoc™ MP Imaging System (Bio-rad, USA).

Statistical analysis

All data, including the antibody responses and cytokine production, were compared between the three groups with one-way ANOVA using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered to indicate significantly different.

5.3 Results
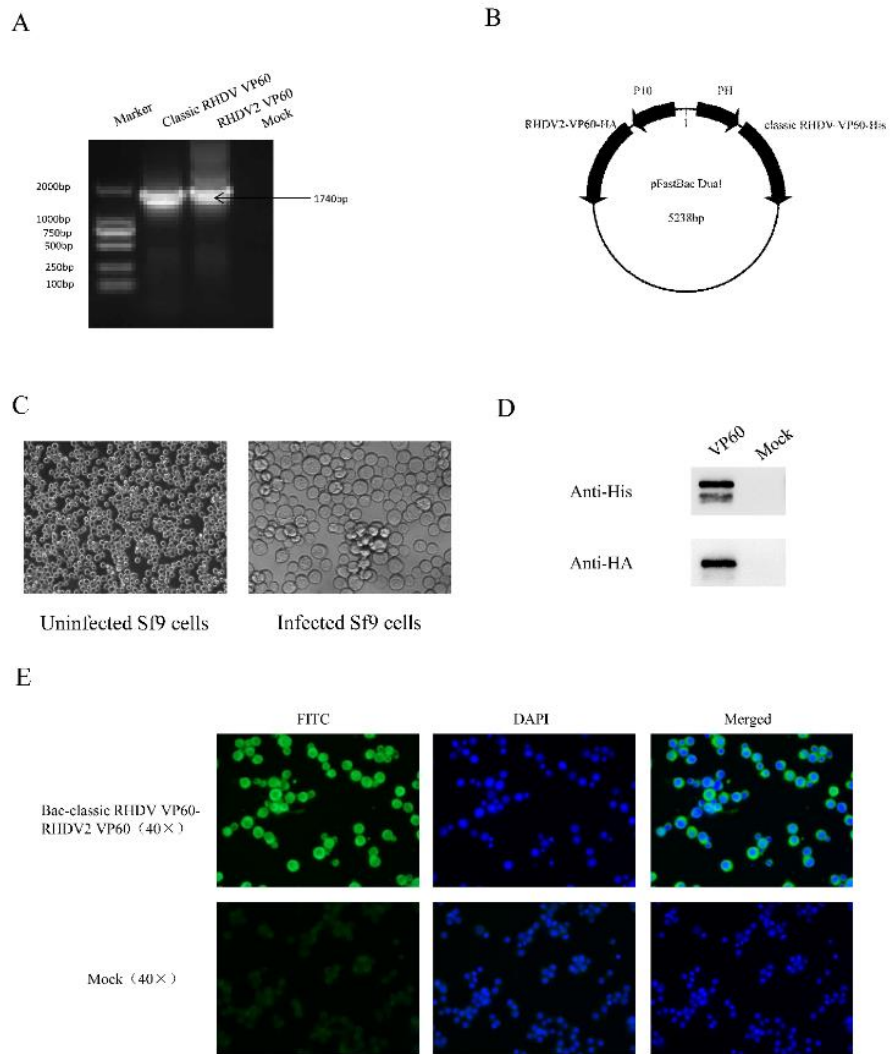
Figure 5.1 Construction of the recombinant plasmid and expression of the recombinant baculovirus in Sf9 cells.

(A) Amplification of classic RHDV and RHDV2 VP60 genes. (B), Schematic diagram of the recombinant plasmid. (C) Cytopathic effect in Sf9 cells infected with the recombinant baculovirus or control (40×). (D) Western blotting analysis of the expression of classic RHDV VP60 and RHDV2 VP60 protein in Sf9 cells infected with the recombinant baculovirus. (E) VP60 protein expression in infected Sf9 cells by IFA.

Construction and expression of recombinant plasmid

Both VP60 genes were correctly amplified and subcloned into the pFastBac Dual Expression Vector (**Figure 5.1A-B**). A recombinant bacmid was generated by transforming DH10Bac competent *E. coli* cells with the recombinant vector, which was used to create a recombinant baculovirus by transfection on Sf9 cells. After Sf9 cells were infected with the recombinant baculovirus, the diameter and volume of the infected Sf9 cells were much larger than other cells. The infected cells were filled with cell nuclei and their adhesive ability was declined at 24-48 hpi (**Figure 5.1C**). According to the one-step growth curve of the recombinant baculovirus, the titer of the recombinant baculovirus was highest at 72 hpi (**Figure 5.2A**). The recombinant VP60 proteins were efficiently and simultaneously expressed in the infected Sf9
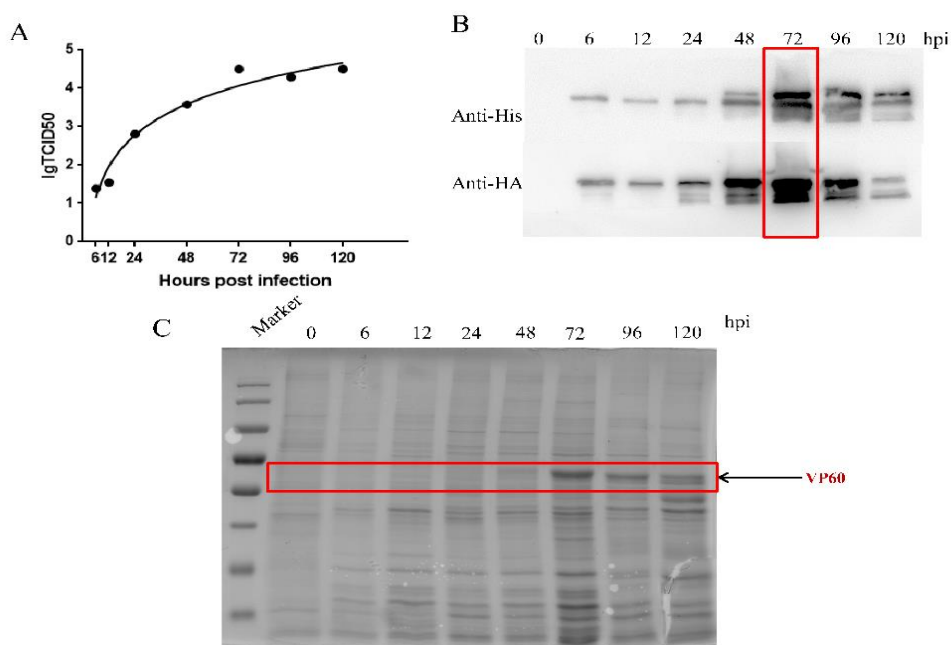
Figure 5.2 Analysis of recombinant baculovirus and expression of recombinant proteins.

(A) One-step growth curve of the recombinant baculovirus. The titer of the recombinant baculovirus was highest at 72 hpi. (B) Western blotting and (C) SDS-PAGE was used to detect the expression of the recombinant VP60 proteins at different time points post-infection.The expression level of VP60 proteins peaked at 72 h post-infection.

cells according to IFA and western blotting analyses (**Figure 5.1D-E**). The expression levels of the VP60 protein were also highest at 72 hpi and began to decrease from 96 hpi, according to the western blotting and SDS-PAGE analyses (**Figure 5.2B-C**).

Characterization of VLPs

TEM was used to characterize the self-assembly of the classic RHDV and RHDV2 VP60 proteins in Sf9 cells. The supernatants of the infected Sf9 cells were clarified with cesium chloride density gradient centrifugation, and a TEM analysis showed that the VP60 proteins could assembled into VLPs with diameters around 35-40 nm, thus demonstrating the self-
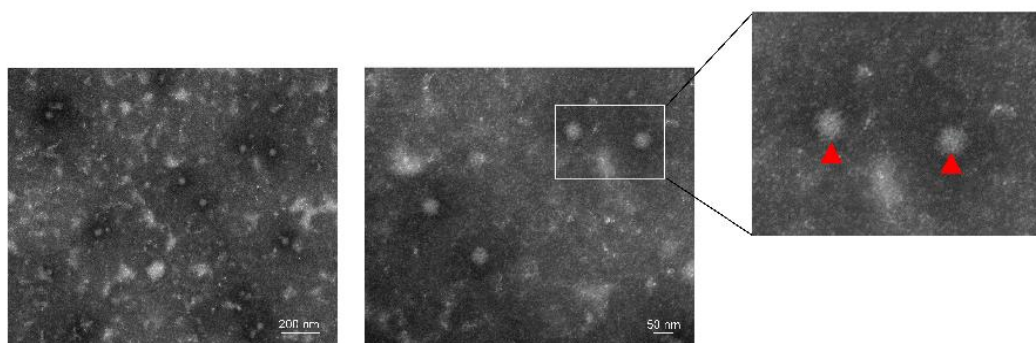
Figure 5. 3 TEM images of classic RHDV and RHDV2 VP60 VLPs.

Supernatants of infected Sf9 cells were clarified with cesium chloride density gradient centrifugation. TEM analysis showed that VP60 proteins assembled into VLPs with diameters of 35-40 nm, demonstrating the self-assembly of the VP60 proteins in Sf9 cells.

assembly ability of the VP60 proteins in Sf9 cells (**Figure 5.3**).

Immunogenic effects of the bivalent VLPs vaccine

Serum samples from the immunized rabbits of each group were collected to measure the level of specific antibodies directed against RHDV, the neutralizing capacity for the virus, and level of the cellular immune responses induced by the bivalent VLPs vaccine at 0, 7, 14, and 21 days post-immunization. The humoral and cellular immune responses were detected with indirect ELISA. In detail, the antibody titers were much higher in the rabbits immunized with the bivalent VLPs vaccine than in the rabbits immunized with the control, and they increased with

time. At 7 days after immunization, the titers in the test rabbits differed significantly from those in the control rabbits, reached their highest levels at 14 day post-immunization. The specific antibody titer of the group immunized with 0.5 mg of VLPs was lower than that of the rabbits immunized with 2 mg of VLPs, as expected (**Figure 5.4A**).
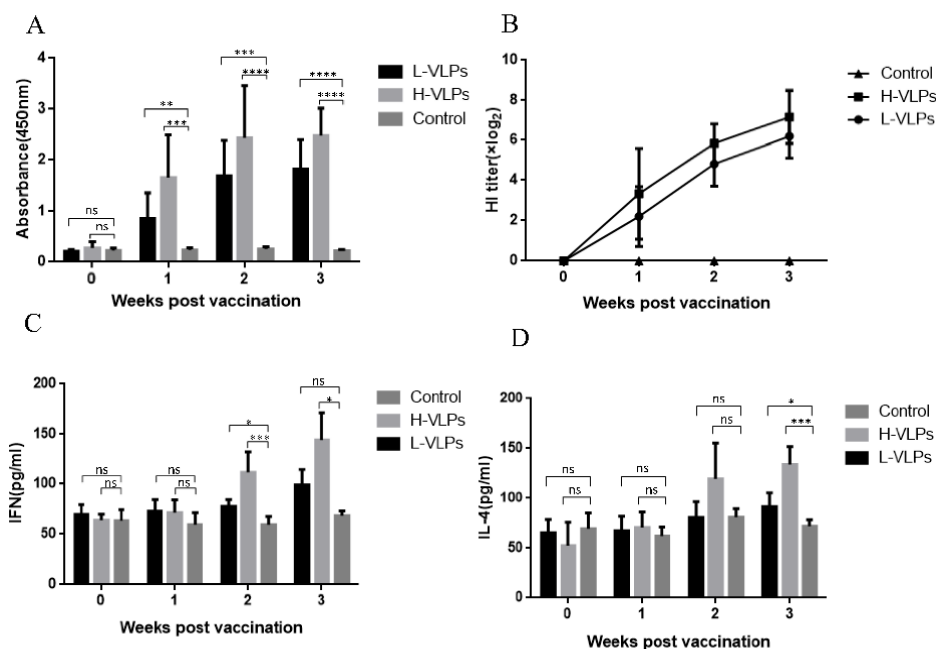
Figure 5.4 Immunogenicity of the bivalent VLP vaccine in rabbits.

(A) Specific antibodies against classic RHDV and RHDV2. (B) The HI titers of serum samples from immunized rabbits. (C) Production of Th1-related cytokine IFN-γ and (D) Th2-related cytokine IL-4 in immunized rabbits. The rabbits in each group were immunized with L-VLPs (0.5 mg of VLPs/rabbit), H-VLPs (2 mg of VLPs/rabbit), or control (2 ml of Sf-900 II SFM medium/rabbit). Serum samples were collected from each immunized rabbit at 0, 7, 14, and 21 days post-immunization. Humoral and cellular immune responses were efficiently induced by the bivalent VLP vaccine and the specific antibodies neutralized the virulence of RHDV.
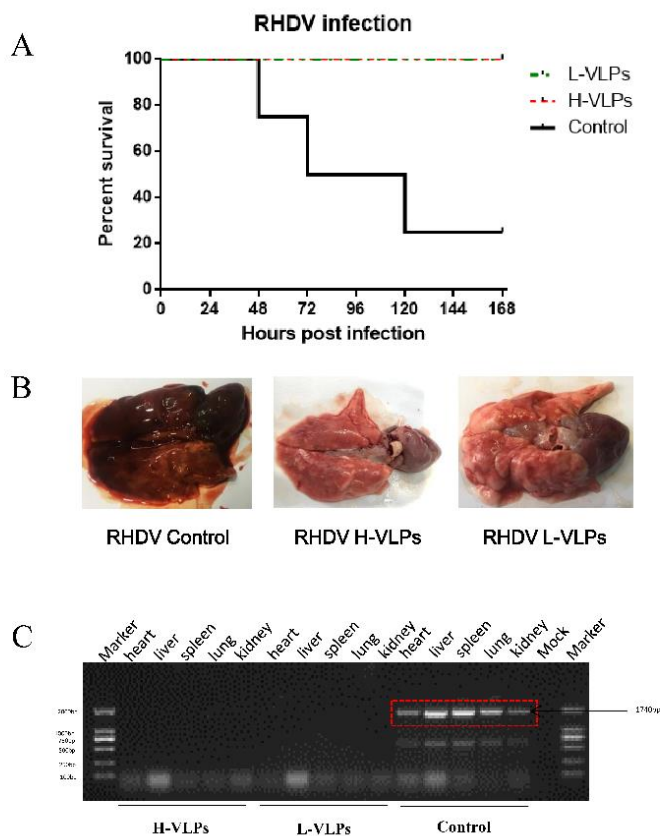
Figure 5.5 Protective efficacy of the bivalent VLP vaccine in rabbits.

(A) Percentage survival of rabbits in the three groups infected with classic RHDV. All rabbits immunized with the bivalent VLP vaccine survived, whereas three rabbits immunized with the control died at 48, 72, or 120 h post-infection. (B) Pathological changes in the rabbits of each group. The lungs of rabbits immunized with the control appeared hemorrhagic, hyperemic and edemic, whereas those of rabbits immunized with VLPs showed no typical clinical signs of RHD. (C) RT-PCR analysis of organs of the rabbits in each group to detect the spread of classic RHDV. RHDV VP60 gene was only detected in rabbits immunized with the control, indicating that the recombinant VLPs could inhibit the replication of RHDV.

HI assay was used to detect the ability of each serum sample to neutralize the virus. The HI titers of the rabbits immunized with the bivalent VLPs vaccine were much higher than those of the rabbits immunized with the control, and increased over time, as did the levels of specific antibodies against RHDV. At 7 days post-immunization, the serum samples from the rabbits immunized with the bivalent VLPs vaccine inhibited the hemagglutination ability of RHDV

and the HI titers were highest at 21 days post-immunization. The HI titers of the serum samples from the rabbits immunized with 2 mg of VLPs were much higher than those of the rabbits immunized with 0.5 mg of VLPs (**Figure 5. 4B**).

The Th1-related cytokine IFN-γ and Th2-related cytokine IL-4 of each serum sample were measured with indirect ELISA to determine the cellular immune response induced by the bivalent VLPs vaccine. As shown in **Figure 5.4C-D**, the IFN-γ and IL-4 levels in the groups immunized with VLPs differed significantly from those in the group immunized with the control group at 14 and 21 days respectively post-immunization, and increasing over time. This demonstrates that the Th1 and Th2 cellular immune responses were both induced by the bivalent VLPs vaccine.

