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STUDIES ON THE FUSION AND ATTACHMENT ENVELOPE GLYCOPROTEINS
OF MOJIANG HENIPAVIRUS: FUNCTION, STRUCTURE AND SEROLOGIC
APPLICATIONS IN RODENTS

by

Sofia Cheliout Da Silva

Dissertation submitted to the Faculty of the
Emerging Infectious Disease Graduate Program of the
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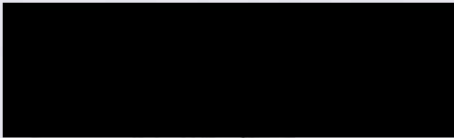
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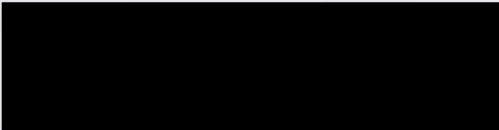
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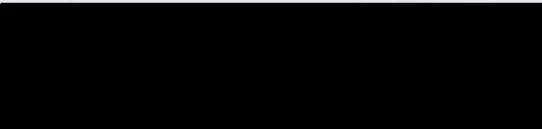
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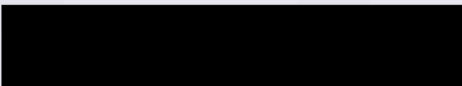
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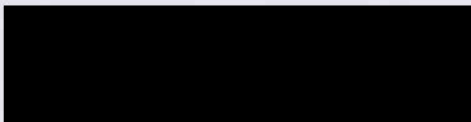
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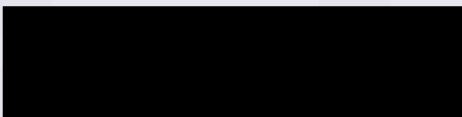
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DEDICATION

Pour Maman et Sarah:

Malgré le temps qui passe et la distance, votre soutien indéfectible et votre affection continue de me porter dans les moments difficiles comme dans les petites victoires de tous les jours. Cet accomplissement est aussi le votre.

To all curious and inquisitive minds:

Scientia vincere tenebras (motto, Université Libre de Bruxelles, Belgium)

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August 4, 2020

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ABSTRACT

Studies on the Fusion and Attachment Envelope Glycoproteins of *Mojiang henipavirus*: Function, Structure and Serologic Applications in Rodents.

Sofia Cheliout Da Silva, Ph.D., 2020

Thesis directed by: Dr. Christopher C. Broder, Professor and Chair, Department of Microbiology and Immunology

Mojiang virus (MojV) is a recently discovered paramyxovirus of the genus henipavirus, which includes highly pathogenic zoonotic species. Henipaviruses employ the concerted action of their fusion (F) and attachment (G) glycoproteins to mediate host cell entry by utilizing ephrin ligands as entry receptors. While the natural reservoirs for all henipaviruses have been pteropid bats, MojV was the first henipavirus identified in rodent specimens, which raises important questions about its receptor tropism and host range. Due to the unknown pathogenic potential of MojV and the absence of any viral isolate, alternative experimental approaches to study the molecular virology of MojV were required. Here, recombinant MojV F and G glycoproteins were functionally and structurally characterized. A highly sensitive cell-cell fusion assay using a Nano luciferase reporter was developed to investigate the species tropism and the role of ephrin ligands in MojV F and G mediated fusion as a surrogate of viral infection. Immunogenic soluble MojV F and G glycoproteins were also integrated into a multiplex microsphere

immunoassay (MMIA). The present work demonstrates that MojV F and G are functionally competent and structurally similar to other henipavirus F and G glycoproteins, despite lower levels of fusion, slower kinetics, and a relative instability of the pre-fusion conformation of MojV F. Cells expressing murine ephrin-A1, rat ephrin-A4 and human ephrin-A5 were susceptible to MojV F and G mediated fusion. However, the cognate primary entry receptor for MojV G was not conclusively identified and the particular role of these ephrin ligands in MojV entry remains unknown. This study also established that MMIA can be applied to assess the reactivity of rodent antibodies to F and G glycoproteins, and that fecal samples can be used as a non-invasive collection alternative to blood serum in serologic rodent studies. Overall, these data confirm the close relatedness of MojV to other henipaviruses, but also highlight unique characteristics of this emerging virus. The tools and findings established here provide additional means for serologic surveillance, and a better understanding of the mechanisms of MojV cell entry and tropism. These assays may also prove valuable in crafting adequate responses should a zoonotic outbreak occur.

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CHAPTER 1: General Introduction

HENIPAVIRUS HISTORY AND DISEASE

Hendra and Nipah viruses: prototypical pathogenic henipaviruses

History and emergence

Hendra (HeV) and Nipah (NiV) viruses were discovered in the 1990s (1; 59; 110; 166; 175; 231; 253). HeV was identified during an outbreak that occurred in Hendra in the suburbs of Brisbane, Australia in September 1994 (175). During this episode, a respiratory disease originally thought to be caused by a new equine morbillivirus killed 14 horses as well as the racehorse trainer, Vic Rail, who had been working with these animals (175; 231). Although the stablehand fell ill, he was able to recover (175; 231). Due to its unique genomic, molecular and pathogenic characteristics, this new pathogen was later reclassified as a member of the novel paramyxovirus genus *Henipavirus* and named Hendra virus (253). Two cases of fatal viral encephalitis in horses recorded in August 1994 in Mackay, Queensland, 970 km north of Hendra, were retrospectively attributed to HeV infection as well (110; 221). The owner of these horses, who seemed to recover from a brief aseptic meningitis in August, succumbed to relapsed HeV severe encephalitis in 1995 (186). Sporadic HeV outbreaks have been reported between 1994 and 2004, then became yearly occurrences until 2010, and further accelerated in 2011 when 18 outbreaks occurred involving 22 horses, requiring the monitoring of 70 at-risk individuals and providing the first report of natural infection in dogs (10; 11; 191). Another 30 separate outbreaks were reported between 2012 and June 2019, with multiple isolated reports every year (90). Since the discovery of HeV a total of 84 confirmed and

20 untested or unresolved horse cases resulted in death; however, human infections remain rare with a total of only seven cases, four of which resulted in fatalities (90; 191).

In contrast, NiV outbreaks exact a notably heavier toll on human lives. NiV emerged in 1997 near Ipoh, Malaysia, during an epidemic that involved the death of pigs afflicted with respiratory illness and encephalitis (57). At first these cases were believed to be caused by classical swine fever (57). Then, the first major outbreak of NiV occurred in 1998/1999 and likewise affected pigs and pig farmers. Symptoms of febrile encephalitis and respiratory illness observed in human cases were attributed to Japanese encephalitis virus (JEV), which is endemic to the region and for which pigs can serve as amplifying hosts (58). Measures to curb the suspected JEV epidemic, including a vaccination campaign and mosquito control programs, failed to prevent the spread of the outbreak which reached Singapore by export of infected animals to abattoirs (57). Virus isolated from cerebrospinal fluid from a patient of Seremban General Hospitals was shown to cause syncytia formation, a hallmark cytopathic effect of paramyxoviruses infections, in Vero cells, and electron microscopy further suggested that this novel pathogen was closely related to paramyxoviruses (58). Confirmation that an emerging unknown paramyxovirus had been identified came from immunofluorescence studies demonstrating cross-reactivity to HeV and sequence analysis by the Center for Disease Control and Prevention (CDC) (58). NiV was isolated for the first time from a patient residing in Sungai Nipah, Malaysia, after which the virus was named (57; 58; 60; 191). The Malaysian outbreak of 1998/1999 resulted in 265 reported human infections, 105 of which were fatal, and had severe economic consequences; over one million pigs were culled to contain the epidemic (57). Since 2001, subsequent outbreaks of a second

distinct strain of NiV have occurred yearly in regions that span over part of Bangladesh and the neighboring West Bengal area of India, and that are now referred to as the “Nipah belt” (146; 147). Surprisingly, a recent outbreak that claimed 21 lives out of 18 confirmed and 4 suspected cases was contained in Kerala, India which is located on the southwestern Malabar Coast of India, more than 1800 km away from the regions where the recurring NiV epidemics have been recorded in the last decade (15). Once more an isolated case was reported in Kerala in June 2019 (12). Lastly, an outbreak of NiV or NiV-like infections involved 17 human cases in 2014 in two villages in Mindanao, Philippines (53). Interestingly, this was the first report of the possible involvement of horses as transmission hosts of NiV as 10 horses, most of which presented an acute neurological illness, died and seven patients had slaughtered horses or consumed their meat (53).