Bivalent VLPs vaccine conferred protection against classic RHDV challenge

At 21 days after immunization, all the rabbits in the three groups were challenged with classic RHDV to determine the protective efficacy of the bivalent VLPs vaccine. Rabbits immunized with the bivalent VLPs vaccine survived and showed no clinical signs of RHD post-infection, whereas three rabbits immunized with the control antigen died at 48, 72, or 120 h post-infection, which demonstrated that the bivalent VLPs vaccine provides rabbits with full protection against classic RHDV challenge (**Figure 5.5A).**

After observation for 7 days, all the surviving rabbits were euthanized with intravenous injection of sodium pentobarbital. All the rabbits were autopsied to detect any pathological changes. The tissues were stored in 4% paraformaldehyde, and later tissue sections were prepared and analyzed by IHC. The tracheae of the control group displayed typical ring hemorrhage, hemorrhage, and hyperemia. Edema were detected in all substantial viscera, especially in the lung and kidney. As expected, the organs of the rabbits immunized with the bivalent VLPs vaccine displayed none of the pathological changes specific to RHD (**Figure 5.5B**).

In further analysis of tissue sections, we found the lungs of the rabbits immunized with the control appeared hemorrhagic and endemic, and the alveoli had been destroyed, in addition, the liver cells were degenerate and apoptotic, and the number of white blood cells had decreased. Hemorrhage was apparent within the glomerular loops and renal medulla of the kidney. Furthermore, the number of white blood cells and splenic corpuscles had declined in the spleen, and swollen airways and hemorrhage were observed in the trachea, the organs of the rabbits immunized with the bivalent VLPs vaccine showed no pathological changes (**Figure 5.6**).

Detection of classic RHDV in rabbits

RT-PCR and IHC analyses were performed to detect the virus distribution in the rabbits of the three groups. In detail, all the solid organs of the rabbits immunized with the control group
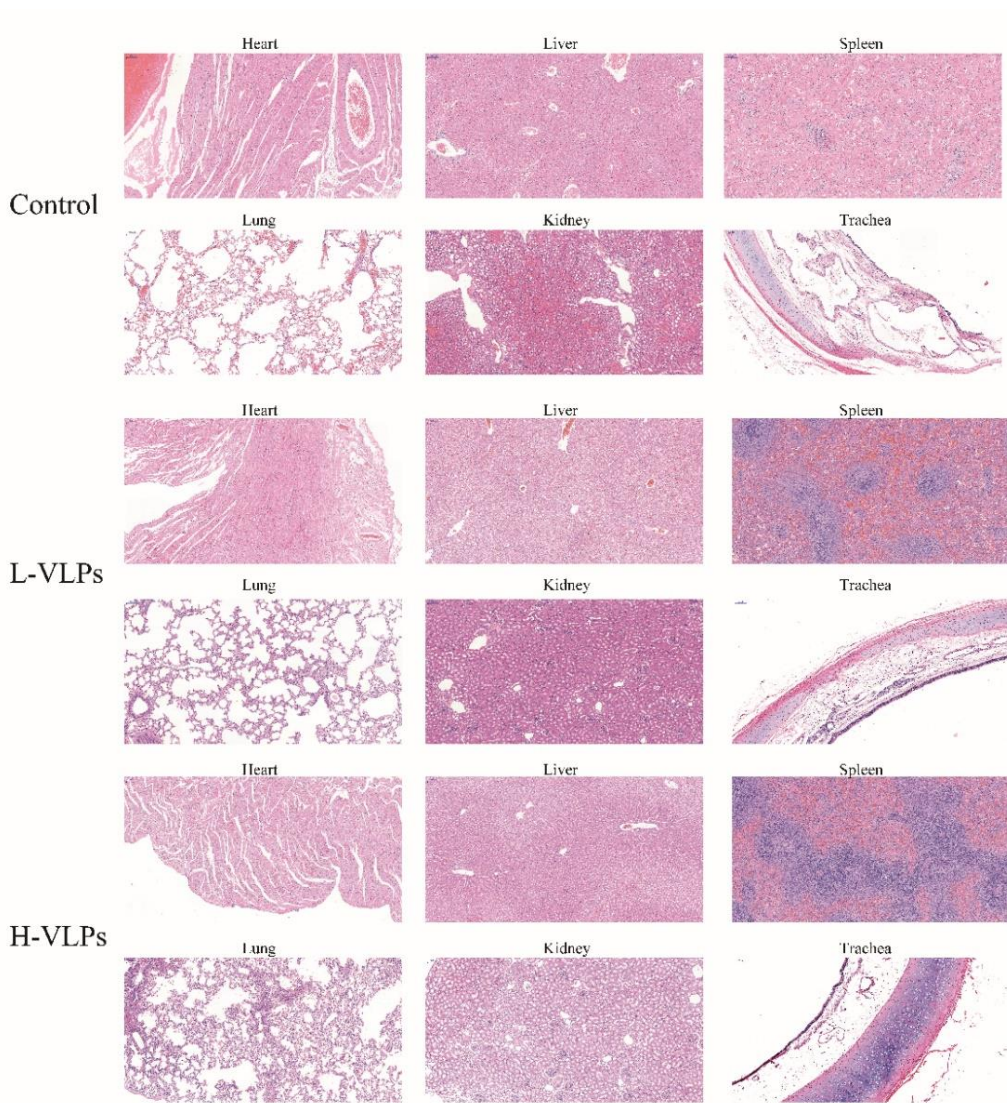
Figure 5.6 Pathological section analysis of tissues from immunized rabbits.

The solid organs of rabbits immunized with the control showed pathological changes specific to RHDV, whereas those of rabbits immunized with the bivalent VLP vaccine showed no pathological changes.
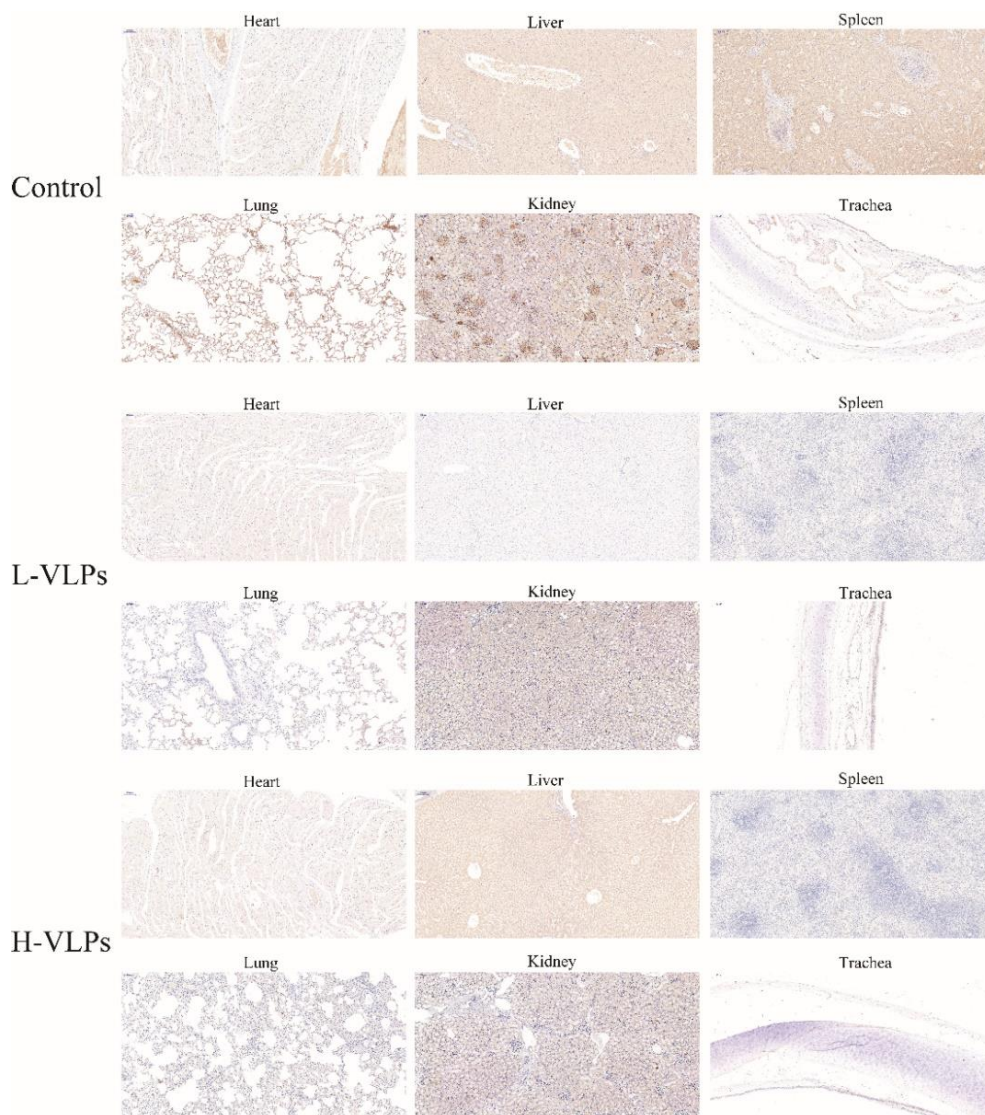
Figure 5.7 Immunohistochemical (IHC) analysis of tissues from immunized rabbits.

Images of IHC analysis presented that classic RHDV VP60 was predominantly located in the cellular cytoplasm of rabbits immunized with the control, but not in that of rabbits immunized with VLPs. This demonstrates that the bivalent VLP vaccine inhibited the spread of classic RHDV.

showed the presence of the classic RHDV VP60 gene, whereas no VP60 gene was detected in the rabbits immunized with the bivalent VLPs vaccine by RT-PCR (**Figure 5.5C**). The IHC analysis detected the classic RHDV VP60 most located in the cellular cytoplasm of the rabbits

immunized with the control, but not in those immunized with the VLPs (**Figure 5.7**). These results demonstrated that the bivalent VLPs vaccine inhibit the spread of classic RHDV.

5.4 Discussion

RHD is a highly infectious and fatal disease that mainly affects rabbits or hares. Since the first report in China in 1984, it has spread to many countries or regions in Asia, Europe, Africa, and the Americas, and has caused serious losses in hare resources and the rabbit industry (Xu 1991, Mitro and Krauss 1993, Delibes-Mateos *et al.* 2007). RHDV contains a positive-sense single-stranded genomic RNA with a high mutation rate, which causes variations in its antigenicity and the emergence of novel genotypes. In 2010, RHDV2 was first reported in France. Because classic RHDV and RHDV2 differ in their antigenicity, the traditional RHDV vaccine does not completely protect rabbits against RHDV2 challenge, which allows continuing outbreaks of RHDV2 infection. Therefore, it is necessary to develop a new vaccine against classic RHDV and RHDV2 infections to avoid the spreading of this virus all over the world.

Tissue-inactivated vaccines are the major commercially available vaccines against RHDV (Arguello Villares 1991, Huang 1991, Smid *et al.* 1991). However, to reduce the ecological risk of traditional vaccine, RHDV VP60 is widely used to develop subunit vaccines against RHD. The BEVS has been widely used to develop new subunit vaccines against many different kinds of virus, such as human papillomavirus, norovirus, and *Hepatitis B virus* (Zuckerman *et al.* 2001, De Vincenzo *et al.* 2014, Shouval *et al.* 2015, Atmar *et al.* 2016), because of the advantages of its high-level protein expression, safety, and simplicity. There have been many reports of individual classic RHDV VLPs or RHDV2 VLPs produced with BEVS, which induce efficient humoral and cellular immune responses and confer protection against classic RHDV or RHDV2 challenge (Nagesha *et al.* 1995, Plana-Duran *et al.* 1996, Muller *et al.* 2019). However, no vaccine that protects rabbits simultaneously from both classic RHDV and RHDV2 infection has been reported. Therefore, a new vaccine is urgently required to counter the spread of classic RHDV and RHDV2 in a single region.

VP60, as the capsid protein of RHDV, can self-assemble into VLPs. So, we subcloned the classic RHDV and RHDV2 VP60 genes together into the pFastBac Dual Expression Vector, with the expression of the classic RHDV VP60 gene under the PH promoter and the expression of the RHDV2 VP60 gene under the p10 promoter. The recombinant baculovirus was produced by transfecting Sf9 cells with the recombinant bacmid. Both VP60 genes were strongly expressed and then self-assembled into VLPs in the infected Sf9 cells. We then produced a bivalent VLPs vaccine and immunized rabbits with it. The outcomes suggested that the humoral and cellular immune responses of the rabbits immunized with the VLPs were efficiently

induced and differed significantly from those of rabbits immunized with the control group.

To evaluate the protection efficiency afforded by the bivalent VLPs vaccine against classic RHDV challenge, all the rabbits were infected with 2 LD_{50} of classic RHDV (JX/CHA/97) at 21 days post-immunization and observed for 7 days. Three rabbits immunized with the control died, whereas all the rabbits immunized with the bivalent VLPs vaccine survived, demonstrating that the bivalent VLPs vaccine confers full protection against classic RHDV challenge in rabbits. Furthermore, we found that a low dose (0.5 mg) of VLPs was enough and sufficient to induce efficient immune responses. The rabbits were not challenged with RHDV2 because there has been no report of RHDV2 in China. According to the animals' test results after infection with classic RDHV and their high humoral and cellular immune responses, we speculate that the bivalent VLPs vaccine would also protect rabbits against RHDV2 as well as classic RHDV. A number of studies have demonstrated that VLPs of RHDV2 VP60 induce efficient humoral and cellular immune responses and protect rabbits against RHDV2(Miao *et al.* 2019, Muller *et al.* 2019, Muller *et al.* 2019). Nevertheless, whether the bivalent VLPs vaccine does confer complete protection on rabbits against classic RHDV and RHDV2 must be verified with further experiments.

In conclusion, to restrict outbreaks and the spread of RHDV2, we successfully constructed a recombinant baculovirus containing the VP60 genes of both classic RHDV and RHDV2. The VP60 proteins highly expressed in Sf9 cells which infected with this baculovirus. In addition, VP60 proteins could self-assemble into VLPs in the infected Sf9 cells. Then we produced a bivalent VLPs vaccine that efficiently induced humoral and cellular immune responses and protected rabbits from challenge with classic RDHV. All these results indicate that the recombinant baculovirus is a candidate construct from which to develop a new subunit vaccine against both classic RHDV and RHDV2 infection.

Ethics approval and consent to participate: Specific-pathogen-free New Zealand white rabbits were treated humanely and with regard to the alleviation of their suffering. The use of

all rabbits in the immunization trials was approved by and in compliance with the guidelines of the Animal Research Ethics Board of Shanghai Veterinary Institute (CAAS).

Chapter 6

Getah virus-like particle vaccine candidate produced in insect cells

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Getah virus-like particle vaccine candidate produced in insect cells

Abstract: Getah virus (GETV) is an emerging arthropod-borne virus of the genus *Alphavirus* in the family *Togaviridae*. This virus causes economic losses associated with infections in horses and pregnant sows. In this study, we successfully produced Getah virus-like particles (VLPs) in insect cells using the baculoviruses expression vector system by expressing the GETV structural gene cassette in *Spodoptera frugiperda* Sf9 cells. We show that the resulting VLPs were glycosylated in both cells and supernatants, and that the expressed E1 envelope protein localized to the cell surface. The capsid protein assembled in the form of core-like particles, which accumulated in the nucleus of the cells as seen with transmission electron microscopy. Furthermore, VLPs with a diameter of 60~80 nm were observed after discontinous sucrose gradient purification. With this strategy we produced a candidate GETV vaccine which after further testing may prevent animals from GETV infection.

6.1 Introduction

Getah Virus (GETV) is an emerging arthropod-borne (arbo) virus (family *Togaviridae*, genus *Alphavirus*) that is transmitted by mosquitoes. The virus was first discovered in Malaysia in 1955 (Srivastava and Igarashi 1986) and the word "getah" is derived from Malay, meaning rubber, because the virus was first discovered in rubber plantations. Economic losses related to piglet abortions have been reported associated with an recent outbreak of GETV in China(Yang *et al.* 2018, Xing *et al.* 2020), but GETV has also been reported in Japan (Kamada *et al.* 1980), Australia (Doherty *et al.* 1963, Spradbrow 1972), Eurasia (Lvov *et al.* 1991), Korea (Rhee *et al.* 1986), India (Brown and Timoney 1998), and Russia (Gur'ev *et al.* 2008), in mosquitoes, pigs or horses. Moreover, GETV infections have also been seen in other hosts, such as cattle (Liu *et al.* 2019) and blue foxes (Shi *et al.* 2019), and in areas in China (Liu *et al.* 2019, Shi *et al.* 2019, Cheng *et al.* 2020) that are geographically far away from each other, suggesting that GETV has spread throughout China. The increasing frequency of GETV outbreaks is also becoming a public health concern, because of potential co-circulation of GETV with other mosquito-transmitted viruses (Tajima *et al.* 2014, Vogels *et al.* 2019, Lu *et al.* 2020).