Clinical presentation of HeV and NiV disease

HeV and NiV are categorized as BSL-4 agents and cause disease presentations that share similar characteristics (263). The incubation period can last up to two weeks and the initial stage of the HeV or NiV disease tends to present as a flu-like illness with fever, headaches and drowsiness (263). The infections can then progress to severe respiratory syndrome, encephalitis or, in some cases, a combination of both, and have high fatality rates reaching on average 50% in HeV outbreaks or 40% and up to 100% in the NiV Malaysia outbreak and the Bangladesh/India outbreaks, respectively (234; 263). Pathogenesis of henipaviral disease is characterized by severe respiratory distress, CNS involvement and vasculopathy (263). During the Malaysia outbreak only 25% of NiV illnesses presented as respiratory syndrome. Most patients, instead, showed neurological

symptoms, including seizures, dizziness, vomiting, hypotonia, reduced consciousness and coma (263). In contrast, pulmonary syndrome was more common than encephalitis during the Bangladesh/India outbreaks with symptoms such as cough, abnormal chest X-rays, atypical pneumonia, and at times diarrhea and vomiting (22; 111; 263). Systemic thrombosis and vasculitis resulting from the infection of endothelial cells can cause microinfarction, necrosis or hemorrhage in major organs (263). The combination of endothelial cells presenting syncytia, vasculitis with microinfarction and infection of neuronal cells with plaque-like lesions in the brain are characteristic of henipaviral neurological disease compared to encephalitis caused by other viral infections (261; 263). Less than 10% of patients who recover from NiV infection can suffer from relapsing encephalitis on average 8 months after they recovered from the initial illness, although one case was reported to have relapsed 11 years later; relapsing NiV disease is restricted to encephalitic symptoms with viral replication limited to the central nervous system (CNS) without any pulmonary involvement (191; 263).

Cedar virus: a non-pathogenic henipavirus

Cedar virus (CedV) was isolated from urine samples collected in 2009 during a surveillance program of a flying foxes (*Pteropus* fruit bats) colony in Cedar Grove, South East Queensland, Australia (149). Syncytia formation was observed in *Pteropus alecto* kidney cells (PaKi) and African green monkey kidney cells (Vero) infected by the virus suggesting that it might be a new paramyxovirus, while genomic analysis confirmed its classification as henipavirus (149). Interestingly, the phosphoprotein (P) gene of CedV lacks the RNA editing sites required for the expression of the accessory proteins V and W, known antagonists of the type I interferon response to henipaviral infections (further

discussed below) (149). In addition, rabbit anti-HeV and anti-NiV antibodies were found to be cross-reactive, but did not cross-neutralize CedV in cell culture; the converse was observed as well (149). Moreover, Marsh et al. (149) established that CedV infection, unlike HeV and NiV infection, did not result in significant pathology in guinea pigs and ferrets, despite CedV being able to replicate in these animals. In support of the original findings from Marsh et al. (149), the P protein of CedV was shown to be unable to interact with STAT1/2, unlike HeV P and NiV P, and unable to inhibit IFN α production (143; 230). These non-pathogenic characteristics of CedV offer an interesting opportunity for use in vaccine development and studies of henipavirus virulence.

Henipaviruses identified from sequencing data alone

Ghana virus

Reports of the existence of additional distinct henipaviruses have highlighted the presence of these emerging viruses in geographical areas other than South East Asia. For instance, Ghana virus (GhV), formally named African bat henipavirus Eid hel/GH-M74a/GHA/2009 (M74) or Kumasi virus (KV), was identified by full genome sequencing of viral nucleic acids extracted from fecal samples from *Eidolon helvum* bats collected in Ghana (76; 128). Although GhV was shown to use the canonical henipavirus entry receptor ephrin-B2 ligand (further discussed below), no viral isolate was recovered from the samples (128; 136; 256). Interestingly, early reports established that co-expression of GhV F and G glycoproteins in baby hamster kidney cells (BHK-21) and African green monkey cells (Vero76) failed to produce syncytia, but did so in *Hypsignathus monstrosus* kidney and lung cells (HypNi/1.1 and HypLu/2, respectively), and *Eidolon helvum* kidney cells (EidNi/41), unlike transiently co-expressed NiV F and

G glycoproteins, which mediated cell-cell fusion in all three cell lines (127; 128; 256). Weis et al. (256) proposed that impaired trafficking and inadequate proteolytic processing caused a functional defect in the recombinant GhV F glycoprotein. Pernet et al. (200) subsequently demonstrated that a single nucleotide addition near the N terminus of the GenBank reference sequence of GhV F restored proper function of glycoprotein, suggesting that a RT-PCR error may have resulted in the report of an inaccurate reference sequence and the defective phenotype of recombinant GhV F previously observed by Weis et al. (256). Studies based on the corrected version of GhV F found that co-expression of GhV F and G resulted in syncytia formation in human U87 glioblastoma cells and BHK-21 cells (136; 215). Receptor usage and ability of GhV F and G to mediate syncytia formation in rodent and human cells suggest that GhV has the potential to spill over into human populations. In addition, as of now the possibility that GhV may express functional V and W accessory proteins has not been investigated, which leaves unanswered questions about the pathogenic potential of this emerging African henipavirus.

Mojiang virus

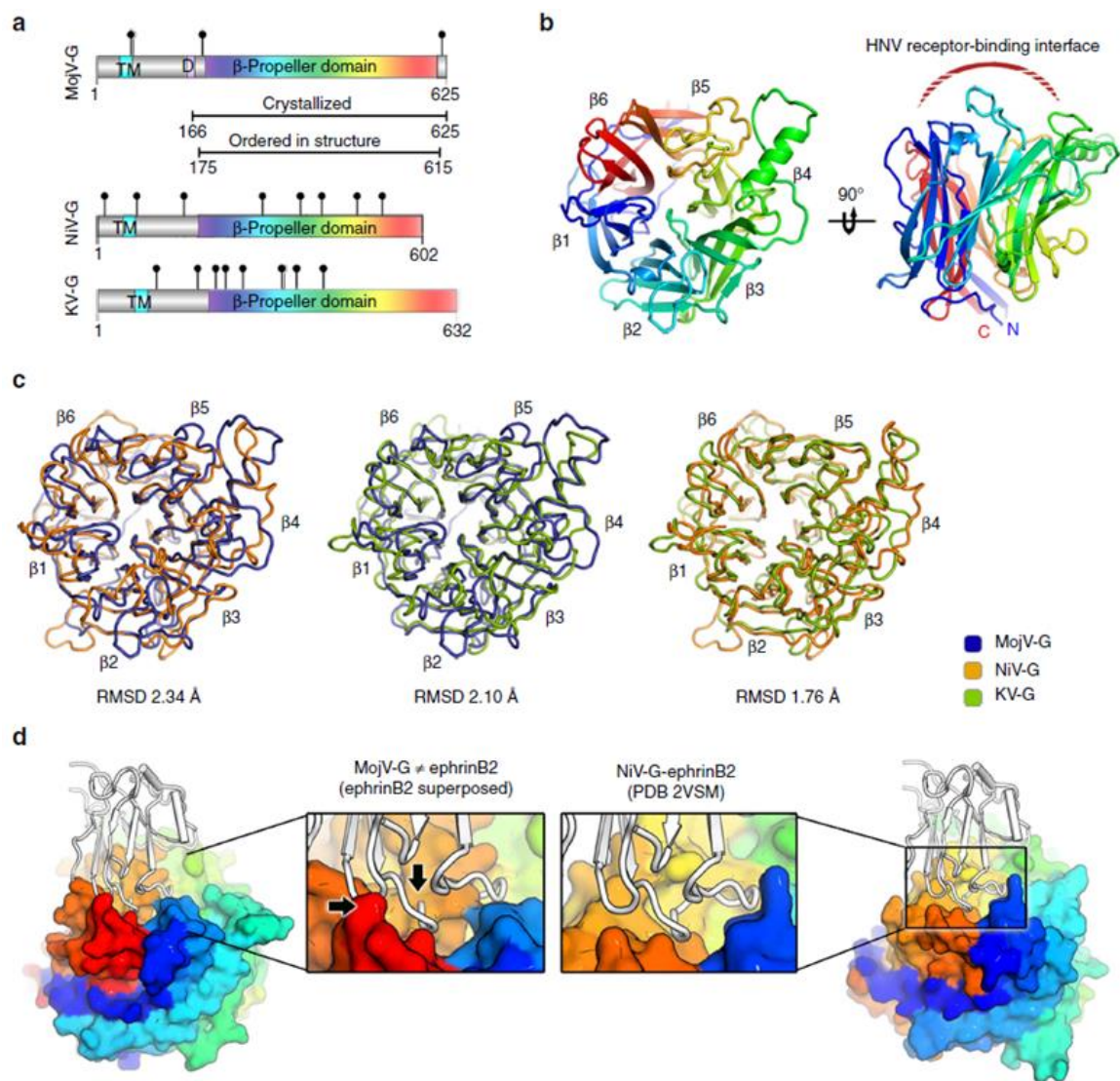
Mojiang virus (MojV) was identified in 2012 by PCR detection of viral RNA in *Rattus flavipectus* anal swabs from specimens collected in an abandoned mine in Mojiang Hani Autonomous County, Yunnan Province, China, six months after three individuals who had worked in the mine died of pneumonia of unknown etiology (266). MojV was classified as henipavirus due to its genomic organization and conserved sequences for gene starts, intergenic regions and gene stops amongst henipaviruses (266). The identification of MojV as a henipavirus is further supported by studies on nuclear

localization and secretion of henipavirus M proteins (154). MojV M, like other henipavirus M proteins, shares a bipartite nuclear localization signal (NLSbp1 and NLSbp2) region with HeV, NiV and CedV M, allowing its trafficking to the nucleus before MojV M locates to the plasma membrane and induces filamentous extensions (154). Interestingly, MojV G glycoprotein is unable to bind ephrin-B2 and -B3 ligands, the viral entry receptors for prototypical henipaviruses HeV and NiV (further discussed below) (**Figure 1-1d**) (215). Moreover, sequence and crystal structure analysis of MojV G revealed that, unlike other henipavirus G glycoproteins, MojV G lacks N-linked glycan modifications throughout its β propeller domain, which may be relevant in term of immune evasion or folding and assembly of the protein, as well as triggering of MojV F and activation of the fusion cascade (mechanism further discussed below) (**Figure 1-1a**) (215). Nevertheless, the overall structure of MojV G closely resembles that of other henipavirus Gs (**Figure 1-1**) (215). Although no viral isolate was obtained from the rodent samples tested, the presence of the RNA editing site required for the expression of the V and W proteins precludes the rescue of a recombinant MojV outside of BSL-4 containment, and suggests that MojV may be an emerging pathogenic henipavirus (266). Furthermore, while no direct link can be made between the death of the miners and MojV infection due to the lapse of time between the moment these cases were reported and the sample collection occurred, it is possible that these patients may have been exposed to MojV. Intriguingly, MojV is the first henipavirus detected in a rodent species, raising to the possibility that henipaviruses may also utilize mammals other than bats as animal reservoirs and could naturally infect a wider range of hosts than originally anticipated.

Figure 1-1: Structural analysis of MojV-G.

(a) Domain schematics of MojV-G, NiV-G and KV-G indicate the locations of the transmembrane domain (TM), aspartic acid-rich domain D in MojV-G, b-propeller domain and N-linked glycosylation sequons (black spikes). **(b)** Crystal structure of the MojV-G b-propeller domain (cartoon representation, coloured rainbow with the N terminus in blue and the C terminus in red). **(c)** Structural comparison of MojV-G with other henipavirus-G glycoproteins. Overlays of MojV-G (coloured blue) with the equivalent domains from NiV-G (orange, PDB 2VWD) and KV-G (green, PDB 4UF7), rendered in Ca trace representation. **(d)** Comparison of MojV-G with the crystal structure of the NiV-G b-propeller domain in complex with ephrinB2 (PDB 2VSM), right, reveals that MojV-G lacks the ephrin binding pocket found in classical henipavirus. Indeed, the interaction between MojV-G and ephrinB2/B3 would be obstructed (clashes indicated by black arrows) by the ‘closed’ arrangement of loops in first (blue) and fifth (orange) blades of the MojV-G b-propeller. NiV-G and MojV-G are rendered in a surface representation and coloured rainbow with the N terminus in blue and the C terminus in red. EphrinB2 is shown as a white ribbon.

From: Rissanen, I., Ahmed, A., Azarm, K. et al. Idiosyncratic Mòjiāng virus attachment glycoprotein directs a host-cell entry pathway distinct from genetically related henipaviruses. *Nat Commun* 8, 16060 (2017)
doi:10.1038/ncomms16060 (creative commons license).



THERAPEUTICS

Currently, no licensed NiV and HeV therapeutics approved for human use are available. Consequently, the medical management of human henipaviral disease remains primarily supportive. A number of groups have been working on active immunization strategies, including live recombinant and subunit vaccines, as well as passive immunization strategies as countermeasures to these emerging pathogens. Virtually all virus-neutralizing antibodies against paramyxoviruses such as measles (91), mumps (222) and parainfluenza viruses (119) target the attachment and fusion glycoproteins of these pathogens. Therefore, vaccine and therapeutic research for henipaviruses has also been focusing on the immunogenic properties of the F and G glycoproteins.

Live recombinant vaccines

Various viral vector-based NiV live recombinant vaccines have been evaluated in animal models. Guillaume et al. (93) demonstrated that a recombinant vaccinia virus expressing NiV F or NiV G, or a combination of both, administered to golden hamsters was fully protective upon lethal intraperitoneal challenge. However, the use of a vaccinia strain, although attenuated, for human applications remains unlikely (44). Similarly, a canarypox-based recombinant vaccine (ALVAC) expressing NiV F or NiV G was developed and tested in a pig model, and provided full protection and sterile immunity to the animals whether they were subjected to the F, G or F/G vaccine regimens (255). ALVAC G elicited the strongest response, whereas the F/G vaccine combination afforded minimal improvement compared to the administration of ALVAC NiV G alone (255). Ploquin et al. (208) generated an adeno-associated recombinant vaccine (AAV8-NiV.G) which protected golden hamsters from lethal challenge after a single injection and

allowed for 50% survival in animals challenged with HeV. A measles virus-based recombinant NiV vaccine (rMV-NiV-G) also provided protection to hamsters and African green monkeys challenged with NiV (277). Another vaccine candidate was recently developed utilizing a recombinant rabies virus platform expressing NiV G (NIPARAB) based on a culture-attenuated strain also containing a mutation in RABV G to reduce its neurovirulence (122). However, this vaccine was only assessed in C57BL/6 mice and warrant further testing in established models of henipaviral disease that recapitulate more closely human infection and pathology (122). Likewise, a candidate recombinant vaccine based on the non-virulent lentogenic strain of Newcastle disease virus (NDV) LaSota (126) was shown to induce neutralizing antibodies in mice, pigs and chicken, but was not assessed in term of protection to lethal challenge after immunization. Recent studies comparing infection with the Bangladesh strain (NiV-B) and the Malaysia strain (NiV-M) of NiV in African green monkeys confirmed that NiV-B is more pathogenic (165). Thereupon, Mire et al. (164) explored the use of a recombinant vesicular stomatitis virus (VSV) expressing of F and G glycoproteins from the Bangladesh strain of NiV rather than the Malaysia strain, upon which vaccine development had been focused until then. Single dose vaccination with rVSV NiV-B vaccines allowed all animals challenged to survive (164).