GETV is an enveloped virus with a diameter around 60~80 nm and a positive sense, single-stranded RNA genome, which is almost 12 kb in length. The genome contains two open reading frames (ORFs), each encoding a polyprotein. The 5'ORF (ORF1) is translated directly into the polyprotein nsP123/4, which is processed by nsP2 into the four non-structural proteins (nsPs) to form viral replication complexes (VRCs). The 3'ORF (ORF 2) is translated from a 26S subgenomic mRNA into five structural proteins: capsid (C), E3, E2, 6K, and E1. Like other alphaviruses, the GETV budding process is thought to occur at the plasma membrane, initiated by capsid-glycoprotein interactions. Finally, virus particles bud out of the cells, thereby obtaining an envelope from the cell membrane in which E1/E2 trimers have formed (Owen and Kuhn 1997, Garoff *et al.* 2004).

GETV was first identified in *Culex gelidus* mosquitoes, but later on the virus was successfully detected in various other mosquitoes in the family *Culicidae*, including other *Culex* and *Aedes* mosquito species, as well as in *Armigeres subalbatus* and *Anopheles sinensis* (Takashima *et al.* 1983, Takashima and Hashimoto 1985, Kobayashi *et al.* 2016, Xia *et al.* 2018, Liu *et al.* 2019). GETV induced symptoms in horses may last for 1~2 weeks and are characterized by self-limiting rash and fever, hind limb edema, and lymph node swelling (Fukunaga *et al.* 2000). In swine, GETV infection often result in the abortion of piglets (Xing *et al.* 2020). Recently, GETV was detected in a commercial live-attenuated vaccine preparation aimed at preventing infections with Porcine reproductive and respiratory syndrome virus (PRRSV) (Zhou *et al.*

2020). The contamination occurred during the manufacturing and might result in PPRSV vaccination-related GETV infections (Zhou *et al.* 2020). Pigs immunized with these commercial live PPRSV-vaccines might be a source for further GETV spread to other vertebrate species that likely serve as reservoir host for GETV.

Thus, there is a great need for a safe and effective GETV vaccine. However, at present there is only an inactivated GETV vaccine on the market (Nisseiken, Tokyo, Japan), derived from the Japanese GETVMI-110 horse isolate of 1978 (Nemoto *et al.* 2015). In a combination with the inactivated Japanese encephalitis virus (JEV), it is used to protect racehorses against JEV- and GETV-related disease (Bannai *et al.* 2016). Unfortunately, several outbreaks of GETV in horses in Japan have been seen in recent years despite the vaccination program (Nemoto *et al.* 2015, Kuwata *et al.* 2018), which might indicate that the vaccine is not potent enough. The potency of this vaccine was recently analyzed in a mouse model of GETV-induced arthritic disease, which demonstrated that the vaccine was only effective in an immunization regime with two shots 20 ug of antigen (Rawle *et al.* 2020). How this translates to effective formulations for horses or pigs needs to be determined, but it becomes clear that there is a rising need for a safe and efficacious GETV vaccine that can easily be adapted to new strains if needed.

Virus-like particles (VLPs) are noninfectious antigens that have been demonstrated as a powerful tool for developing a growing number of vaccines (Roldao *et al.* 2010). VLPs structurally mimic the native virus and present viral spikes or other immunogenic surface components, which can be recognized by B cells to stimulate protective antibody responses to the authentic viruses. The baculovirus expression vector system (BEVS) has been recognized as a safe and versatile way to express viral structural genes often resulting in VLPs with high immunogenicity. A number of examples of approved veterinary (and human) vaccines produced in this system exist(van Oers *et al.* 2015). In this study, we describe the generation of a GETV VLP vaccine candidate by expressing the entire GETV structural cassette (C-E3-E2-6K-E1) from a bacmid-based baculovirus vector. We characterized the production of the VLPs in Sf9 insect cells and determine the optimal harvesting time. Overall, our data demonstrate that BEVS can be used as an effective large-scale production method for the expressed GETV VLPs in order to develop a new generation of veterinary GETV vaccines.

6.2 Material and methods:

Cells and viruses

Sf9 insect cells (Invitrogen) derived from *Spodoptera frugiperda,* were maintained at 28ºC in SFM900II serum-free medium (Invitrogen) supplemented with 200 µg/ml gentamicin (Gibco). Sf9-easy titration (ET) cells (Hopkins and Esposito 2009) were grown in the same medium, but now supplemented with 5% FBS (Gibco) and 200 µg/ml gentamicin. Vero cells infected with the GETV MM2021 (RNA provided by Michael S. Diamond from Washington University) were used to isolate total RNA, which was then subjected to reverse transcription to obtain cDNA of the GETV genome. Primers (**Supplementary Table 6.S1**) were used to amplify the GETV genome, and the structural protein cassette was amplified from the cloned cDNA with specific primers (**Supplementary Table 6.S1**) using Q5 high fidelity polymerase (NEB). The resulting 3762 bp GETV structural fragment was cloned into the pFastBac1 plasmid (Invitrogen) between *EcoR*I and *Xba*I sites. The insert was verified by sequencing (Macrogen, Europe). Recombinant baculoviruses were generated using the Bac-to-Bac expression vector system, with an adapted Autographa californica nucleopolyhedrovirus (AcMNPVΔp10ΔccΔE56) bacmid in which the attTn7 transgene insertion site was relocated, to the *odv-e56* locus to achieve a more stabµle recombinant baculovirus genome (Pijlman *et al.* 2020). In addition, the chitinase and cathepsin genes were deleted to achieve higher and stable protein expression levels and lower changes of glycoprotein degradation (Pijlman *et al.* 2020). Transposition of the GETV structural cassette into the AcMNPVΔccΔE56 backbone, resulted in the recombinant bacmid Bac-MM2021. The bacmid was verified by PCR with M13F in combination with M13 Reverse or with Genta Reverse primers (Supplementary Table.S1). This bacmid was used to transfect Sf9 cells using ExpresS2 TR transfection reagent (ExpreS2ion Biotechnologies) to obtain the recombinant baculovirus AcGETV. Finally, AcGETV titers were determined by End Point Dilution Assays (EPDA) using Sf9-ET cells (Hopkins and Esposito 2009), and expressed as tissue culture infectious dose 50 % ($TCID_{50}$)/ml culture. AcGFP baculovirus was used as negative control.

GETV VLPs production and purification from Sf9 cell culture

Sf9 cells were grown in suspension in Erlenmeyer shake flasks and infected with AcGETV with a multiplicity of infection of 0.5 $TCID_{50}$ units/per cell (cell density of 1.5×10^6/mL) to produce GETV VLPs. After 5 days of infection, cells and medium were harvested. Cells were collected by centrifugation at 4700 rpm (Thermo fisher) for 30 min and then stored in PBS at -20ºC. Supernatants were filtered by using 0.22 nm filters (Minisart, Thermo fisher). Secreted VLPs in the culture medium were precipitated by adding 7% (w/v) polyethylene glycol PEG-

6000 and 0.5 M NaCl and incubating on a roller bench at 4°C overnight. After centrifugation at 4700 rpm for 20 min, protein pellets were resuspended in GTNE buffer (200 mM glycine, 50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH 7.3), and then loaded on discontinuous 70% (w/v)/ 40% (w/v) sucrose for ultracentrifugation at 30,000 rpm at 4°C for 2 h (SW 55 Ti, Beckman). The interphase between 40% sucrose and 70% sucrose was harvested and resuspended in GTNE buffer. The VLPs in the GTNE buffer were further concentrated by centrifugation at 30,000 rpm (SW 55 Ti, Beckman) for 30 min to remove the sucrose. Each pellet was finally resuspended in 50 µL GNTE at 4°C overnight and then stored at -80ºC for future use.

Time course assays

Sf9 cells were seeded in 6-well plates, and infected with MOI 1 or MOI 5. The production level of GETV VLPs was followed over time by harvesting the supernatants at different time points post-infection. The baculovirus titer in the culture medium was determined at the same time points by EPDA assays. For western blot analysis, 500 µl of the culture medium was concentrated by adding three times cold acetone and stored at -20 ºC for 1 h. After centrifugation at 4700 rpm for 20 min, the pellets were air-dried and diluted in 50 µl PBS and subjected to western blot analysis to quantify viral protein levels.

SDS-PAGE and Western Blot analysis

Infected cells, sucrose purified VLPs, and the protein content of the concentrated culture medium were analyzed by SDS-PAGE (Biorad). Protein samples were denatured in Laemli buffer with β-mercaptoethanol at 95°C for 10 min. Following electrophoresis, the proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) at a constant amperage of 0.05A per gel by semi-dry blotting (Bio-Rad). Membranes were blocked with 1% skimmed milk (Campina) in PBS-0.05% Tween-60 (PBST) for 1 h at room temperature (RT) or overnight at 4°C, and then washed 3 times with PBST. Membranes were further incubated with primary antibodies for 1 h. GETV-MM2021-infected mouse serum (1:1000), rabbit polyclonal anti-CHIKV E1 (1:5000) (Metz *et al.* 2013), and rabbit polyclonal anti-CHIKV capsid (1:50) were diluted in 1% skimmed milk. After incubation, membranes were rewashed with PBST for 3 times (5 min each). Next, the membranes were incubated for 1 h with alkaline phosphatase (AP) conjugated goat anti-rabbit or goat anti-mouse IgG (Sigma) 1:2500 times diluted in PBST. Finally, the secondary antibodies were removed, and membranes were washed three times with PBST and then subjected to incubation with AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) for 10 min. Membranes were developed with NBT/BCIP solution (Roche) and later imaged using a Bio-Rad imaging station.

Glycosylation analysis

PNGase-F (NEB) was used to determine the glycosylation status of the baculovirus-expressed GETV glycoproteins E1 and E2. PEG6000 precipitated medium and cell fractions were treated for 10 min with 2 µl denaturing buffer (NEB) at 95 °C. Subsequently, 0.5 µl (250 units) PNGase-F (NEB) was added to a total volume of 20 µl in G7 reaction buffer with 1 µl NP40 (NEB). The samples were incubated at 37°C for 1 h. Treated and non-treated protein samples were analyzed in parallel by SDS-PAGE and western blotting.

Immunofluorescence assays

Sf9 adherent cells were seeded in an 8-well Lab-Tek® Nunc chamber (Thermo Fisher) and infected with AcGETV at an MOI of 5 $TCID_{50}$/ml in SF900 SFM medium (Invitrogen). Cells were harvested at 60 h p.i by fixation with 4% paraformaldehyde (PFA) for 15 min and washed with PBS. Cells were then subjected to permeabilization with 1% SDS in FBS for 20 min. and then incubated with rabbit anti-CHIKV-E1 diluted 1:500, mouse anti-CHIKV-capsid diluted 1:50, or mouse anti-GETV polyclonal antibodies diluted 1:200 in 5% FBS in PBS for 1 h at RT. After incubation, cells were washed four times with PBS, after which they were incubated with 1:100 diluted Rhodamine Red™-X (RRX) AffiniPure Goat-anti-rabbit IgG (H+L) (Jackson Immuno Research) or Goat-anti-mouse TRITC (Nordic) for 1 h at RT. Cells were washed with PBS four times after incubation and nuclei were stained with Hoechst (1:100 dilution in PBS) for 3 min at RT. Finally, images were taken with a Zeiss Axio Observer Z1 inverted microscope.

Electron microscopy of VLPs and to detect GETV-core like particles

Aluminium grids were discharged for 30 s with Argon gas and then loaded with 5 µl sucrose purified VLPs (5 times diluted in GTNE buffer) and incubated for 2 min at RT. The grids were washed with MilliQ water and the excess liquid was removed by blotting paper. Grids were subsequently stained with 2% ammonium malate (AM) (pH 6.8) for 30 s at RT, and the extra liquid was removed again from the edges of the grids. Finally, the grids were air-dried and analyzed by transmission electron microscopy (TEM) (Jeol JEM 1400) at the Wageningen Electron Microscopy Centre. For visualization of core-like particle (CLPs) with TEM, Sf9 cells were seeded in T25 flasks and infected with AcGETV or AcGFP at MOI 5. Sixty hours after infection, cells were harvested by centrifugation for 10 min at 1500 rpm, resuspended in 1 ml of 2.5% glutaraldehyde in 0.1 M phosphate/citrate buffer and incubated for 1 hour at room temperature. After pelleting the cells, they were washed two times with 0.1 M phosphate/citrate buffer, before being resuspended in 100 µl of 3% gelatin in 0.1 M phosphate buffer. The gelatin was solidified by incubation for 30 minutes at 4ºC, after then samples were post-fixed 1 hour

in 1% osmium tetroxide in 0.1M phosphate/citrate buffer, then samples were dehydrated using different percent of ethanol in the following order: 30% for 5 minutes, 50% for 5 minutes,70% for 5 minutes, 80% for 5 minutes, 90% for 5 minutes, 96% for 5 minutes, 100% for 10 minutes, and 100% for 10 minutes. And the specimen were transferred to BEEM (Spurr) capsules completely filled with resin and then polymerized in an oven for 8 h at 70 °C , and then ultrathin sections approximately 50nm thick were cut from the capsules. Sections were incubated for 2 minutes in UranyLess (EMS) or 10 minutes in 2% uranyl acetate, then wash five times with MilliQ. Incubate sections for 2 minutes in ready to use lead citrate (EMS) or 10 minutes in 1:3 diluted lead citrate stock (done in a CO_2-free environment), and then washed twice in 0.01 N CO_2-free water. Following with wash three times in MilliQ, and finally examined by TEM (Jeol JEM-1400 120kV).

6.3 Results

Recombinant baculovirus expression of GETV structural proteins in insect cells
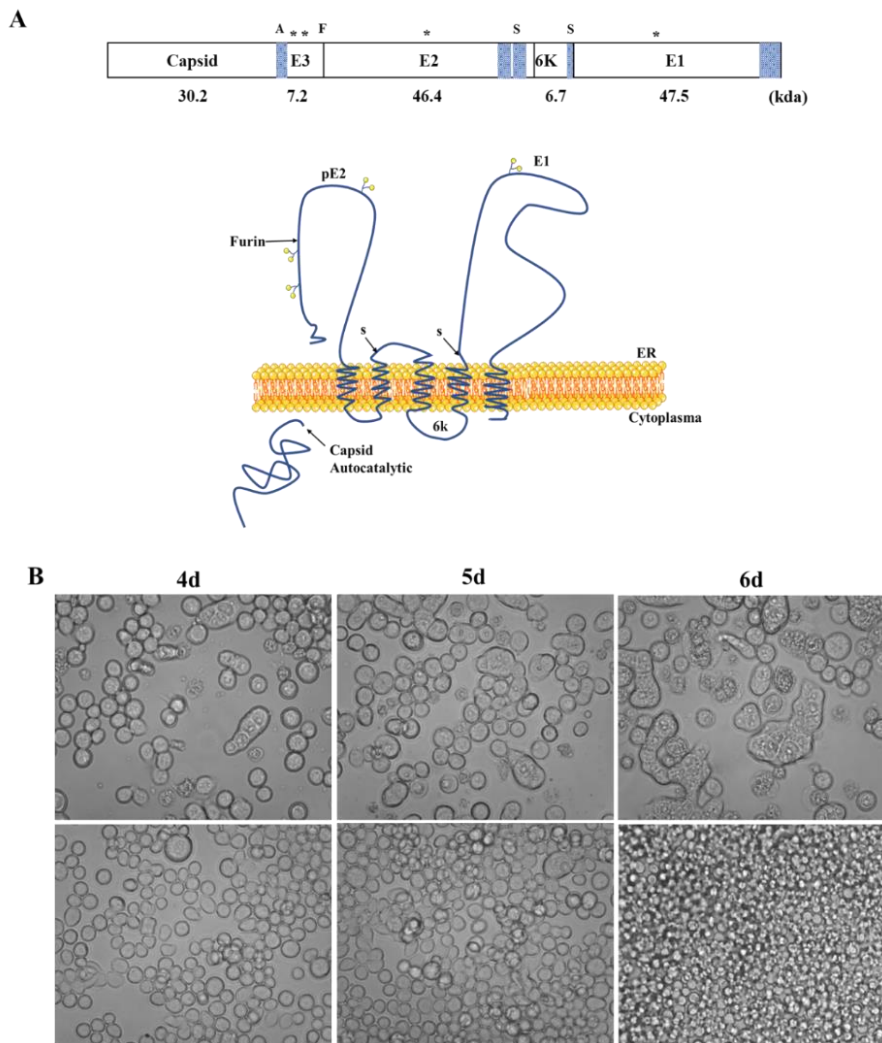
Figure 6.1. Baculovirus expression of GETV structural proteins in Sf9 cells

(A) Schematic representation of the GETV structural cassette. Asterisks indicate predicted N-glycosylation sites and the molecular mass (in kDa) of individual proteins is presented. Transmembrane domains or signal sequences (ss). Autocatalytic (A), furin (F) and signalase (S) cleavage sites are indicated, After capsid is autocatalytic released, the envelope cassette inserted into ER and further processed by signalase. (B) The Sf9 cells infected with AcGETV show clear cytopathological effects with syncytia formation and nuclear core-like particles from 4 days post-infection. At 6 days post-infection, the syncytia formation was much more severe and the cells started to show disruption.