Subunit vaccines

Soluble ectodomain subunit henipavirus G glycoproteins have been generated in the context of studies on cell entry and fusion mechanism and present an alternative to live and replication competent viral vaccines (30). An engineered soluble HeV G (sG) was shown to oligomerize similarly to the native conformation of full length HeV G, bear

proper N-glycosylation, bind to its cognate receptors and elicit potent cross-reactive neutralizing antibodies against HeV G and NiV G (30). NiV sG and HeV sG were tested in various animal models and proved to be protective against disease in cats (153; 172), hamsters (94), ferrets (190), African green monkeys (34) and non-human primates (163), paving the way for the development of the Equivac®HeV (Zoetis Inc.) vaccine currently in use for horses in Australia. The deployment of Equivac®HeV illustrates how a “One Health” approach can support protection of livestock and prevent exposure and transmission to human (159).

Passive immunotherapy

Passive immunotherapy has proven protective against henipaviral infection and disease in various studies. Prophylactic administration of polyclonal anti-NiV F and G antibodies protected golden hamsters challenged with NiV (93), while cross-reactive monoclonal anti-NiV F antibodies prevented death in hamsters infected with HeV (95). In the same animal model, monoclonal anti-NiV F and G antibodies administered 24h post-NiV infection resulted in 100% and 50% survival, respectively (94). A humanized monoclonal anti-NiV F antibody, h5B3.1, was shown to prevent lethal disease in ferrets when administered post- HeV and NiV infection even if provided several days after the animals were challenged (162). Studies in ferrets and African green monkeys have also demonstrated that postexposure treatment of NiV infection by administration of the human monoclonal IgG antibody m102.4 offers full protection from disease in these animal models (31; 37). Although large scale production of such therapeutic agent may not be economically viable, passive immunotherapy presents an interesting option as a stockpiled resource for limited emergency use and, to date, has been offered on a

compassionate use basis to 13 individuals exposed to HeV in Australia and one person exposed to NiV in the United States (207). Recently, a phase 1 clinical trial involving 40 participants was conducted in Australia to assess the safety of m102.4 and reported that single and multiple intravenous administration of this antibody was well tolerated in healthy human adults (207). Such studies can educate future dosing regimen for this postexposure therapeutic, which shows great potential both as a prophylactic agent and to treat HeV or NiV infections (207).

HENIPAVIRUS RESERVOIR AND TRANSMISSION

Reservoir

Bats of the *Pteropodidae* family, also known as flying foxes or old world fruit bats, have been identified as the natural reservoir of HeV and NiV (100; 276; 279). These bats are distributed throughout a wide geographic range following tropical and subtropical environments that include the northern and western coasts of Australia and South Asia, but also South Africa, Madagascar, the southern coast of the Arabian peninsula and eastern Mediterranean, which explains some of the serologic evidence and viral nucleic acid detection of henipavirus or henipa-like viruses in bats sampled not only in South East Asia, where HeV and NiV outbreaks have been taking place, but also in Africa (Ghana, Zambia Cameroon) (75; 76; 171; 201; 224). Interestingly, Brazilian *Phyllostomidae* frugivorous and/or insectivorous bats sampled in the tropical savanna Cerrado tested positive for exposure to henipa-like viruse(s) in enzyme-linked immunosorbent assay (ELISA) confirmed by indirect immunofluorescence assay (IFA) (70). Furthermore, serologic evidence of henipa-like virus exposure in insectivorous *Myotis* vesper bats collected in the Southern Chinese provinces of Yunnan and Hainan

suggests that additional bat families may serve as natural reservoirs of henipaviruses (140). The extended geographic range of possible reservoir hosts and their overlapping habitats may provide increased risk of zoonotic spillover beyond the known at-risk regions of South East Asia.

Of note, MojV, the first henipavirus detected in rodent field samples, was also identified by nucleic acid genome assembly in the Yunnan Province of China (266). This discovery raises further questions about the range of possible mammalian reservoir species for henipaviruses. Yet, it is not known if *Rattus flavipectus*, which has a wide geographic distribution in South East Asia (**Figure 1-2**), acted as a natural reservoir host, or simply an accidental spillover host. Lastly, in 2014 henipa-like viral nucleic acid was detected in rodent and shrew samples from Zambia (*Rattus rattus* and *Crocidura hirta*, respectively) suggesting novel henipaviruses may indeed be circulating in rodents and shrews (224).

Figure 1-2: Geographic distribution of *Rattus flavipectus (tanezumi)*.

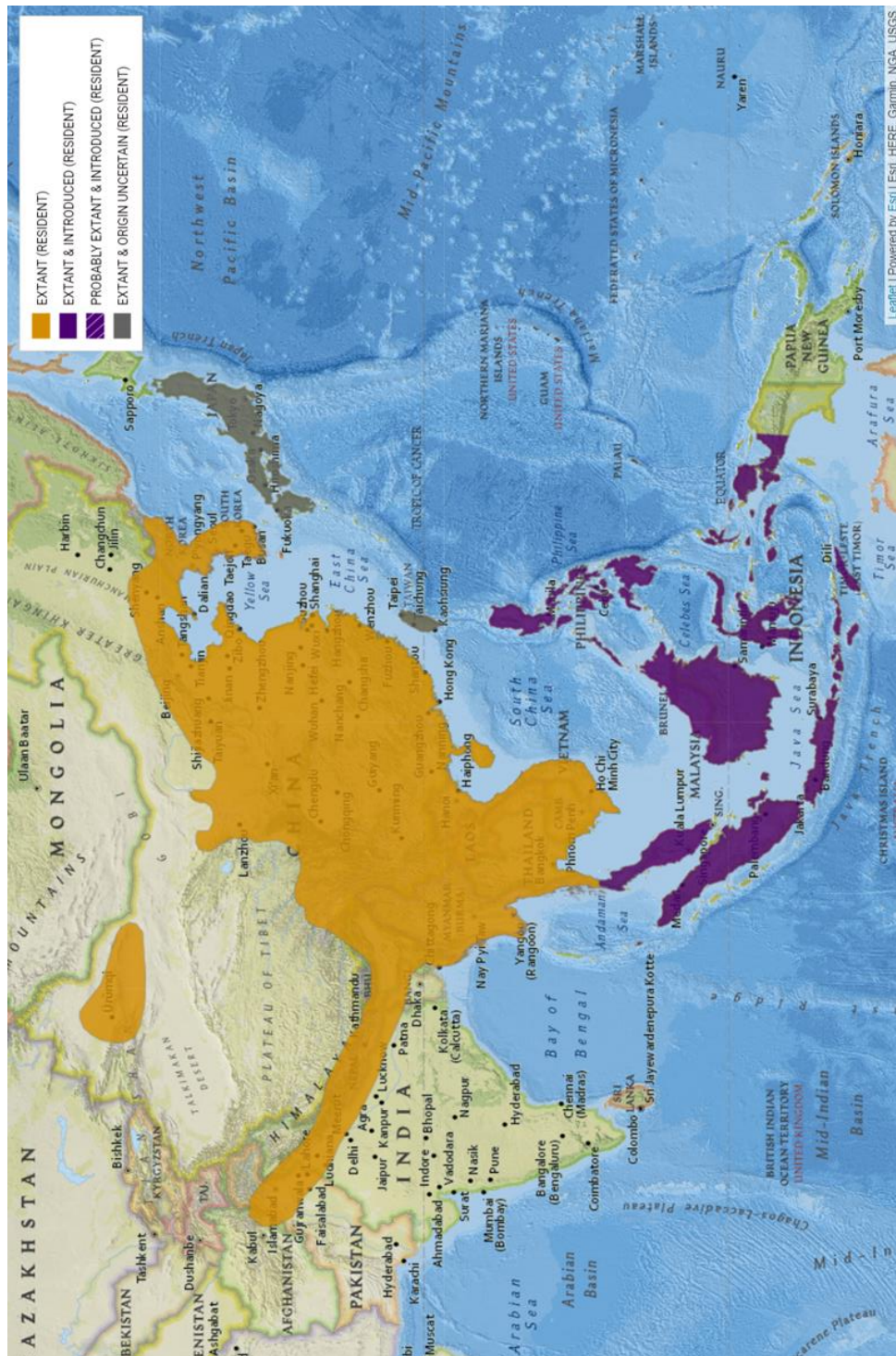
Range description: Both the native and introduced geographic distribution of this very widespread species remains somewhat unclear. Musser and Carleton (177) consider the species native distribution to range from eastern Afghanistan (183) through central and southern Nepal (below about 2,000 m above sea level), Bhutan, northern India, northern Bangladesh and northeastern India (distributional relationships between *R. rattus* and *R. tanezumi* along the northern portion of the Indian subcontinent still requires resolution) into southern and central China (including Hainan Island), the Korean Peninsula, and mainland Southeast Asia (including offshore islands) south to the Isthmus of Kra, being also probably native to Mergui Archipelago. Musser and Carleton (177) report that it is presently unclear whether the species is native or introduced to Taiwan and Japan. But is most likely introduced to Peninsular Malaysia and islands on the Sunda Shelf and nearby archipelagos, including the Mentawai Islands (176; 178). It has been introduced to Little Nicobar and Great Nicobar, in the Andaman and Nicobar Islands of India. It has been widely introduced throughout the Philippines (105; 177) (Larry Heaney pers. comm.). It appears to have been widely introduced throughout insular Southeast Asia, including the Greater and Lesser Sunda Islands and the Molucca Islands, to western New Guinea (range map very provisional for this island) (85; 86), and from here into Micronesia to the islands of Eniwetok and Fiji (177). Native extant to: Afghanistan; Bangladesh; Bhutan; Cambodia; China; India; Japan; Korea, Democratic People's Republic of; Korea, Republic of; Lao People's