119

A recombinant baculovirus (AcGETV) was generated to express the complete GETV-MM2021 structural polyprotein sequence (C-E3-E2-6K-E1) (**Figure 6.1A**) in Sf9 insect cells. Clear cytophatic effect (enlarged nuclei, floating cells) and syncytium formation was observed 4 days postinfection of Sf9 cells with AcGETV, with syncytia becoming larger at 5 and 6 dpi (**Figure 6.1B**). To confirm the expression of the individual GETV structural proteins in insect cells, AcGETV infected cells, PEG-precipitated medium, and sucrose-purified samples were all subjected to western blot analysis. AcGFP infected medium was used as negative control. The GETV capsid protein with a size of approximately 35 kda, the glycoproteins E1 and E2 both around 47 kda, and the E2 precursor E3E2 of approximately 52 kda were all detected in infected cells with the anti-GETV polyclonal mouse serum (**Figure 6.2A**, upper panel). Polyclonal rabbit antibodies against CHIKV-E1 cross-reacted with GETV E1 and clearly showed GETV-E1 protein expression in supernatants (**Figure 6.2A**, lower panel).
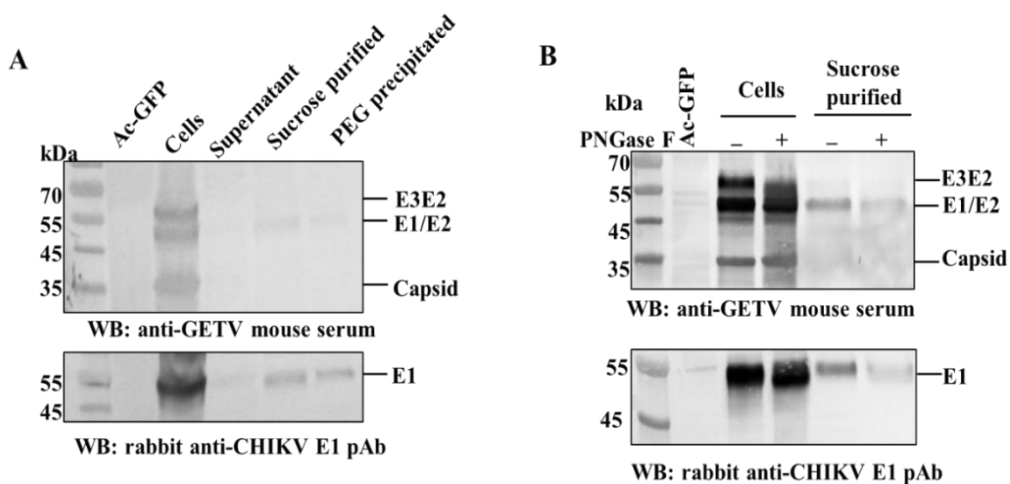
Figure 6.2. GETV structural protein production and glycosylation analysis

(A) GETV E1/E2 and capsid expression in Sf9 cells was analyzed. Anti-GETV mouse serum was used to determine GETV proteins in cells and medium after PEG precipitated and sucrose purification. CHIKV-E1 polyclonal antibodies were applied for the detection of E1 in cells and culture medium. Ac-GFP was used as negative control. (B) Glycosylation analysis was performed by treatment of samples with PNGaseF. Ac-GFP was used as negative control.

Glycosylation analysis of the GETV structural glycoproteins

It is known that N-linked glycosylation is the most common posttranslational modification in insect cells, which also occur during baculovirus production of CHIKV VLPs (Metz *et al.*

2013). We therefore studied whether the GETV glycoproteins E1 and E2 are also glycosylated. First, the N-linked glycosylation sites in the GETV structural cassette were predicted by online software (http://www.cbs.dtu.dk/services/NetNGlyc/) and indicated with asterisks at location 279, 325 (both in E3), 532 (in E2), and 956 (in E1) (see **Figure 6.1A**). To study the glycosylation status of the expressed GETV glycoproteins in both insect cells and culture medium during baculovirus infection, we harvested the cells by centrifugation and concentrated the proteins in the supernatant with PEG6000/NaCl a hundred-fold, and then performed sucrose gradient ultracentrifugation to purify the VLPs. Both the cells and the sucrose-purified material were incubated with PNGase-F for 1 h. Western blot analysis using anti-GETV mouse serum and anti-CHIKV E1 antibody showed that the E1, E2 and E3E2 proteins decreased in size after PNGase-F treatment, in both the cell and the sucrose-purified medium samples. This indicates that the GETV glycoproteins were indeed glycosylated(Figure 6.2B).

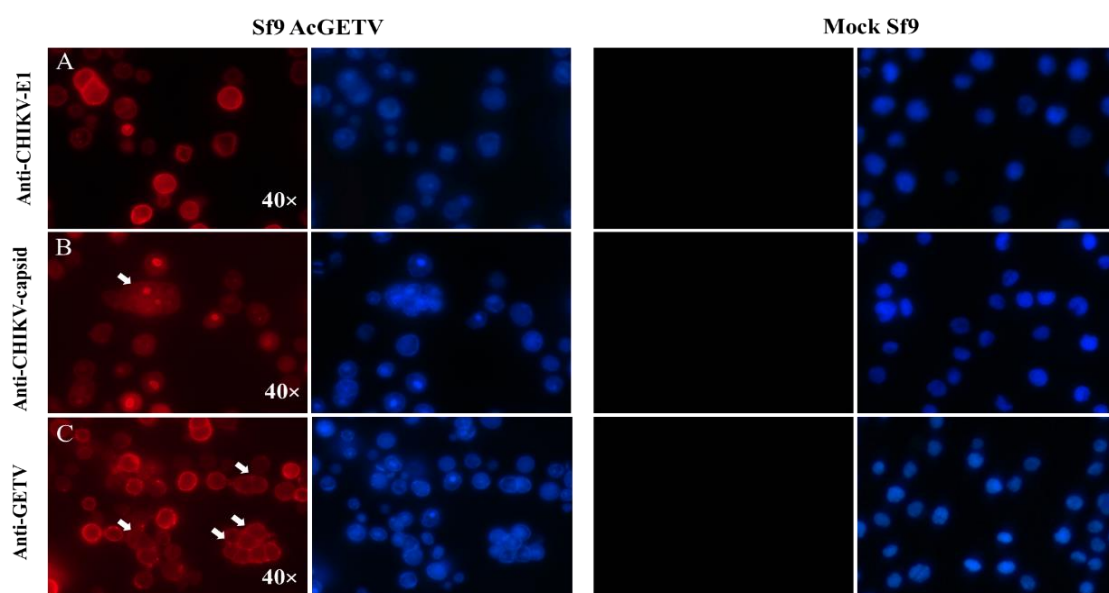**Figure 6.3. Localization of GETV-E1 and capsid in infected Sf9 cells.**

Sf9 cells were infected with AcGETV and subjected to immunofluorescence assay (IFA) using anti-CHIKV-E1 (A), anti-CHKIV capsid antibody (B), and anti-GETV mouse serum (C). Red staining shows detection by antibodies. Nuclei were stained with Hoechst (blue). Mock-infected cells served as negative control.

Localization of GETV glycoproteins and capsid in Sf9 cells

The envelope of alphavirus particles contain trimeric spikes built of three assembled heterodimers of E1 and E2 glycoproteins (Sjöberg and Garoff 2003). These spikes are essential for budding and for virus entry. To further analyze the location of the GETV proteins in infected Sf9 cells, Sf9 cells were fixed and subjected to immunofluorescence assay (IFA) 60 h post infection. IFA conducted with CHIKV antibodies (CHIKV-E1, CHIKV-capsid) showed cross-reactivity with GETV. The GETV E1 protein localized to the surface of infected cells (**Figure 6.3A**), while the GETV capsid protein was predominantly present in the nucleus of the cells (**Figure 6.3B**). Furthermore, by using an anti-GETV mouse serum, syncytia formation (white arrows) can be seen clearly with a 40× magnification after infection with AcGETV (**Figure 6.3C**). Mock infected Sf9 cells did not show any immunofluorescence signal.

Growth curve of AcGETV infection and time course expression of GETV VLPs in Sf9 cells

To determine the expression of GETV VLPs in the culture medium, Sf9 cells were seeded in 6-well plates with 0.8×10^6 cells per well and infected with an MOI of 1 or 5 when cells were 60-70% confluent. The growth curve of AcGETV was determined to define the optimal harvest time for the GETV VLPs. Cells were harvested every 24 h and stored at 4 °C, and culture
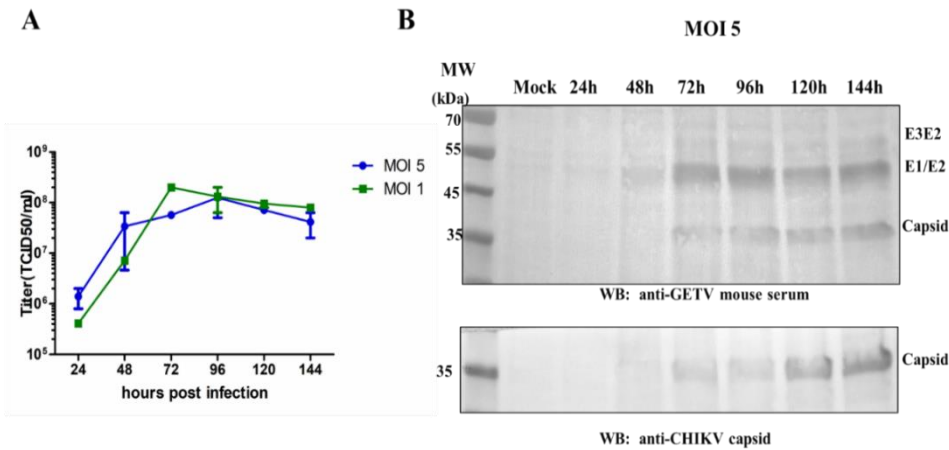
Figure 6.4. GETV growth curve and time course of GETV VLP production in Sf9 insect cells

(A) Sf9 cells were seeded in a 6-well plate and infected with AcGETV at an MOI 1 or 5. Cells and medium were both harvested at different times post infection. Baculovirus titers were determined by EPDA. The experiment was performed in triplicate and error bars represent mean error. (B) The expression of GETV VLPs in the medium was analyzed on western blot . The proteins were detected using anti-GETV mouse serum and anti-CHIKV capsid antibody.

medium collected at the same time points for virus titration by EPDA using Sf9-ET cells. From the growth curve it can be concluded that the titer of AcGETV reaches a peak between 72 and 96 h post-infection when using an MOI of 1 or 5, respectively (**Figure 6.4A**). After the peak, both curves slowly descended. To analyze the amount of secreted GETV VLPs over time, 500 µl cell culture medium was precipitated with ice-cold acetone and subjected to western blot analysis. Results using mouse anti-GETV serum demonstrated that the levels of E1/E2 and E3E2 reached a maximum level around 72 h p.i. (**Figure 6.4B**, upper panel). With CHIKV anti-capsid it became clear that the level of capsid protein accumulated further till about 120 h p.i.

GETV VLP production in Sf9 cells

Baculovirus expression of the entire structural cassette of GETV in Sf9 cells lead to the processing of the structural polyprotein and glycosylation of the GETV glycoproteins E1 and E2 (**Figure 6.3B**). Moreover, analysis of concentrated culture medium samples demonstrated that E1, E2, and capsid could be purified on sucrose gradients and were all present at well-detectable levels (**Figure 6.4B**). To further confirm the assembly of GETV VLPS in the culture medium, sucrose-gradient purified cell culture medium was subjected for TEM analysis. These
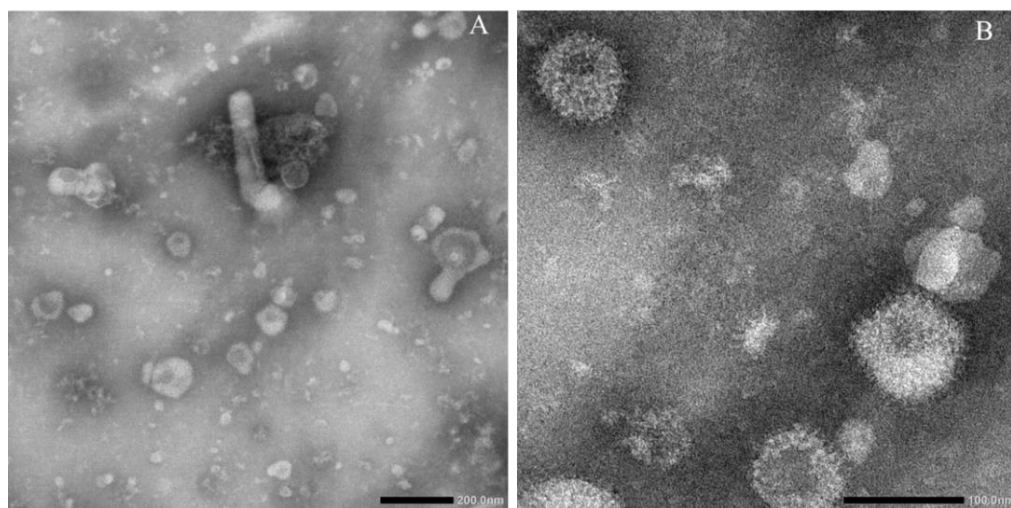
Figure 6.5. Production of GETV VLPs in Sf9 cells

(A) The culture medium of AcGETV infected Sf9 cells was analyzed by transmission electron microscopy (TEM). The supernatants were precipitated with PEG 6000 and NaCl and then purified by discontinuous sucrose gradient centrifugation. A band obtained between 40% and 70% sucrose was resuspended in GTNE buffer and further analyzed by TEM. The size of the GETV VLPs produced in insect cells is estimated at 60~80 nm. The scale bar is 200 nm. (B) GETV VLPs at higher magnification, the scale bar represents 100 nm.

results indicated that VLPs are abundantly produced and secreted into the medium. Some rod-shaped baculovirus particles (~200nm in length) can also be found. The GETV VLPs have a diameter ranging from 60~80 nm in size (**Figure6.5A**) with typical surface spikes (**Figure 6.5B**), and share a similar morphology to CHIKV and other alphavirus VLPs (Noranate *et al.* 2014).