Democratic Republic; Malaysia; Nepal; Taiwan, Province of China; Thailand;
Viet Nam. Extant and introduced to: Fiji; Indonesia; Papua New Guinea;
Philippines.

From Heaney, L. & Molur, S. 2016. *Rattus tanezumi* (errata version published
in 2017). The IUCN Red List of Threatened Species 2016:

e.T19366A115149780. <https://dx.doi.org/10.2305/IUCN.UK.2016->

3.RLTS.T19366A22445589.en. Downloaded on 18 January 2020.



IUCN (International Union for Conservation of Nature) 2014. *Rattus lanezumi*. The IUCN Red List of Threatened Species. Version 2020-1

Transmission

As natural hosts of henipaviruses, bats do not seem to develop clinical signs from henipaviral infection (100). HeV and NiV are shed in bodily fluids and can be detected mostly in infected bats urine, but also in throat and rectal swabs (100). Bat-to-bat transmission is believed to occur via direct contact between bats, and to be facilitated by high density of individuals roosting, which could support transmission not only between bats of the same species but also across species when multiple species share a same roost (80). Horses and pigs can act as amplifying hosts to henipaviruses. HeV is thought to be transmitted from infected bats to horses via urine-contaminated pasture, water or feed (80). Horse-to-horse transmission through close contact can occur through an extended period of time as animals begin shedding HeV as early as 2 days post-inoculation before the onset of clinical signs (150). Additionally, it is possible that human handling of sick horses without adequate protection may have caused cross-contamination of horses as well. Unprotected direct contact with bodily fluids, particularly with copious frothy infectious nasal discharge, of sick horses seems to be a main route of horse-to-human HeV transmission (146). However, horse-to-human transmission of HeV remains rare and human-to-human HeV transmission has not been reported to date (146).

Horses are the principal spillover hosts for HeV, yet they also have been shown to be susceptible to NiV infection. For instance, two polo ponies tested seropositive for NiV during the Malaysian outbreak (59). Horses have also been implicated in NiV transmission as evidenced by the 2014 NiV outbreak that took place in the Philippines (53). During this outbreak, Ching et al. (53) suggest, fruit bats reported near the outbreak locality might have infected horses. Horse-to-human transmission was likely due to the fact that patients had been involved with horse slaughtering and/or meat preparation or

consumption, whereas human-to-human transmission involved health care workers that wore limited personal protective equipment (PPE) (53). Domestic animals that had been fed horse meat, including four cats and a dog, also succumbed to the infection (53). This was the first outbreak caused by the Malaysia strain of NiV (NiV-M) during which confirmed cases of human-to-human transmission were clearly documented; although the strain identification was only based on a short genomic segment of the P gene (53). During the 1998/1999 NiV-M outbreak, bats infected with NiV-M were responsible for the contamination of pigs (146). Pig-to-pig transmission was observed and facilitated by direct contact with infectious nasal secretions with an infection rate of 100% in farms concerned by the outbreak, but a mortality rate of no more than 5% and a majority of swine remaining asymptomatic (160). Similar to what is observed in HeV horse-to-horse transmission, NiV-M-infected asymptomatic pigs can spread the infection to both naïve pigs and human handlers via nasal secretions (160). In contrast, the Bangladesh strain of NiV (NiV-B), which has been circulating in Bangladesh and India since the 2001 outbreaks, was shown to be transmitted from infected bats directly to humans via consumption of fresh or fermented contaminated date palm sap (113; 211). Bats often feed at the site of sap collection and can contaminate the sap with infectious saliva, feces or urine; this can be remedied by protecting the tree taps and collection pots with a bamboo skirt (123). Though this preventive measure seems inexpensive and simple to deploy, a pilot study conducted by Nahar et al.(179) showed that it was not readily implemented; sustained use of the skirts remained low despite some willingness of the participants to engage in the practice. Human-to-human transmission of NiV-B is

believed to occur through close exposure to infectious bodily fluids and particularly respiratory secretions (147; 184).

Knowledge about possible bat-to-bat, bat-to-amplifying host(s), non-bat reservoir-to-amplifying host(s) or direct zoonotic transmission of CedV, GhV and MojV is lacking. To date, most of the work exploring these novel and emerging henipaviruses has been focused on establishing foundational knowledge of their molecular virology which can in turn support viral exposure and circulation studies, as well as the development of animal models, to assess modes of transmission.

MOLECULAR VIROLOGY OF HENIPAVIRUSES

Taxonomy and genome organization

As non-segmented negative-sense single-strand enveloped RNA viruses, henipaviruses belong to the order *Mononegavirales* and are further classified in the *Paramyxoviridae* family because they express a fusion glycoprotein that allows for viral entry via pH-independent membrane fusion (132; 253). According to the current International Committee on Taxonomy of Viruses (ICTV) classification, paramyxoviruses are divided into four subfamilies that include *Avulavirinae*, *Metaparamyxovirinae*, *Rubulavirinae* and *Orthoparamyxovirinae* (214). The *Orthoparamyxovirinae* subfamily is composed of eight genera: *Aquaparamyxovirus*, *Ferlavirus*, *Henipavirus*, *Jeilongvirus*, *Morbillivirus*, *Narmovirus*, *Respirovirus*, and *Salemvirus* (214). Five species of henipaviruses have been discovered to date: *Hendra*, *Nipah*, *Cedar*, *Ghanaian bat* and *Mojiang henipaviruses* (59; 76; 104; 128; 149; 214; 253; 266). GhV and MojV were identified based on genomic sequence data alone; no

viral isolates of these two species have been recovered from their respective hosts, *Eidolon helvum* bats for GhV and *Rattus flavipectus* for MojV (75; 76; 128; 266).