Analysis of GETV core-like particles in Sf9 cells by TEM

Dense protein bodies within the nucleus were becoming clear upon infection of Sf9 cells with AcGETV (**Figure 6.1B**). Staining the nucleus with Hoechst during the IFA indicated that these nuclear bodies contain accumulated capsid protein (**Figure 6.3B**). To visualize the dense nuclear aggregates in more detail, cells were infected with AcGETV at an MOI 5 or with AcGFP as control. The cells infected with AcGFP (**Figure 6.6B**) showed an enlarged nucleus compared with uninfected Sf9 cells, which is typical for baculovirus infected cells in general (**Figure 6.6A**). For cells infected with AcGETV, apparent nuclear bodies were again observed with light microscopy (**Figure 6.6C**, white arrows). When analysed by TEM, the plasma membrane of cells infected with AcGETV was often disrupted and an electron dense area was readily observed. Within this dense area, spherical particles and tubular structures with a diameter of approximately 30 nm were visible at higher magnification (**Figure 6.6D**), a size similar to that of nucleocapsid cores reported for other alphaviruses, such as SFV (Acheson and Tamm 1967), CHIKV (Hikke *et al.* 2016) and Salmonid Alphavirus (SAV) (Hikke *et al.* 2016). These observations demonstrated that the expression of GETV capsid was able to form core-like particles (CLP) in insect cells during expression.
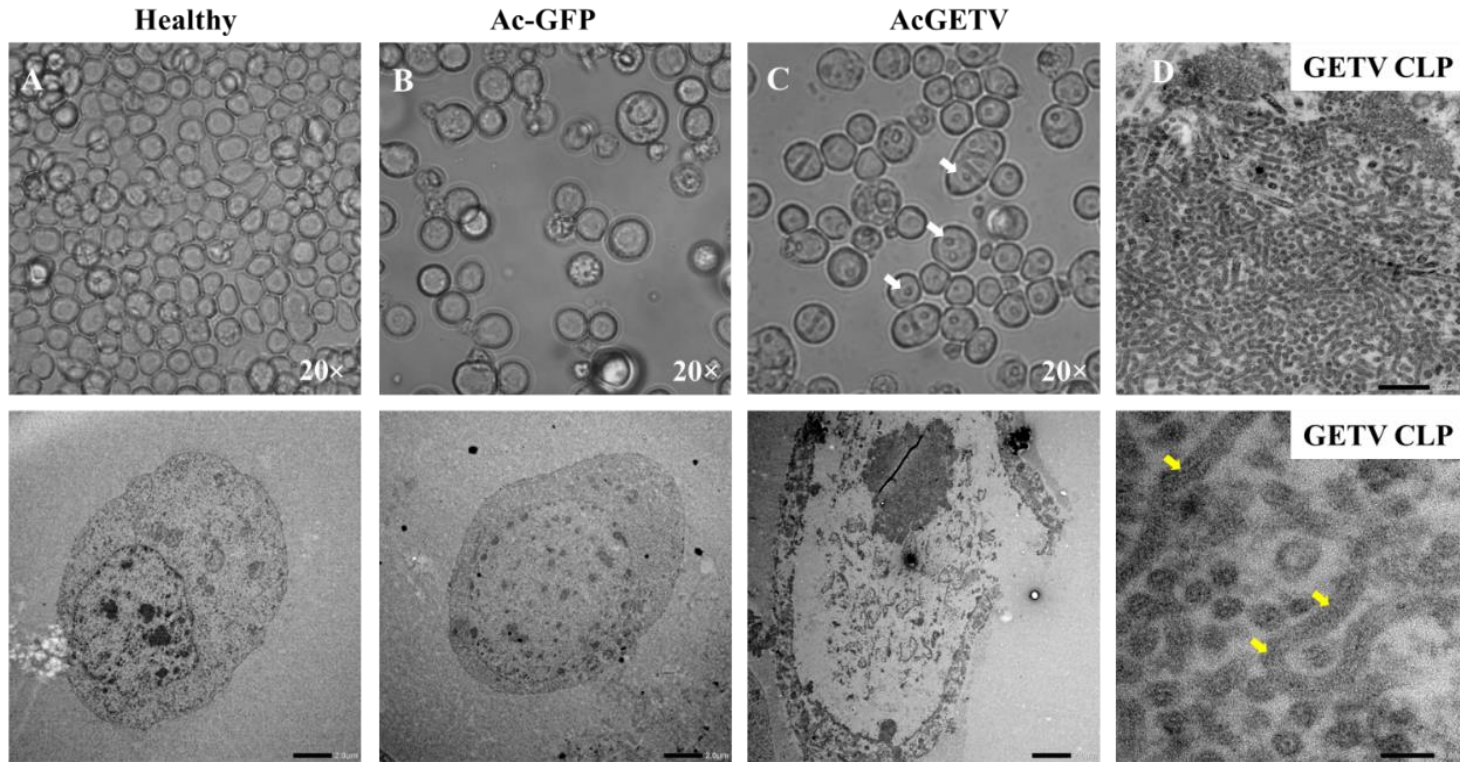
Figure 6.6. Core-like particle (CLP) formation during AcGETV infection in Sf9 cell nuclei

Sf9 cells were infected with Ac-GFP and AcGETV at MOI 5 in T25 flasks. Compared with healthy cells (A), apparent CPE was observed for Ac-GFP infected cells with an enlarged nucleus (B), and clear aggregated core-like particles in the nucleus of AcGETV infected cells (C). The lower panel in A, B and C represent the view of TEM with one cell (scale bar=2µm). At higher magnification, Core-like particles around 30 nm in diameter were observed in the nuclei of AcGETV infected cells by TEM (D), scale bar =200 nm(upper panel), and scale bar =50 nm(lower panel).

6.4 Discussion

GETV infections cause economic losses in the piglet industry and form a serious risk in China (Lu *et al.* 2020). Emerging alphaviruses, like Mayaro virus (MAYV) and GETV, can often use multiple invertebrate vectors and eventually spread to other animals hosts or become zoonotic and infect human beings. The increasing numbers of GETV infection in multiple different species (Kuwata *et al.* 2018, Liu *et al.* 2019, Shi *et al.* 2019) indicates the need of generating a safe and efficient vaccine that can be applied in a variety of animal species. Vaccination is regarded as the most efficient way to reduce GETV infections. In this study, we produced GETV VLPs in Sf9 insect cells. To that aim, the GETV structural cassette (C-E3-E2-6K-E1) as expressed using a recombinant baculovirus under control of the strong polyhedron (polH) promoter. The produced GETV structural proteins were successfully processed and the viral envelope proteins were glycosylated. Spherical VLPs were observed in the culture medium, that were 60~80 nm in diameter and showed typical surface spikes, a structure similar to authentic GETV particles (Ren *et al.* 2020).

During infection with the recombinant baculovirus syncytia were formed, most likely due to the surface expression of active GETV fusion protein. The alphavirus E1 protein is a class II fusion protein (Lescar *et al.* 2001, Roussel *et al.* 2006). Using anti CHIKV E1 polyclonal antibody, we confirmed that the GETV E1 protein localized to the plasma membrane. Syncytia formation has also been observed during CHIKV VLP production in different pH 6.4/5.8/5.5, from which demonstrated that the E1 fusion activity was not pH-dependent (Metz *et al.* 2013). This might explain why syncytium formation occurs without further lowering of the pH in the culture medium. CHIKV E1 also displayed its fusion ability in mosquito cells (Metz *et al.* 2011), and Salmonid Alphavirus (SAV) infection in fish cells also resulted in syncytia formation (Hikke *et al.* 2014), indicative for successful glycoprotein processing in different hosts. Interestingly, in AcGETV infected Sf9 cells syncytia formation was even more evident and severe (**Figure 6.1B**), than found previously when expressing the CHIKV structural cassette in the same cells (Metz *et al.* 2013). This may be due to differences in the pH threshold for E1 conformational change between GETV and CHIKV, but it may also be due to differences in the amount of E1 protein present at the cell surface.

The alphavirus capsid protein can self-assemble into core-like particles (CLPs) when expressed in cell culture (Hikke *et al.* 2016), therefore we hypothesized that the capsid protein of GETV would form a similar structure within the nucleus. During the infection with the recombinant baculovirus, we observed aggregated CLPs with light microscopy and IFA (**Figure 6.1B**). The aggregation of capsid protein in the nucleus was verified with a cross-reacting anti-CHIKV

capsid antibody (**Figure 6.3B**). TEM imaging showed CLPs with a diameter of approximately 30 nm (**Figure 6.6C-D**). In addition, tubular structures were seen in the nucleus, which may be an artefact of the sheer overexpression of the capsid protein. The accumulation of capsid in the nucleus may also explain why its expression still increases beyond 72 h p.i. whereas optimal VLP production in the culture medium has already reached its plateau.

In our study, we applied sucrose gradient purification to isolate the GETV VLPs. On those gradients (irrespective of whether they were continuous or discontinuous) the VLPs showed a distribution over several bands after ultracentrifugation (**Supplementary Figure 6.S1**). Baculoviruses could also be found in every density correlated band with WB (**Supplementary Figure 6.S1**). Thus, during the manufacturing of enveloped VLPs the contamination with baculovirus is inevitable. This is not an obstacle for the further development of GETV VLP as veterinary vaccine, however, for human vaccine applications the purification method needs to be further optimized to effectively remove baculoviruses from the drug substance. In a follow up study we aim to test the immunogenicity and protective ability of the GETV VLPs in a mouse model.

In general, VLPs are self-assembling particles that can efficiently stimulate the host immune system due to similarity in structure with authentic viruses. VLPs do not contain a viral genome, and hence, have lost the capacity of replication, making VLP-based vaccines safer than live-attenuated vaccines (van Oers *et al.* 2015) or even inactivated vaccines that must be produced in higher biosafety level facilities. VLPs are often highly immunogenic and elicit a higher neutralizing antibody response than subunit vaccines, consisting of individual viral proteins (Metz *et al.* 2013). Previously, alphaviruses VLPs have been produced in various expression systems, including CHIKV in insect (Metz *et al.* 2013, Wagner *et al.* 2014) and mammalian cells (Noranate *et al.* 2014, Ko *et al.* 2019), and in yeast expression systems (Saraswat *et al.* 2016), and also Mayaro virus (MAYV) (Rezende *et al.* 2018), SFV (Diatta *et al.* 2005), and Equine encephalitis virus (EEV) (Ko *et al.* 2019) in mammalian cells. Compared with mammalian expression systems, the BEVS may lead to increasing immunogenicity as the glycosylation pattern in insect cells differs from that in mammalian systems, which may stimulate innate immune responses (Dowling *et al.* 2007, Urbanowicz *et al.* 2019). In the insect cell expression system, glycosylation has been proven to be efficient and critical for a wide range of biological processes. Especially for VLPs, modification of asparagine-linked glycan VLPs have been demonstrated to induce earlier and longer-lasting antibody responses than wild type VLPs (Hyakumura *et al.* 2015). Moreover, the excellent scalability of suspension Sf9 cells allows for large scale production of antigens including VLPs. Previously, we have

demonstrated in an animal study with purified CHIKV VLPs that only 1 ug of unadjuvanted VLPs successfully protected mice against CHIKV infections (Metz *et al.* 2013). Commercial VLP-based vaccines are available for humans (Cervarix, GlaxoSmithKline) and for the veterinary market, such as the licensed Porcilis PCV (Fort *et al.* 2009) from Merck and CircoFLEX from Boehringer Ingelheim, making the future of VLPs based vaccines more promising. Except for applying VLPs as vaccines, applications as gene delivery vehicle or protein carrier for presenting antigens for vaccination have also been reported (Urakami *et al.* 2017, Qian *et al.* 2020). CHIKV VLPs have been engineered, for instance, to display malaria antigens. These recombinant VLPs induced high antibody titers against malaria and conferred protection in animal trials (Urakami *et al.* 2017). By generating GETV VLPs we will also gain insights and tools for future swine vaccine development, which is important in light of emerging mosquito-borne viral diseases in the field.

Supplemental files
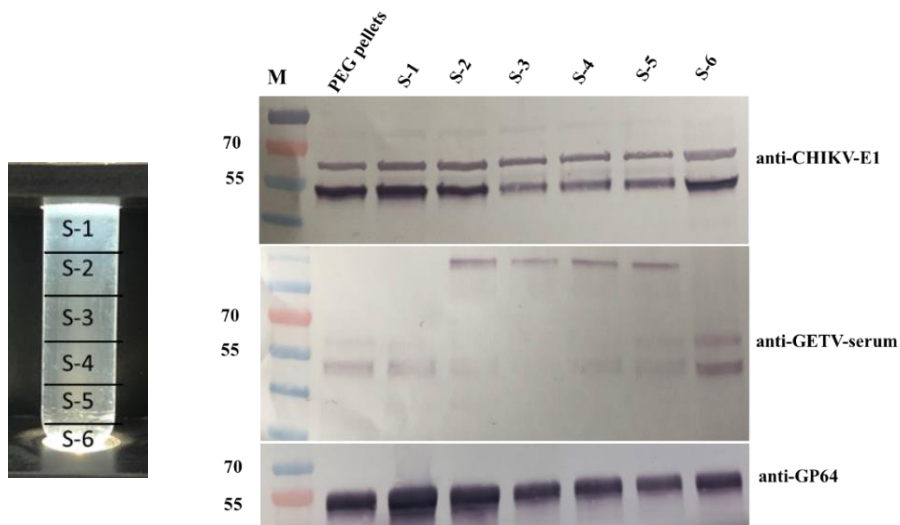
Figure 6.S1: WB detection of each pellets after continuous sucrose ultracentrifugation
PEG precipitated protein samples were subjected to continuous sucrose gradient centrifugation(30%-70%). continuous sucrose gradient was prepared with 1mL respectively gradient sucrose, and leave at 4 ℃ to form continuous gradient. Each pellet (1mL) was harvested and resuspended in GTNE buffer to remove the sucrose by ultracentrifugation at 30000rpm for half an hour, and finally diluted in 50µl GTNE buffer for WB analysis. With using anti-GP64, baculoviruses were detected in each pellet, and anti-CHIKV-E1 detected E1 protein in each pellet, and also using anti-GETV antibody, capsid, E1/E2, and E3E2 were all detected in each pellet.

Table. 6.S1 .Primers used in this study

Primer name	Primer sequence
Ge1F	ATGGCGGACGTGTGACATCACCG
Ge1R	GATGGGTGCCAGCTTCTCGCTTTTC
Ge2F	CTGCCACCGCTAGTGAGCGGCTC
Ge2R	TCCTGATATATCAGCGCGGCGGA

Ge3F	GCACCAAGCTAACCAGAAACTT
Ge3R	TAGGTGACCCGGGCCAGTGTCA
Ge4F	AGCCACGCCGCCGGAACACCAGG
Ge4R	AGATGTAGTGTAATCCTGCATC
Ge5F	AATAGATGCAGGATTACACTACA
Ge5R	TACGGTTTCGTGGCCTTGTA
Ge6F	TCGAGGCCACGATGACGTGTAAC
Ge6R	CCACCACATTCGGGATCGTTG
Ge7F	GAGCCTGGGAACTCCCGTCGTAA
Ge7R	TAATCAAGTAGATCAAAGGGCTA
Ge8F	CCTCAAGTTGTCAAGACCTTCGTC
Ge8R	GTAAAATATTAAAAAAACAAATTAGACGCC
SC-F	AA*GAATTC*accATGAATTACATCCCATCT
SC-R	GC*TCTAGA*TTAGCGGCGCATGGTCACACA
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC
Genta-Rev	AGCCACCTACTCCCAACATC

Chapter 7

General discussion

Qiuhong Miao

7.1 Introduction

In a world with increasing international global trade, climate changes and urbanization, and the increasing demand for livestock animals such as chickens, goats and pigs for protein supply, the incidence of veterinary disease outbreaks increases. The emerging animal viruses Peste des Petits ruminants virus (PPRV), Rabbit Hemorrhagic Disease Virus (RHDV2), and Getah virus (GETV) mentioned in this thesis cause significant economic losses in China. Therefore, the virulence factors of these viruses, the antiviral response of their hosts and the development of safe and effective vaccines had become scientific issues that urgently needed to be addressed.

In response to virus infection, innate and adaptive immunity work together to limit virus spread, neutralize infectious virus particles and remove infected cells. As many details of emergence, pathogenesis and control of emerging animal viruses are still largely unknown, it is important to connect existing knowledge with new insights to give a perspective for the future. Studying the interactions between viruses and the innate immune system in host cell lines leads to better understanding of virus pathogenesis (**Chapter 2**). Identification of antiviral host-restriction factors, such as BST2, may aid in development of novel broad-spectrum antivirals (**Chapter 3**). Meanwhile, stimulation of adaptive immunity via administration of virus-like particle vaccines can be applied as an effective way to control emerging virus outbreaks (**Chapters 4-6**).

7.2 Innate immunity and host restriction factors

7.2.1 RIG-I-like receptors and MAVS

RIG-I-like receptors have been studied extensively because of their essential role during RNA virus infection as sensors of cytoplasmic viral dsRNA. Three members of the RLR family are, the retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and Laboratory of Genetics and Physiology protein 2 (LGP2). Mitochondrial antiviral-signaling protein (MAVS) is a necessary adaptor required for RLR-recognized RNA viruses signaling. MAVS is also known as IPS1, VISA, and CARDIF (Kawai *et al.* 2005, Meylan *et al.* 2005, Seth *et al.* 2005, Xu *et al.* 2005), and is characterized by interactions with RIG-I and MDA5. MAVS induces the downstream activation of transcription factors, including
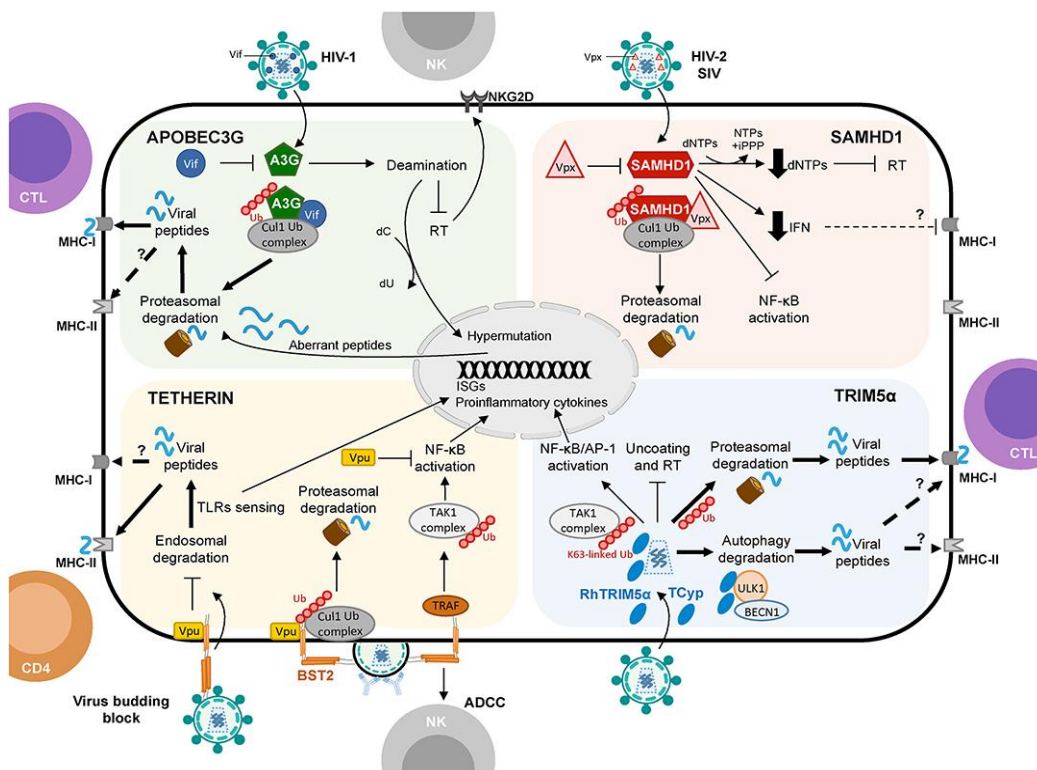
Figure 7.1. Prominent host restriction factors (HRFs) and their participation in cellular process during virus infection.

APOBEC3G, SAMHD1, Tetherin, and TRIM5a proteins involved in cellular innate and adaptive immune responses. This figure shows is the innate immune pathways activated after infection with human immunodeficiency virus (HIV) as an example. Pictures used with permission from Marta Colomer-Lluch *et al*, 2018.

interferon regulatory factors 3 and 7 (IRF3/7) and nuclear transcription factor-κB (NF-κB), which leads to the expression of interferons (IFNs) and IFN-stimulated genes (ISGs), which display antiviral activity in multiple ways.

During the innate immunity response, MAVS has been demonstrated to be a central adaptor protein to control virus infection in a number of species (Liu *et al.* 2014, Xu *et al.* 2015, Wu *et al.* 2016, Huang *et al.* 2017, Sun *et al.* 2019). In **Chapter 2** we observed that PPRV infection of caprine cells leads to degradation of MAVS, which suggests that PPRV can escape the host antiviral response by interfering with MAVS signaling pathways.

Normally, MAVS activation leads to the downstream expression of IFNs, which are considered the first line of defense against viral infections. For that reason, IFN is used in antiviral

treatment strategies, although IFN is associated with substantial side effects, because all cell types express interferon-α/β receptors (IFNAR) (Sleijfer *et al.* 2005) and have the ability to respond to this treatment. In addition, and often in response to IFNs, antiviral host response factors (HRFs) are produced by host cells to counteract crucial processes involved in the viral life cycle, such as entry, uncoating, replication, or translation. Examples are BST2/Tetherin (Neil *et al.* 2008), IFIT (interferon-induced protein with tetratricopeptide repeats) (Fensterl and Sen 2015), TRIM5α (Tripartite motif-containing protein 5) (Nakayama and Shioda 2010), ADAR-1 (adenosine deaminase, RNA-specific 1) (Samuel 2011), APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 3) (Okeoma *et al.* 2007), SAMHD1 (SAM-domain- and HD-domain-containing protein) (Chen *et al.* 2018), PKR (Protein kinase R) (García *et al.* 2006) and Viperin (Mattijssen and Pruijn 2012) (**Figure 7.1**). These antiviral proteins are crucial for regulating cell signaling and maintaining cellular homeostasis (Colomer-Lluch *et al.* 2018).

Moreover, while most viruses evolve much quicker than their hosts, these HRFs are rapidly evolving as well. The fact that species have HRFs that are slightly different from that of other species, but with similar structures, explains why the innate immune system is either susceptible or resistant to the viruses present in the modern world (Duggal and Emerman 2012). However, whether HRFs from different species have played a role in the modulation of different viruses remains unknown. Thus, the identification of HRFs from animal hosts, will help to understand better the conserved functions and associated characteristics of these proteins, which sheds light on how the virus and the host interact and co-evolve, and might provide clues for the design of possible antiviral molecules. Taken together, the research on HRFs may find future applications in the development of broad-spectrum antiviral drugs.

7.2.2 Re-examining MAVS regulation during PPRV infection

After viral RNA recognition by RLR sensors, MAVS is triggered. MAVS activation leads to the increased production of cytokines (including IFN) and expression of interferon-stimulated genes (ISGs) (Vazquez and Horner 2015). During this activation, many other host proteins are interacting with MAVS, such as tumor necrosis factor receptor-related factor 2,3,5,6 (TRAF2/3/5/6), TNFR1-associated death domain protein (TRADD), phosphorylation of NF-κB inhibitor-a (IkBa) (**Figure 7.2**), which all contribute to downstream RLR-mediated signal transduction. However, many additional cellular factors such as TRIM21, RNF125, TRIM25 have been identified to interact with MAVS. These proteins are involved in e.g. post-translational modifications (PTMs) and promote RLR-mediated innate immune responses. For example, lysine 63 (K63) (Liu *et al.* 2017) as well as K48 linked polyubiquitination of MAVS

has been reported in response to virus infection(You *et al.* 2009). Furthermore, K27-linked ubiquitination of MAVS promotes the recruitment of TANK binding kinase 1 (TBK1) to MAVS to enhance downstream signaling (Xue *et al.* 2018). Several other PTMs, including O-GlcNAcylation (Song *et al.* 2019), phosphorylation (Li *et al.* 2019), and dephosphorylation (Xiang *et al.* 2016), have also been demonstrated to functionally regulate MAVS. In **Chapter 2**, we found that in host EEC cells infected with PPRV at a high multiplicity of infection (MOI) MAVS degradation was mediated by the proteasome pathway in response to PPRV-V
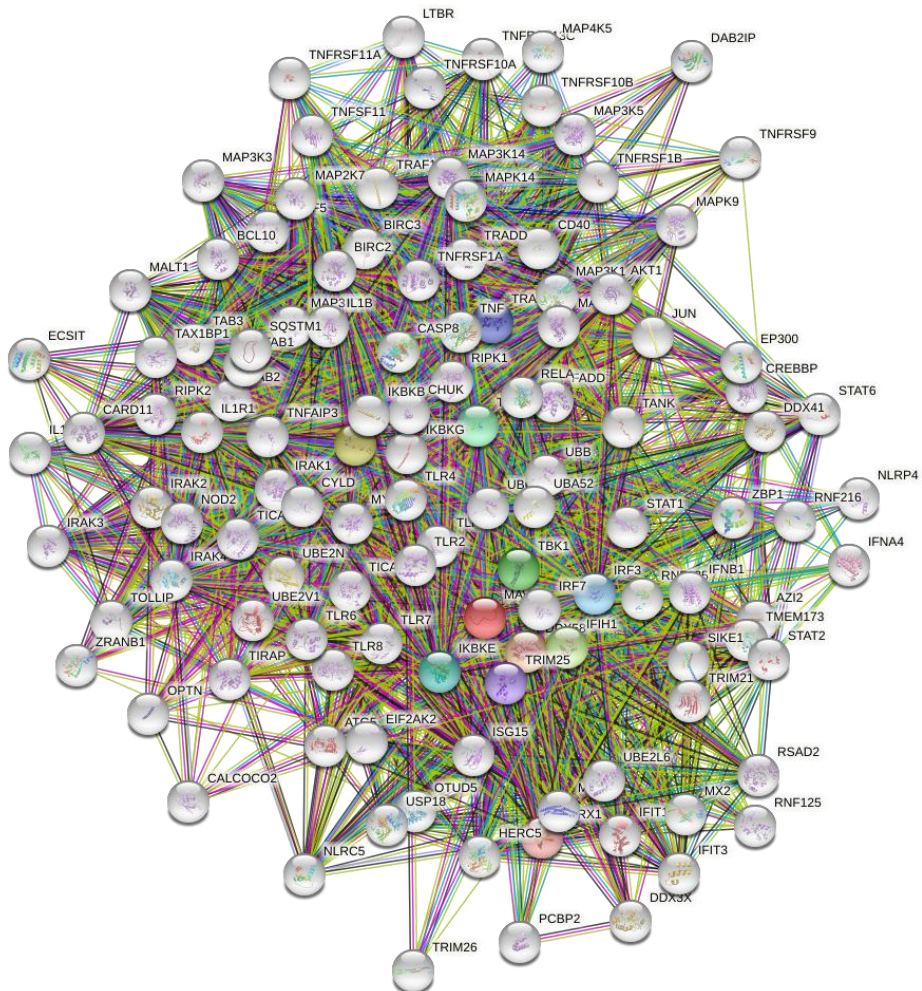

Figure 7.2. Complex molecular interactions of MAVS with other cellular proteins

Pictures obtained from https://string-db.org/ by chose human MAVS (which is in dark green in the middle)

expression. A variety of molecules might be involved in this process; therefore, it needs to be investigated which ubiquitination enzyme accounts for the observed PPRV-V mediated MAVS degradation, for example using immunoprecipitation (IP) and mass spectrometry (MS) analysis. In addition, the putative interactions between MAVS and PPRV-V protein we observed in **Chapter 2** were found in overexpression conditions for both the co-immunoprecipitation (Co-IP) and Immunofluorescence assay (IFA). However, it is still not entirely clear how and when viral V protein interacts with MAVS, given the fact that the subcellular localization of viral proteins changes during the course of infection. So how and when the virus protein localizes to mitochondria could not be concluded directly from this colocalization. In addition, we cannot exclude a physiological interaction between PPRV-V and MAVS occurring in the cytoplasm under some circumstances. Nevertheless, our results likely imply that newly synthesized viral protein V translocate into mitochondria, where it induces MAVS degradation, thereby limiting the function of this adaptor protein, which then to facilitates virus replication. Moreover, it remains uncertain whether the mitochondrial marker pDsRed-Mito is good enough to show the exact site of the MAVS regulation by PPRV-V, in view of the complex interaction network known for MAVS (**Figure 7.2**).

While speculating about the exact mechanism of how PPRV antagonizes MAVS, we also need to consider the possibility of interactions between MAVS and other viral proteins, such as PPRV viral protein P. During PPRV infection, the V protein of PPRV is derived directly from an edited P gene RNA (**Figure 7.3**). The N-terminus of the V protein, therefore, is identical to that of the P protein, and the C protein is also derived from the same coding sequence. Previous studies found that the PPRV non-structural C protein inhibits the induction of interferon-β by potentially interacting with MAVS (Li *et al.* 2021), and P protein has also been demonstrated to block IFN by interacting with IRF3 (Zhu *et al.* 2019) and STAT1 phosphorylation (Sanz Bernardo *et al.* 2017), so its role in innate immunity remains to be fully elucidated. With a further Co-IP experiment, we recently demonstrated that PPRV-P did not interact with MAVS. As it is known that both V and C protein were derived from the P gene, it becomes interesting to investigate the detailed molecular mechanism of how PPRV utilizes the viral V and C protein, instead of the P protein, to be involved in MAVS mediate innate immunity pathway (**Figure 7.4**).

Taken together with PPRV-V and PPRV-P, I am also curious to examine the interaction of MAVS with PPRV-H. PPRV-H is known to mediate virus binding to cellular receptors and initiates virus entry and later virus release. Recently, PPRV-H was shown to be involved in innate immunity signaling as well. It was demonstrated to be responsible for TLR2 activation, which results in the transcription of pro-inflammatory cytokines and Th1-polarized cytokine IL-12 secretion (Rojas *et al.* 2021). In addition, PPRV-H was also demonstrated to be inhibited by bone marrow stromal antigen 2 (BST2) proteins (Kelly *et al.* 2019). In our additional experiment, the apparent MAVS expression level decreased when PPRV-H was co-expressed (**Figure 7.4**). The interactions between PPRV-H and MAVS shown within this thesis raises the question if PPRV-H takes part in regulation during MAVS degradation, may be together with other viral proteins, and as well what the mechanism is behind the regulation process.
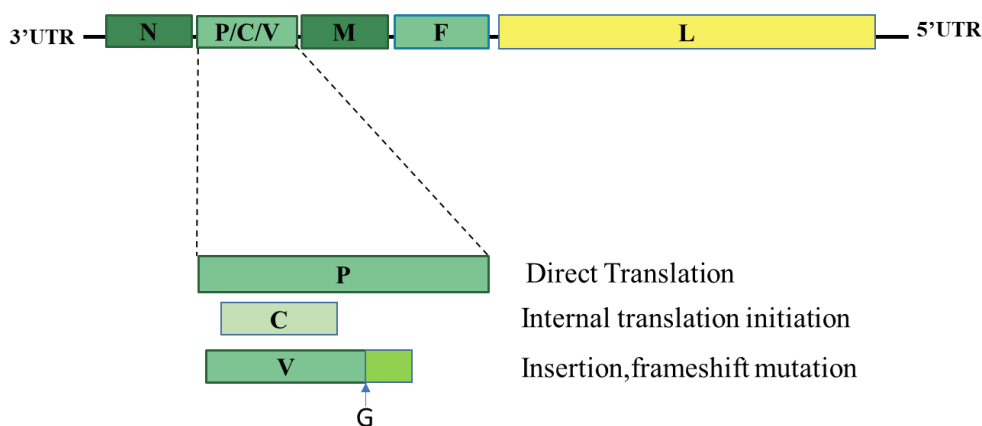
Figure 7.3. Schematic diagram of the genome organization of PPRV and how to derive three gene products encoded by the P gene.

P protein is the full-length gene product; C protein is translated from an internal open reading frame; V protein arises by the insertion of a non-templated G resulting in a frameshift which resulted the V protein share a common N-terminal region as P protein.

As we know, mice lacking MAVS tend to be viable and fertile, but MAVS-deficient mice have an abolished induction of the IFN, and lack NF-κB and IRF3 activation in multiple cell types during viral infection (Sun *et al.* 2006), which all together demonstrates the important role of MAVS in immune responses. Although caprine MAVS was identified with an adaptor function in several critical processes in **Chapter 2**, in the future, after generating MAVS knock-out

caprine cell lines, studies may show to which extent MAVS normally downregulates PPRV proliferation, despite the activity of PPRV-V.

7.2.3 Role of caprine BST2 paralogs in PPRV budding
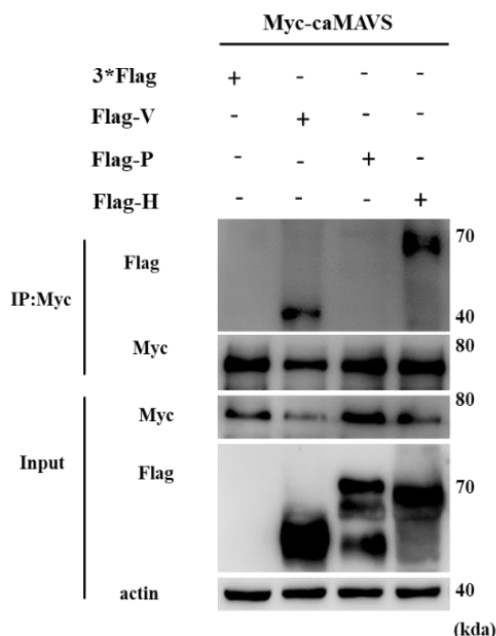
Figure 7.4. caMAVS interacts with both PPRV viral protein H and V, but not with P

HEK293T cells were transfected with equal amount of caMAVS together with PPRV viral protein V, P, H respectively, for CO-IP assays. The results indicated the direct interaction of caMAVS with PPRV-V, and PPRV-H, but not with PPRV-P.

Numerous studies have proven that BST2/tetherin displays a broad ability to restrict the budding of viruses from multiple virus families, whereas other reports showed that BST2 could inhibit virus replication directly (Liberatore and Bieniasz 2011, Hammonds *et al.* 2012, Hoffmann *et al.* 2019). However, for BST2 from ruminant species, researchers found that BST2 does have several gene duplications, including the two paralogs from sheep termed oBST2A and oBST2B, and three bovine paralogs (Takeda *et al.* 2012). oBST2A limited virus budding by BST2 particles on the membranes, but oBST2B was demonstrated to be limited to the Golgi apparatus and disrupted the trafficking of the virus envelope (Env) by sequestering the virus. Thus, oBST2B leads to the release of less infectious virions (Murphy *et al.* 2015). **Chapter 3** identified two paralogs from goat cells, named GBST2 and GBST2like, and showed their

localization within cells with IFA. Unluckily, we did not precisely identify their co-localization with cellular organelles because of the shortage of applicable markers.