Henipaviruses have some of the largest genomes amongst paramyxoviruses, ranging from 18,162 nucleotides in the case of CedV to 18,530 nucleotides for GhV (76; 149), and follow the rule of six which requires that the number of nucleotides of the genome be divisible by six in order to accommodate binding of each nucleoprotein monomer (N) to six nucleotides (99; 132; 253). The genome of henipaviruses is organized to encode six structural proteins, nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), attachment (G) and large polymerase (L), in the following order: 3'-leader-N-P-M-F-G-L-trailer-5' (214; 217) (**Figure 1-3**). The promoter for the transcription of positive sense mRNA to support viral protein production is located in the 3' leader sequence; the 5' trailer sequence contains the promoter that initiates the synthesis of negative sense RNA during replication (252). Additionally, the open reading frame (ORF) for P can encode three accessory viral proteins: V, W and C (101). RNA editing of the ORF for P generates the V and W proteins by insertion of one or two G nucleotides, respectively (101; 217), while the C protein is the product of leaky ribosomal scanning and translational initiation at a weak downstream initiation codon resulting in the synthesis of a smaller protein product (132). Wang et al. (252) have also reported the existence of a smaller ORF flanked by the coding sequences of the V and C proteins in the genome of HeV, but not NiV. The gene product of this ORF was termed small basic protein (SB) due to its pI of 11.9 (151; 252). To date, the function of this putative protein has not been established, and expression has not been reported in infected cells (252).

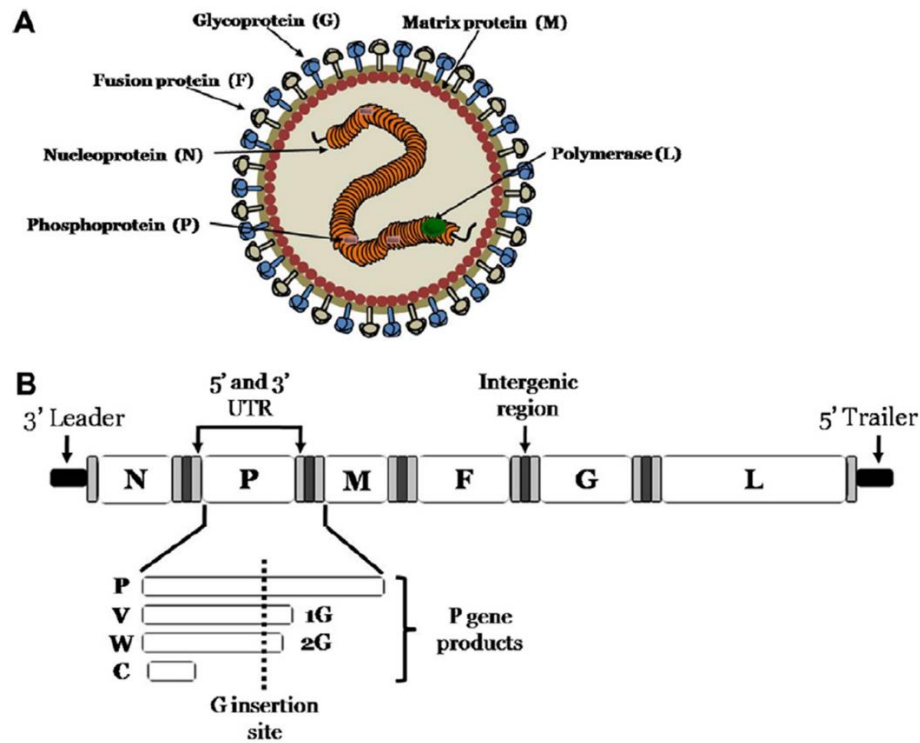


Figure 1-3: Henipavirus virion structure and genome organization.

(A) Schematic representation of a henipavirus virion, illustrating the M protein lining the inner surface of the lipid envelope, the viral glycoproteins decorating the virion surface, and the helical ribonucleoprotein (RNP) complex in association with the polymerase and phosphoprotein enclosed within. (B) Schematic representation of henipavirus genome organization. The polycistronic nature of the P gene is indicated. From Rockx, B., Winegar, R., & Freiberg, A. (2012). Recent progress in henipavirus research: Molecular biology, genetic diversity, animal models. *Antiviral Research*, 95(2), 135-149. <https://doi.org/10.1016/j.antiviral.2012.05.008>. License number: 4744861480100.

Viral proteins and replication cycle

Henipaviruses, like other members of the *Paramyxoviridae* family, begin their replication cycle after gaining cell entry by releasing their genomic material into the host cell cytoplasm following fusion between the viral and cellular plasma membranes (discussed in more details below) (132). Disruption of the contact between the M protein shell, which lines the inner side of the viral membrane, and N proteins, which are associated with the L and P proteins in ribonucleoprotein (RNP) complexes that coat the viral genome, is required for the helical nucleocapsid to be released into the cytoplasm (132). During the first part of the viral replication cycle, termed primary transcription, the pre-packaged viral RNA-dependent RNA polymerase, which functions as one L protein associated with a homotetramer of P proteins, transcribes the negative sense viral genomic RNA into mRNAs encoding each viral proteins (132; 217). Gene transcription starts at the 3' end of the genome and takes place sequentially, terminating and re-starting at each gene junction in a “stop-start” manner (**Figure 1-3**) (132; 217). Due to the viral RNA polymerase occasional failure to reinitiate transcription at gene junctions further away from the 3' end, the viral genes are transcribed following a gradient with more abundant transcripts being generated from ORFs located closer to the 3' end (132). When the amount of N proteins reaches a certain concentration, genomic replication begins as the viral polymerase now ignores gene junction signals and editing sites to produce positive sense antigenomes used as intermediates in genome replication to prepare for virion assembly (132). During virion assembly, N proteins associate with the negative sense genomic RNA and recruit P and L proteins into RNP complexes coating the viral genome (132). M proteins lining the inner side of the cellular plasma membrane facilitate the budding of infectious virions by supporting the interaction of the N proteins with F

and G proteins, which roles are further discussed below (132). Exogenous expression of viral proteins can lead to spontaneous budding of virus-like-particles (VLPs) which are replication incompetent and behave as viral empty shells. VLPs can be produced by independent expression of henipaviral M, F and G proteins, or in co-expression systems to investigate numerous viral processes, including for example the role of the M protein in budding, interactions between F and M, G receptor tropism, or applied to vaccine development research (61; 62; 195; 196; 241; 250; 251).

Henipavirus P gene products and innate immune response inhibition

Viral RNA synthesis in the cytoplasm during the replication cycle of henipaviruses triggers innate immune responses in infected cells (20). The type I interferon (IFN) response is an important antiviral pathway to control infection. RIG-I-like receptors (RLRs), such as RIG-I and MDA-5, can sense cytoplasmic viral nucleic acids and signal through the mitochondria-associated adaptor protein MAVS to activate IKK ϵ and TBK-1 kinases, which in turn phosphorylate the transcription factors IRF3 and 7 (20; 174). The activated transcription factors can then translocalize to the nucleus to initiate the expression of the IFN α/β genes (20; 174). IFN α/β are released by the infected cell and bind to IFN α/β receptor (IFNAR) which signals through the JAK/STAT pathway to activate tyrosine kinases Jak1 and Tyk2, which allows for the phosphorylation STAT1 and STAT2 (20; 174). Phosphorylated STAT1 and STAT2 can then form heterodimers that translocate to the nucleus where they associate with IRF9 to bind to interferon-stimulated response element (ISRE) and initiate the transcription of antiviral interferon-stimulated genes (ISGs) encoding antiviral effectors such as protein kinase RNA-

activated (PKR), virus inhibitory protein endoplasmic reticulum-associated interferon-inducible (viperin), tetherin, tripartite motif protein (TRIM) or Mx, for example (228).