The effect of both goat BST2 paralogs on virus budding is quite similar, which was determined by Western blot analysis (WB) and End-point Dilution Assays (EPDA). We also showed that GBST2 interacted with PPRV-V. using Co-IP and IFA (**Chapter 3**). However, further studies did not find any interactions or co-localization of GBST2like with PPRV-V protein. Hence, the molecular mechanism of how PPRV antagonizes ISGs remains undiscovered in the presented thesis. Moreover, how the two individual paralogs respond to the viral protein V needs to be further examined, as well. Previously, PPRV-V has been shown to inhibit IFN induction pathways and type I IFN response (Chinnakannan *et al.* 2013, Rojas *et al.* 2016)). Moreover, as one of the unnecessary proteins during viral replication, other paramyxovirus V proteins have been proven to interact with the RIG-I/TRIM25 complex via a conserved C-terminal domain, thus inhibiting the RIG-I signaling pathway (Sanchez-Aparicio *et al.* 2018). The discovery of interactions between the PPRV-V protein and GBST2 will contribute to the understanding of PPRV-V mediated innate immunity.
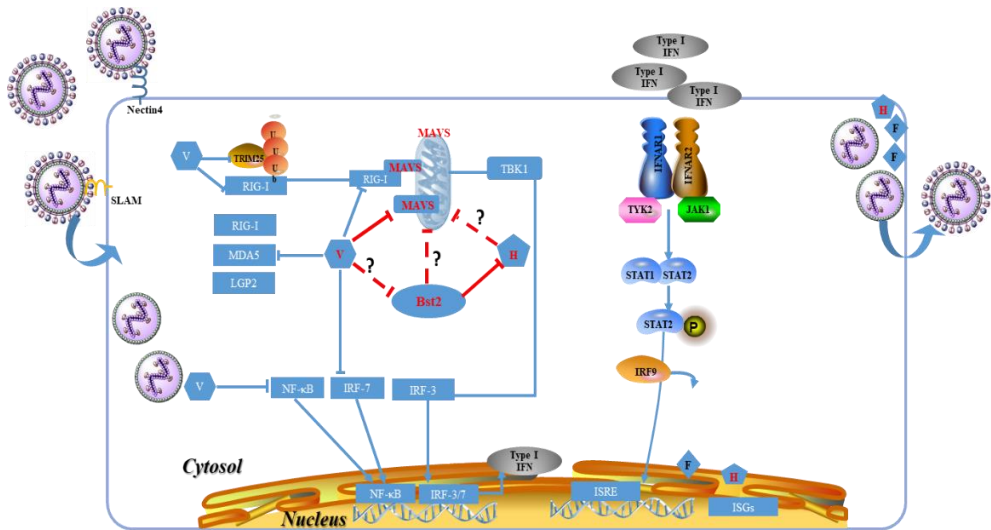
Figure 7.5. Morbilliviruses and host factor interactions.

Signaling Lymphocytic Activation Molecule (SLAM), Tripartite Motif Containing 25 (TRIM25), melanoma differentiation-associated protein 5 (MDA5), TANK Binding Kinase 1(TBK1), interferon-α/β receptor (IFNAR), Tyrosine kinase 2(TYK2), Janus kinase 1(JAK1), Interferon (IFN), Interferon Regulatory Factor (IRF), Signal transducer and activator of transcription 2 (STAT), Interferon-sensitive response element (ISRE)

Moreover, surprisingly, BST2's role as a negative regulator of RIG-I like receptor (RLR)-mediated type I IFN signaling by targeting MAVS was demonstrated recently. BST2 recruits E3 ubiquitin ligase membrane associated ring-CH-type finger 8 (MARCHF 8) to catalyze K27-linked ubiquitin chains on MAVS at lysine 7, which serves as a recognition signal for NDP52-dependent autophagic degradation (Jin *et al.* 2017). In **Chapter 3**, focusing on the study of BST2's function on PPRV budding, we also evaluated the interactions of caprine BST2 with caprine MAVS (data not shown). Indeed, we observed the interactions between BST2 and MAVS by Co-IP but the IFA assay was less convincing. It must be noted that these interactions were observed in experiments of exogenous overexpression. If the interactions are also observed with the endogenous proteins during virus infection remains to be studied. The research results from **Chapter 3** suggested the antiviral property of caprine BST2 towards virus budding, but the mechanism needs to be studied in greater detail. In all, the interaction of GBST2A with caMAVS provide indications on the possible functions of ISGs in regulating adaptor proteins. Normally the activation of RLR mediated signaling pathways result in the expression of ISGs, while at a certain point, the feedback loop of ISGs on MAVS or other adaptor proteins indicate a versatile function of ISGs during innate immunity responses.

7.3 Adaptive immunity and vaccines

7.3.1. Novel vaccine technologies

Compared with traditional inactivated vaccines, the live-attenuated and viral vector vaccines, or innovative mRNA vaccines can provide more robust immune responses against viral infections. Inactivated virus vaccines often require multiple immunizations or the use of adjuvants to boost the immune response. Live-attenuated vaccines, on the other hand, result in very limited viral replication, but a very strong immune response including both humoral immunity and cell-mediated immunity (Dudek and Knipe 2006). However, the development and registration process for new live-attenuated vaccine candidates takes a long time. For example, the alphavirus non-structural protein 3 (nsp3) is known to participate in a number of cellular processes through interactions with host cellular proteins (Götte *et al.* 2018). Abolishing these interactions could potentially result in the generation of replication-defective viruses with deletions of nsp3 domains (Götte *et al.* 2018). In 2014, a novel attenuated chikungunya virus (CHIKV vaccine candidate was designed by an in-frame deletion of 61 amino acid of coding sequence within the nsP3 hypervariable domain (CHIKVΔ5nsP3) (Hallengärd *et al.* 2014). After successful preclinical testing in mice and nonhuman primates (Hallengärd *et al.* 2014, Roques *et al.* 2017), the CHIKVΔ5nsP3 vaccine candidate entered a

clinical phase 1 trial, and proved to be well tolerated in the low-dose groups, but fever was common in the high-dose groups, the vaccine was highly immunogenic (Wressnigg *et al.* 2020). In 2020, this candidate finally turned out to be the first chikungunya vaccine candidate to enter clinical phase 3, but in total, it took six years since the first experiments had started. Hopefully, successful clinical results from phase 3 will provide the basis for the licensure of the vaccine. However, although the CHIKVΔ5nsP3 vaccine has been rigorously tested (pre)clinically and found to be generally safe, the mechanism of the observed attenuation is still unclear, as the engineered deletion is just downstream of the CHIKV nsP3 conserved, zinc-binding, alphavirus unique domain, but upstream of important regulatory motifs within the HVD. The potential for reversion to virulence, which is an essential aspect to be addressed for any live-attenuated vaccine, has not been studied extensively beyond a serial passage experiment. Fortunately, no indications for reversion to virulence have so far come forward from the (pre)clinical trials. Ideally, the attenuation strategy should be based on solid theoretical knowledge combined with experimental safety data. The potential for reversion virulence remains the biggest concern in developing vaccines for emerging viruses based on a random attenuation strategy (Minor 2015). Research on virus-host interactions might lead to a more targeted approach to develop safe and effective vaccine candidates in the future.

A new era in vaccinology has recently commenced with vaccines based on nucleic acids, based on DNA or mRNA (Hobernik and Bros 2018, Pardi *et al.* 2018). RNA vaccines have gained particular attention in the current SARS-CoV-2 pandemic (Corbett *et al.* 2020, Jackson *et al.* 2020), because mRNA-based vaccines were generated in record time and proved to be highly efficient at preventing COVID-19. Furthermore, mRNA technology proved to have potential as a platform for clinical development in the treatment of tumors and other diseases in the future (Szurgot *et al.* 2020). Compared with traditional vaccines, mRNA/DNA vaccines are easier to design and produce. However, even though DNA is much more stable than RNA, only a limited number of DNA vaccines have been approved for human or veterinary use (Hobernik and Bros 2018). A reason for this might be that by injecting DNA into the nucleus of cells leads to safety concerns of introducing foreign DNA into host cells. Moreover, because of intramuscular injection, DNA vaccines tend to elicit mostly cell-mediated immune responses instead of antibody mediated responses (Cui 2005), and immunization often requires a large dose of DNA, which will greatly limit the application of DNA-based vaccines. Currently, there are two main types of mRNA vaccines: conventional mRNA vaccines and self-amplifying mRNA vaccines (saRNA). mRNA vaccines deliver a piece of intact mRNA encoding an antigen protein transcribed *in vitro* to the cell by using for instance nanoliposome delivery technologies. The cells use the mRNA to translate the antigen protein *in vivo*, subsequently, to trigger the immune

response. The major advantage of mRNA is that it does not have the risk of integrating into the host genome compared with DNA. Regarding animal infectious diseases, mRNA has also been shown to prevent infections, such as the application of vitro-transcribed foot-and-mouth disease virus (FMDV) RNA that showed protection in a mouse model against FMDV infections (Pulido *et al.* 2010). On the other hand, the saRNA technology, has shown enhanced protection at lower doses compared to conventional mRNA vaccines, for example, the saRNA vaccines expressing influenza hemagglutinin proved to be more effective than the corresponding mRNA vaccine in mice (Démoulins *et al.* 2021). In the future, the development of saRNA vaccines will probably be applied in the development of vaccines against veterinary diseases like porcine epidemic diarrhea virus(PEDV) or other coronaviruses (Démoulins *et al.* 2021), malaria (Baeza Garcia *et al.* 2018) and perhaps alphaviruses as well (Ballesteros-Briones *et al.* 2020). However, in general, RNA vaccines are expensive to manufacture, and special requirements, such as low-temperature storage and transportation, may increase the costs, which is a very important factor in veterinary vaccine development.

Compared with the mRNA vaccine candidates mentioned above, vaccine candidates based on protein subunits produced by different expression system are much more common and widely used because of their safety profile and cost-effectiveness. The baculovirus expression vector system (BEVS) was developed more than 30 years ago as a breakthrough technology, that since then produced thousands of recombinant proteins (van Oers *et al.* 2015). Moreover, it has been reported that baculoviruses can be generated with a single baculoviral expression vector. Multi-promoter technology allows BEVS to express multiple proteins at the same time, which is very suitable for complex structures, such as virus-like particles. Protein subunits can be engineered to assemble into virus-like particles (VLPs), which can be used to generate highly effective vaccines without using adjuvant (Vicente *et al.* 2011). Rapid and robust VLP manufacturing with BEVS-based technology can potentially fill the gap between rapidly emerging infectious animal viral diseases and the desperate need for effective vaccines with much lower cost.

7.3.2 Promoter choice in BEVS and the future of VLPs

During BEVS expression, expression levels of the gene of interest (GOI) are driven by baculovirus promotors, such as the very strong polyhedrin gene (polh) promoter and p10 gene promoter from the type species baculovirus, Autographa californica multiple nucleopolyhedrovirus (AcMNPV). In **Chapter 4-6**, the polh promoter within the expression vectors pDEST10 and pFastBAC1 (Invitrogen) was used for RHDV and GETV VLP expression, while for the bivalent RHDV VLP vaccine, we used both polh and p10 promoters

within a pFastBAC-dual transfer vector **(Chapter 5)**. Differences in expression levels were observed at the various time points post-infection (**Figure 5.2**); p10 promotor driven RHDV2 VLPs were shown earlier and had a higher expression level compared with polh promoter driven RHDV VLPs. In an early study, no significant difference in expression levels between polh and p10 promoters was observed (van Oers *et al.* 1992), but competition between the two promoters was later demonstrated by deletion of the p10 promoter, which resulted in an increase of mRNA transcription from the polh promoter (Chaabihi *et al.* 1993).

In addition to the use of polh and p10 promoters, other promoters or modifications to improve the BEVS expression system should be considered (van Oers *et al.* 2015), especially when yields are lower than expected. Transcriptome studies of the AcMNPV provide several promotor options, such as p6.9, odv-e18, and odv-ec27 for optimizing protein production to an earlier stage the infection cycle (Chen *et al.* 2013). In our study, the expression level of GETV VLPs generated in **Chapter 6** was lower compared with previous results from CHIKV VLPs, therefore further improvements of GETV VLP production might be achieved by choosing a different promotor except for promoters from AcMNPV, the orf46 promoter sequence from Spodoptera exigua multiple nucleopolyhedrovirus could be used (Martínez-Solís *et al.* 2016). Another option is to use promoter combinations with enhancers, such as homologous regions (*hr*) transcriptional enhancers or repeated burst sequences. Such strategies can enhance total production and/or shorten optimal production times (Gwak *et al.* 2020). By expressing transactivation factors IE1 and IE0 under the control of the polh promoter, and a *hr* transcriptional enhancer sequence cis-linked to the p10 promoter, prolonged cell integrity, protein integrity, and around four times increase in production levels of recombinant protein can be reached (Gómez-Sebastián *et al.* 2014). Finally, RNAi-mediated silencing of the pro-apoptotic sf-caspase-1 also lead to improved recombinant protein production (Zhang *et al.* 2018), whereas relocation of the attTn7 transgene insertion from the mini-F replicon increases the stability of the baculovirus expression vector (Pijlman *et al.* 2020).

The future of VLPs may not be restricted to their use as a vaccine candidate. For example, chikungunya VLPs were also used as carrier to display circumsporozoite protein (CSP) of the *Plasmodium falciparum* malaria parasite. These chimeric VLPs were able to elicit strong immune responses against CSP in animals (Urakami *et al.* 2017). Similarly, chimeric RHDV VLPs displaying FMDV antigens can induce FMDV neutralizing antibodies in mice (Crisci *et al.* 2012) and pigs (Rangel *et al.* 2021). In yet another studies, RHDV VLPs were also applied to display B-cell epitope of influenza A virus M2 protein which produce protective humoral immune responses (Moreno *et al.* 2016), or expressing ovalbumin (OVA) or OVA-derived

CD4 (OTII) and CD8 (OTI) tumor epitopes which induce an anti-tumor response (Peacey *et al.* 2008, Win *et al.* 2012). The applications of Hepatitis B virus (HBV)VLPs were furtherly also applied in small molecule delivery (Yamada *et al.* 2003). In addition, VLPs are used for development of diagnostic applications such as for Hepatitis C Virus (HCV) (Prasetyo 2017), porcine circovirus type 3 (PCV3) (Wang *et al.* 2020), and SARS-COV2 (Chan *et al.* 2021). In summary, VLPs technology is a valuable tool for vaccine development, directly or as a carrier, and also finds practical applications in drug delivery and diagnostics.

7.3.3 Cross-reactive immunity

Studying the cross-protectivity of virus vaccine candidates against virus of the same species with different genotypes or closely related viruses will directly provide implications for the clinical veterinary vaccination programs. In 2018, cross-protection and comparative pathogenicity of the lagoviruses GI.1b/RHDV and GI.2/RHDV2/b were demonstrated in a challenge trial. Rabbits were first challenged with GI.1b/RHDV and GI.2/RHDV2/b isolates, respectively, and then a second challenge on survivors with the other strain. The results showed that GI.2/RHDV2/b pathology was highly differential due to unknown individual specimen factors, which mortality by GI.1b/RHDV was associated with age (Calvete *et al.* 2018). Since RHDV2 is also discovered in domestic rabbits vaccinated against RHDV, this may suggest that RHDV immunity does not (fully) protect against RHDV2. In **Chapter 4**, we tested the immunogenicity of RHDV2 VLPs, produced by BEVS, in rabbits. By immunization of rabbits with RHDV2 VLPs ("recRHDV2-vacc"), it was demonstrated that the cross-protection provided by the RHDV2 vaccine against classical RHDV was indeed limited, as only 50% of vaccinated rabbits survived after the RHDV infection . Similar results were observed for rabbits vaccinated with the traditional liver-derived RHDV2 vaccine ("convRHDV2-vacc") (Muller *et al.* 2019). Surprising results were found with the traditional inactivated liver-derived vaccine "Cunivak RHD" developed for RHDV (Muller *et al.* 2019). After a prime-boost vaccination rabbits conveyed full cross-protection against RHDV2, while they were partially cross-protected against RHDV after a single vaccination (Müller 2018). Overall, the research results mentioned above indicate the complexity of cell-mediated immunity among strains with different genotypes. RHDV2 is highly virulent in both young and adult rabbits, while rabbits younger than eight weeks are highly resistant to disease caused by traditional RHDV. It was demonstrated that young rabbits have stronger innate immune responses compared to adult rabbits. When infected with RHDV, high expression of major histocompatibility class II molecules and natural killer cells, macrophages, and cholangiocytes might protect the young rabbits from being infected (Neave *et al.* 2018). Downregulation of multiple genes associated with innate immunity during RHDV2 infection in these young rabbits, may contribute to the

development of disease. However, if vaccination against RHD is only complete with two different vaccines, this will pose higher economic costs and efforts for breeders and farmers. Rabbits reared for meat production are sent for slaughter in at 4-5 months of age, so future studies to confirm the cross-protectivity of RHDV2 VLPs against RHDV infection may provide a basis for the industry to reduce the vaccine costs. In **Chapter 5**, we present a unique bivalent RHD vaccine candidate based on RHDV and RHDV2 VLPs produced with the BEVS that induces significant humoral and cellular immune responses. All rabbits immunized with the bivalent VLP vaccine survived after the challenge with classic RHDV and showed no clinical signs of RHD. The limitation of the study is the virus challenge experiment, which was done with the classical RHDV strain only, because at that time no RHDV2 was discovered in China. So, a potential follow-up study needs to confirm protection against the RHDV2 strain as well.

In **Chapter 6**, we produced a GETV VLP vaccine candidate in insect cells with a similar strategy using BEVS. Earlier reports have described cross-protection between different alphaviruses, by using an anti-Sindbis virus hyperimmune serum (Wust *et al.* 1987). Recently, mice exposed to wild-type CHIKV challenge were later also found with strong cross-protection against Mayaro virus (Webb *et al.* 2019). The cross-reactivity of GETV serum against other alphaviruses was also studied (Nguyen *et al.* 2020). With the established mouse model, the cross protectivity between GETV and Ross River virus (RRV) was investigated and a high level of overlapping cross-neutralization between RRV and GETV antiserum was observed (Rawle *et al.* 2020). By generating GETV VLPs, and further study in relevant animal models might provide insight in its cross-protectivity against closely related alphaviruses, and whether it has potential as cross-protective alphavirus vaccine.

7.4 Future perspectives on control measures against emerging animal viruses

Recently, numerous pathogenic animal viruses significantly damaged the Chinese industry and affected virus disease prevention and control. Emerging animal viruses may also pose a serious threat to human health. Within research, important steps are being taken to enhance our knowledge on virus-host interactions and the design of VLP-based vaccines. On the governmental level, the Chinese central government set up a Veterinary Bureau under the Ministry of Agriculture (now called Ministry of Agriculture and Rural Affairs) and appointed a Chief Veterinarian in 2004 (Zhang 2019). By implementing the Animal Epidemic Prevention Law of the People's Republic of China, the Chinese government is trying to strengthen animal disease prevention, to control and stop animal disease epidemics, in order to promote the development of the animal breeding industry, and protect human health. However, the epidemic

prevention system is can be further strengthened in the area of veterinary disease, by strengthen the communications between the Veterinary Bureau of the Ministry of Agriculture and Rural Affairs and the Chinese Center for Disease Control and Prevention (China CDC), which will directly give suggestions to improve the construction of animal epidemic prevention systems. Moreover, the communication of the Veterinary Bureau with CDC also will assist the research on the relationships between animal viruses and human diseases (zoonoses). Moreover, it is also crucial to implement supporting policies to promote the development of veterinary professional education. Changing the traditional production methods and implementing modern livestock breeding systems will also contribute to prevention of animal virus outbreaks and making control management easier.

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English summary

Frequent outbreaks of emerging animal viruses cause great economic losses and pose potential risks for human health. In my thesis, I focused on emerging animal viruses that affect the veterinary industry and have a significant economic burden in China and beyond. These viruses include Peste des Petits ruminants virus (PPRV), Rabbit hemorrhagic disease virus (RHDV2), and Getah virus (GETV). By studying different aspects of virus infection in relation to innate immunity and a focus on virus-like particle (VLP) vaccine development, important knowledge for future control of emerging animal viruses will be generated.

First, to understand PPRV (family *Orthomyxovirdae*, genus *Morbillivirus*) induced innate immune responses, we demonstrated that the mitochondrial antiviral-signaling protein (MAVS, also known as VISA, IPS-1, or CARDIF) plays an essential role in the type I interferon (IFN) response and in retinoic acid-inducible gene I (RIG-I) mediated antiviral innate immunity in mammalian cells (**Chapter 2**). The caprine MAVS gene (caMAVS, 1566 bp) was identified and cloned. caMAVS shares the highest amino acid similarity (98.1%) with the predicted sheep MAVS. By confocal microscopy analysis of partial deletion mutants of caMAVS, we revealed that the transmembrane and the so-called Non-Characterized domain are indispensable for the intracellular localization of caMAVS to mitochondria. Furthermore, overexpression of caMAVS in caprine endometrial epithelial cells resulted in up-regulation of the mRNA levels of caprine interferon-stimulated genes. We concluded that caprine MAVS mediates the activation of the type I IFN pathway. We further demonstrated that both the CARD-like domain and the transmembrane domain of caMAVS were essential for the activation of the IFN-β promotor. The interaction between caMAVS and caprine RIG-I and the vital role of the CARD and NC domain in this interaction was demonstrated by co-immunoprecipitation studies. Moreover, we noticed the level of MAVS was greatly reduced upon infection with PPRV, and this reduction was prevented by the addition of the proteasome inhibitor MG132. In addition, the PPRV protein V interacted and colocalized with caMAVS. Together, we identified caMAVS as a RIG-I interactive protein involved in activating type I IFN pathways in caprine cells and as a target for PPRV immune evasion.

To further understand the effect of the expression of the interferon stimulated genes (ISGs) on the replication cycle of PPRV, we studied one of the ISGs, tetherin/BST2, in detail (**Chapter 3**). We identified two caprine BST2 isoforms and tested their antiviral activity against PPRV. Ectopic expression of either of the two caprine BST2 isoforms blocked the release of PPRV from Vero-SLAM cells. Moreover, we found that caprine BST2 (GBST2) inhibited PPRV

budding and executed this function independent of the GBST2 N-glycosylation status. Moreover, we found that GBST2 interacted and colocalized with the before mentioned PPRV protein V. By generating a series of mutants of both GBST2 and V, we further mapped the domains crucial for that interaction. We also demonstrated that GBST2 interfered with the ability of PPRV-V to inhibit IFN-β production (**Chapter 3**). The study on the importance of tetherin for PPRV expanded our understanding of the antiviral activity of BST2 orthologs against non-human morbilliviruses.

In light of emerging animal viruses in veterinary industry, we took notice of a Rabbit haemorrhagic disease virus (RHDV) type 2 (GI.2/RHDV2/b) outbreak in Europe. RHDV belongs to the family *Calliciviridae* (genus *Lagovirus*). The outbreak of this emerging pathogen is seen in wild rabbits and in domestic rabbits vaccinated against RHDV type 1 (GI.1). We successfully determined the genome sequence of a RHDV2 strain isolated from the liver of a naturally infected wild rabbit in the Netehrlands. The complete viral genome sequence was assembled from sequenced RT-PCR products. Phylogenetic analysis based on the VP60 capsid gene demonstrated that the RHDV2 NL2016 isolate clustered with other contemporary RHDV2 strains. The VP60 coding sequence was cloned in a baculovirus expression vector to produce VLPs in *Spodoptera frugiperda* Sf9 insect cells. After density-gradient purification, RHDV2 VLPs were visible with transmission electron microscopy as spherical particles with a diameter of approximately 30 nm and with a morphology resembling authentic RHDV. The RHDV2 VLPs were used for the immunization of rabbits, which resulted in high levels of serum antibodies directed against VP60. Furthermore, the production of cytokines (IFN-γ and IL-4) was significantly elevated in the immunized rabbits compared to the control group. The results demonstrated that the recombinant RHDV2 VLPs were highly immunogenic and provide an attractive candidate vaccine that might be further developed to protect domestic rabbits against RHDV2 infection. In addition, these VLPs may find applications in serological detection assays.

However, as both RHDV and RHDV2 are the main causes of rabbit hemorrhagic disease (RHD), we also aimed to develop a new subunit vaccine that could protect rabbits against both classic RHDV and RHDV2 infections by the construction of a recombinant expressing the VP60 genes of classic RHDV and RHDV2 simultaneously. Both VP60 genes were well expressed in Sf9 cells after infection with the recombinant baculovirus, and transmission electron microscopy showed that the recombinant VP60 proteins self-assembled into virus-like particles (VLPs). We further studied the antigenicity and immunogenicity of the bivalent VLP vaccine by animal experiments. Our results demonstrated that both the humoral and cellular

immune responses were efficiently induced in rabbits. In addition, all rabbits immunized with this bivalent VLP vaccine survived after challenged with classic RHDV, and showed no clinical signs, whereas all the rabbits in the negative control group died from classic RHDV infection and showed typical clinical signs of RHD. The immunization data demonstrate the future potential of this bivalent VLP vaccine against both classic RHDV and RHDV2 infections.

More recently, there has been an outbreak of Getah virus (GETV) in China among piglets leading to abortion events, even in fully immunized herds. GETV is an emerging arthropod-borne virus of the genus *Alphavirus* in the family *Togaviridae.* This virus causes economic losses associated with infections in pregnant sows and also horses. We successfully produced enveloped Getah VLPs in insect cells using the baculoviruses expression vector system by expressing the GETV structural gene cassette in Sf9 cells. We showed that the resulting VLPs were glycosylated, and that the expressed E1 envelope protein localized to the cell surface. The capsid protein also assembled in the form of core-like particles, which accumulated in the nucleus of the cells as seen with transmission electron microscopy. Furthermore, VLPs with a diameter of 60~80 nm were observed after discontinous sucrose gradient purification. With this strategy we produced a GETV candidate vaccine which after further testing may prevent animals from GETV infection.

In conclusion, the research described in this thesis aimed to understand how to control emerging animal viruses, thereby focusing on three emerging animal viruses. Data were obtained on several aspects of virus-induced innate immunity in PPRV infection in goats, including the function of caprine MAVS and the ISG, BST2/tetherin, and how PPRV counteracted these factors. In addition, promising vaccine candidates were developed against RHDV1 and RHDV2 in rabbits, and against GETV. Over all the research presented in this thesis contributed to the understanding of virus-host interactions and provided candidates for veterinary vaccine development.

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Last but not least, I would like to thank my parents for endless love and support, your encouragement keep me self-motivated to do what I want, I wish I could have more time to spend with you, and I love you, I hope I make you proud. 爸爸妈妈, 我爱你们!

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Qiuhong,

Wageningen, the Netherlands.

About the author

Qiuhong Miao was born on July 6th, 1989 in Taizhou, Jiangsu, China. After completing her primary and secondary education, she started her studies in Animal Science at Yangzhou University in 2007, where she grew interest in virological research. After completing her BSc, in 2011, she was admitted to the Chinese Academy of Agricultural Science to continue her research in veterinary viruses with the major in veterinary medicine. During her master, she was focusing on caliciviruses and generating a replicon system together with her colleagues. In 2014, she successfully finished MSc study and joined a company affiliated to the Shanghai Veterinary Research Institute to commercialize vaccine candidates from the institute. However, her dream to continue to study on viruses and pursue a doctoral degree had never stopped. In 2016, she got the opportunity to continue her study with a Sandwich PhD position through CAAS-WUR sandwich programme. After receiving her doctoral degree, Qiuhong Miao is looking forward to continue her research in the field of host innate immune responses to virus infections and the development of novel viral vaccines as a postdoctoral researcher in Pei-Yong Shi's group at the University of Texas Medical Branch at Galveston.

List of Publications

1. Miao, Q., Qi, R., Meng, C., Zhu, J., Tang, A., Dong, D., Guo, H., van Oers, M. M., Pijlman, G. P., and Liu, G. (2021) Caprine MAVS Is a RIG-I Interacting Type I Interferon Inducer Downregulated by Peste des Petits Ruminants Virus Infection. *Viruses* , 13(3), 409

2. Miao, Q., Qi, R., Veldkamp, L., Ijzer, J., Kik, M. L., Zhu, J., Tang, A., Dong, D., Shi, Y., van Oers, M. M., Liu, G., and Pijlman, G. P. (2019) Immunogenicity in Rabbits of Virus-Like Particles from a Contemporary Rabbit Haemorrhagic Disease Virus Type 2 (GI.2/RHDV2/b) Isolated in The Netherlands. *Viruses* Jun; 11(6): 553.

3. Qi, R. #, Miao, Q. #, Zhu, J., Tang, J., Tang, A., Wang, X., Dong, D., Guo, H., and Liu, G. (2020) Construction and immunogenicity of novel bivalent virus-like particles bearing VP60 genes of classic RHDV(GI.1) and RHDV2(GI.2). *Vet. Microbiol*. 240, 108529 (# co-first author)

4. Guo, H.#, Miao, Q.#, Zhu, J., Yang, Z., and Liu, G. (2018) Isolation and molecular characterization of a virulent systemic feline calicivirus isolated in China. *Infect. Genet. Evol*. 65, 425-429 (# co-first author)

5. Zhu, J., Miao, Q., Tang, J., Wang, X., Dong, D., Liu, T., Qi, R., Yang, Z., and Liu, G. (2018) Nucleolin mediates the internalization of rabbit hemorrhagic disease virus through clathrin-dependent endocytosis. *PLoS.Pathog*. 14, e1007383

6. Zhu, J., Miao, Q., Tan, Y., Guo, H., Liu, T., Wang, B., Chen, Z., Li, C., and Liu, G. (2017) Inclusion of an Arg-Gly-Asp receptor-recognition motif into the capsid protein of rabbit hemorrhagic disease virus enables culture of the virus in vitro. *J. Biol. Chem*. 292, 8605-8615

7. Zhu, J., Miao, Q., Tan, Y., Guo, H., Li, C., Chen, Z., and Liu, G. (2016) Extensive characterization of a lentiviral-derived stable cell line expressing rabbit hemorrhagic disease virus VPg protein. *J Virol. Methods*. 237, 86-91

8. Dong, D., Zhu, S., Miao, Q., Zhu, J., Tang, A., Qi, R., Liu, T., Yin, D., and Liu, G. (2020) Nucleolin (NCL) inhibits the growth of peste des petits ruminants virus. *J. Gen. Virol*. 101, 33-43

9. Guo, H., Zhu, J., Miao, Q., Qi, R., Tang, A., Liu, C., Yang, H., Yuan, L., and Liu, G. (2020) RPS5 interacts with the rabbit hemorrhagic disease virus 3' extremities region and plays a role in virus replication. *Vet. Microbiol*. 249, 108858

10. Qi, R., Zhu, J., Miao, Q., Tang, A., Dong, D., Wang, X., and Liu, G. (2019) Bioinformatics analysis of capsid protein of different subtypes rabbit hemorrhagic disease virus. *BMC. Vet. Res*. 15, 423

PE&RC Training and Education Statement

With the training and education activities listed below
the PhD candidate has complied with the requirements
set by the C.T. de Wit Graduate School for Production
Ecology and Resource Conservation (PE&RC) which
comprises of a minimum total of 32 ECTS (= 22
weeks of activities)

Review of literature (6 ECTS)

- Host restriction factors limiting the budding of enveloped viruses

Writing of project proposal (4.5 ECTS)

- Host restriction factors limiting the budding of enveloped viruses

Post-graduate courses (4.4 ECTS)

- Microscopy and spectroscopy in food and plant sciences; EPS&VLAG (2017)
- Statistics for the life sciences; WIAS (2017)
- Bioinformatics analysis of biological sequences, from sequence to structure;
  Departamento de Biologia Vegetal (FCUL); Lisboa (2021)

Invited review of (unpublished) journal manuscript (2 ECTS)

- BMC Veterinary Research: Rabbit hemorrhagic disease virus 2 infection in captive
  mountain hares (2020)
- Transboundary and Emerging Diseases: Intergenic recombination in feline
  calicivirus (2020)

Deficiency, refresh, brush-up courses (4.2 ECTS)

- Fundamental and Applied Virology; Laboratory of Virology (2017)
- Academic English for PhD candidates; WGS (2017)

Competence strengthening / skills courses (5.6 ECTS)

- Academic writing and presenting in English; WGS (2016)
- Efficient and effective academic development; WGS (2016)
- Research ethics; WGS (2017)

- Data management planning; WUR Library (2017)
- Reviewing a scientific paper; WGS (2017)
- Competence assessment; WGS (2017)
- Scientific artwork, data visualization and infographics with Adobe Illustrator; WUR Library (2020)
-

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- PE&RC First years weekend (2017)
- PE&RC Midterm weekend (2020)

Discussion groups / local seminars or scientific meetings (11.5 ECTS)

- Animal health immunology discussion group (2017)
- Wageningen Evolution & Ecology Seminar (WEES) (2017)
- Wuhan international symposium on modern virology (2017, 2019)
- Dutch annual virology symposium (2017, 2022)
- Dutch arboviral network meeting (2017, 2022)
- Annual meeting of medical virology (2018)
- Natural conferences: viral Infection and immune response (2018)
- 13th Annual meeting Chinese society for immunology (2018)
- Yangtze river delta doctorial forum in preventive veterinary medicine (2018, 2019)
- 9th Annual Edinburgh infectious diseases symposium (2020)
- International workshop on biosafety Laboratory Management and Techniques (2020)
- International virtual seminar on arbovirus biology (2020, 2021)
- American Society of Virology (ASV) (2021)

International symposia, workshops and conferences (3.5 ECTS)

- American Society of Virology (ASV); online (2020)
- FEMS Conference on microbiology; online (2020)

Supervision of MSc students (3.0 ECTS)

- Interactions of Getah virus with stress granules and ZAP
- Function analysis of HVD domain from Getah virus NSP3