The P protein and the alternative accessory proteins V, W and C generated by transcriptional RNA editing (V and W) and ribosomal leaky scanning (C) of the P ORF are known to support immune evasion mechanisms involved in the pathogenicity of henipaviral disease (20; 199). The V protein has been shown to inhibit type I IFN production by interacting with MDA-5 and RIG-I (9; 20; 52; 223), and prevent JAK/STAT signaling by sequestering STAT1 and 2 in the cytoplasm (199; 219; 220). Similarly, Shaw et al. (236) demonstrated that NiV P protein shares a STAT1-binding domain with the V and W proteins in their common N-terminal domain, suggesting that P can also inhibit IFN α/β signaling by interacting with STAT1 in the cytoplasm. Interestingly the W protein was found to localize to the nucleus rather than the cytoplasm, and inhibits type I IFN signaling by interacting with STAT1, but also IFN α/β gene expression by preventing the accumulation of phosphorylated IRF3 (20; 235; 236). In addition, the infection of golden hamsters with a recombinant NiV (rNiV) unable to express the W protein is lethal and the outcome of disease is reached within the same time frame observed with wild type rNiV; however, golden hamsters inoculated with a rNiV lacking the V protein do not display clinical signs and survive the infection (278). Studies in the ferret model of NiV infection further investigated the contributions of NiV V and W proteins to disease course and lethality. Satterfield et al. (227) confirmed that a rNiV lacking the V protein was replication competent yet non-lethal in infected ferrets. While these studies highlighted the role of NiV V as a determinant of pathogenesis and lethality, they also established that NiV W can modulate the course of disease (227).

Indeed, infection of ferrets with a rNiV lacking the W protein, although still fatal, resulted in a delay of the onset of clinical signs and altered course of disease with decreased respiratory signs and increased neurological manifestations (227). Recent findings also suggest that STAT1 inhibition by NiV P, V and W proteins contributes to pathogenesis and course of disease, but is not necessary for the infection to be lethal in the ferret model (226). The role of the C protein as a virulence factor is less well-defined than that of the V and W proteins. Yoneda et al. (278) have shown that the C protein plays a role in pathogenicity in the Syrian hamster model of NiV infection. Though the exact mechanism involved is yet to be identified, the C protein has been reported to regulate viral replication and seem to limit the expression of IFN β and antiviral chemokines such as CXCL10 and CCL5 (145; 152).

FUSION (F) AND ATTACHMENT (G) GLYCOPROTEINS

Attachment (G) glycoprotein

The F and G glycoproteins of henipaviruses are structural proteins expressed on the cell surface of infected cells or on the surface of the virion. The function of the G glycoprotein is to interact with its cognate receptor(s) to initiate the membrane fusion mechanism that permits viral entry into host cells or viral spread between neighbor cells. Although the G glycoprotein of henipaviruses lacks the hemagglutinin and neuraminidase activities observed in paramyxoviruses bearing a H or HN attachment glycoprotein, it is, like all paramyxovirus attachment proteins, a type II transmembrane protein. The G glycoprotein is composed of a N-terminus cytoplasmic tail preceding its single transmembrane domain and an ectodomain presenting a stalk region topped with a globular head (**Figure 1-4**) (38; 132; 238; 270). Modeling of the stalk region of HeV G

based on alignment with the stalk of the Newcastle disease virus (NDV) attachment glycoprotein (HN) revealed a discontinuous helix structure with a helix break separating two helical regions spanning T77 to K95 and T98 to S135 (238). The globular head of henipavirus G glycoproteins is folded as six-blade (β 1- β 6) β -propeller structure with a central cleft that accommodates receptor interactions (further discussed below) (39; 131; 136; 209; 215; 238; 270). Each blade is made of four anti-parallel β -strands and the overall structure of the head of the G glycoprotein is stabilized by disulfide bonds linking the blades (39; 131; 136; 209; 238). The surface-expressed native conformation of G oligomerizes as tetramers formed by dimers of dimers resulting from disulfide bonds between stalk regions of G monomers (**Figure 1-4**) (30; 38; 215; 238). In addition, all henipavirus G glycoproteins bear multiple N-linked glycosylation modifications in their stalk and β propeller domains (63; 131; 136; 270), except for MojV G which lacks such modifications in its β propeller domain (**Figure 1-1**) (215). N-linked glycosylation modifications of the G glycoproteins have been shown to impact fusion activation and shielding from neutralizing antibody (23; 40; 284). Moreover, reports of O-glycosylation of HeV G and NiV G suggest that O-glycans located on stalk residues are involved in modulating fusogenicity and may affect intracellular interactions between F and G and the endocytosis and processing of F (63; 239).

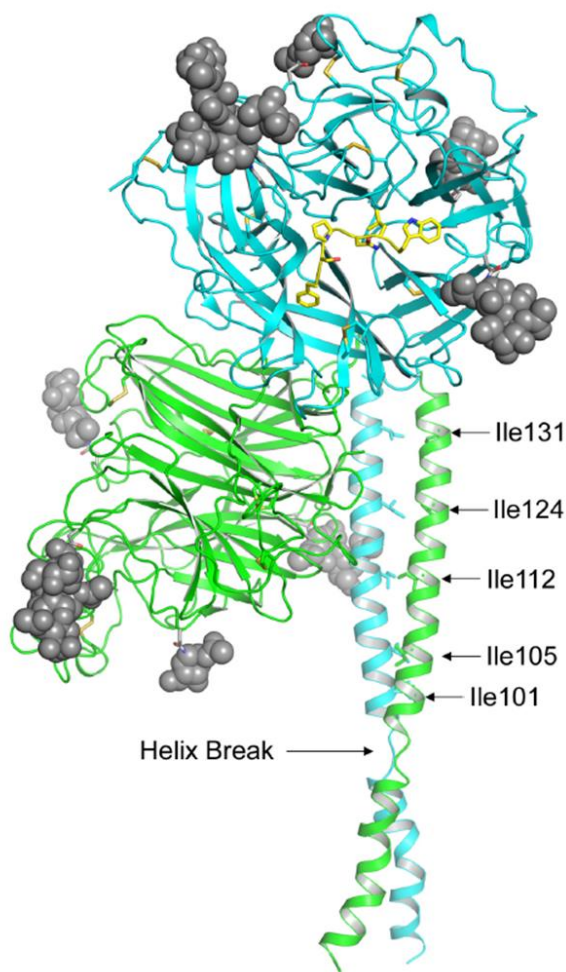


Figure 1-4: Model of the Hendra virus attachment G glycoprotein.

The HeV-G ectodomain is shown in its dimer conformation. The secondary structure elements of the two globular head domains, colored in green and blue, are derived from the crystal structure, which also revealed the five predicted N-linked glycosylation sites (N306, N378, N417, N481 and N529) occupied by carbohydrate moieties (gray spheres). However, N378 was not modeled in the figure due to weak electron density. The G glycoprotein head domain folds as a six-blade β -propeller with disulfide bonds illustrated as yellow sticks. The residues of the ephrin-B2 G-H loop are shown in yellow. While the entire structure of the HeV-G stalk domain (residues 71–173) has not been determined, residues 77–136 are modeled for each monomer suggesting this region forms a discontinuous helix (Helix Break). The position of the HeV-G head dimer and stalks are oriented based on the alignment with the NDV structure and the receptor binding face of the blue monomer is facing out and the green monomer is facing left. The Ile residues in the HeV-G stalk domain that can modulate conformational changes associated with receptor binding are indicated. From Steffen, D.L.; Xu, K.; Nikolov, D.B.; Broder, C.C. *Henipavirus Mediated Membrane Fusion, Virus Entry and Targeted Therapeutics*. *Viruses* 2012, 4, 280-308. (creative commons license).

Fusion (F) glycoprotein

The F glycoprotein of henipaviruses is a type I transmembrane protein with a C-terminus cytoplasmic tail. It is initially expressed by the cellular machinery as an inactive precursor, F₀, which is trafficked to the cell surface (72). Of note, henipavirus F glycoproteins do not have the cleavage recognition site R-X-K/R-R, and are not activated by the Golgi protease furin during this trafficking step (167; 169). However, the F₀ glycoprotein still requires proteolytic processing to become biologically active. After it reaches the cell surface, F₀ is endocytosed via clatherin-coated pits and cleaved by pH-dependent endosomal enzymes cathepsin L or B to generate a mature functional form of the protein now composed of two disulfide linked subunits F₁/F₂. The F₁/F₂ form of henipavirus F glycoproteins is then recycled to the cell surface where it is integrated in the virion envelop during assembly (73; 74; 158; 188; 189; 247; 257). Typical domains found in paramyxovirus fusion proteins are present in the henipavirus F glycoprotein. The F₁ subunit includes a C-terminus cytoplasmic tail followed by a single helical transmembrane domain, two heptad repeat domains, HR1 and HR2, and a N-terminus hydrophobic fusion peptide. Henipavirus F glycoproteins have been shown to oligomerize as homotrimers (**Figure 1-5**) (260; 269). Xu et al. (269) have also demonstrated that NiV F assembles as a hexamer of trimers, and that destabilization of this oligomeric structure reduces fusion efficiency when tested in cell-cell fusion and pseudotyped virus entry assays (**Figure 1-5**) (269). N-linked glycosylation of the henipavirus F glycoprotein is implicated in modulating the kinetics of fusion (7; 46; 168). Interestingly, studies by Aguilar et al. (7) established that the removal of N-glycans from NiV F results in hyperfusogenicity of the NiV F mutants but increases their vulnerability to neutralizing antibodies, suggesting N-glycans on NiV F can act to shield the protein

from antibodies. Moreover, avidity of the F protein for the G protein decreases as fusogenicity increases in NiV F mutants lacking N-glycans (7). Additional mechanisms at play in regulating fusion seem to involve the F glycoprotein. A polybasic KKR motif located in the membrane-proximal region of the cytoplasmic tail of NiV F has been implicated in modulating fusion (6). Mutations of these residues revealed changes in the conformation of the ectodomain of NiV F, which could impact F/G avidity and the ability of F to transition from a pre- to post-fusion conformation to enable the formation of fusion pores (6).

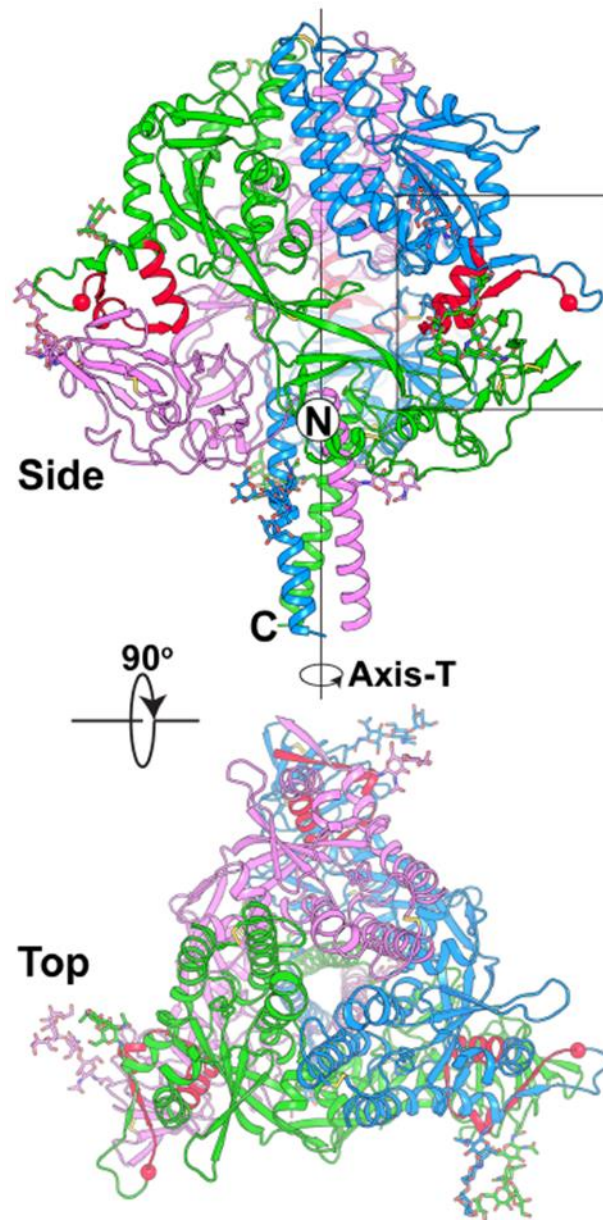


Figure 1-5: Structure the NiV-F trimer.

The NiV-F trimer has a tree-like overall shape with the three copies of the F glycoprotein (colored in blue, green and violet) twined around a central axis (Axis-T). The fusion Peptide (FP) is colored in red. The NiV-F trimer is shown viewed from side, and from the top. Glycosylation moieties and disulfide bonds are shown as sticks. Adapted from Xu K, Chan Y-P, Bradel-Tretheway B, Akyol-Ataman Z, Zhu Y, Dutta S, et al. (2015) Crystal Structure of the Pre-fusion Nipah Virus Fusion Glycoprotein Reveals a Novel Hexamer-of-Trimers Assembly. PLoS Pathog 11(12): e1005322. <https://doi.org/10.1371/journal.ppat.1005322> (creative commons license).

Interactions between the F and G glycoproteins

Interactions between henipavirus F and G glycoproteins that are required for successful fusion events to occur are not fully understood. However, a number of studies have started to shed some light on the subject and provided knowledge suggesting that one of the two models of paramyxovirus fusion debated in the field, the “clamp” model, is more likely adopted by henipaviruses rather than the other, the “provocateur” model (further discussed below). Bishop et al. (25) demonstrated that 9 isoleucine residues between positions 83 and 174 in the stalk domain of HeV G were important in preserving the structure of HeV G in a conformation receptive to receptor-binding. Premature change in HeV G conformation, before G associates with F, could prevent conformation changes in G subsequent to receptor-binding and ultimately result in inability of G to trigger F, through dissociation from F (according to the “clamp” model), precluding fusion (25). Aguilar et al. (4) have presented congruent data and identified a hyperflexible region, $\beta 6S4/\beta 1H1$, at the base of the globular head of NiV G that, upon host receptor binding, interacts with the stalk domain of G. This receptor-induced allosteric signal triggers the required conformational change in F that exposes the fusion peptide of F and allows for fusion to take place (4). The critical role of the stalk domain of NiV G in promoting fusion was further illustrated in studies indicating that a soluble version of the ectodomain region of NiV G alone could not induce cell-cell fusion in the presence of a full length cell-surface-expressed NiV F (240). Of note, HeV G trafficking through the Golgi and to the cell surface is much slower than that of HeV F; the differing rates of oligomerization and transport through the secretory pathway for F and G indicate that the F/G complex likely does not form in the ER, but rather that G might associate with the mature F₁/F₂ glycoprotein at the cell surface (135; 258).

HENIPAVIRUS CELL ENTRY

Fusion mechanism

Henipaviruses, like other paramyxoviruses, gain host cell entry via a fusion mechanism mediated by the coordinated actions of the attachment and fusion glycoproteins. Upon engagement of the G glycoprotein with its cognate receptor(s) (further discussed below), the F glycoprotein is triggered into undergoing an irreversible conformational change from metastable pre-fusion to a post-fusion structural organization requiring a transient conformation termed pre-hairpin intermediate (PHI) (5; 48). Triggering of F allows for the exposure of the highly hydrophobic fusion peptide at the N-terminus of the glycoprotein and its insertion into the host cell plasma membrane as F adopts the PHI conformation (5; 28; 135). In this conformation, the HR1 and HR2 are available to interact with each other and fold into a 6-helix bundle formation which brings the viral and host cell membranes in close proximity to facilitate lipid mixing and the subsequent formation of a fusion pore, resulting in the delivery of the viral genome into the host cell (5; 28). This pH-independent mechanism involves crucial interactions between the F and G glycoproteins.

Two prevailing models of paramyxovirus fusion have been debated in the field and referred to as “dissociation” versus “association” models or using more vivid and descriptive imagery, “clamp” versus “provocateur” models (3; 135). The “clamp” model proposes that G interacts with F to stabilize it in its pre-fusion conformation until G binds its cognate receptor, at which point G releases F, allowing it to undergo the required pre- to post-fusion conformational changes that cause membrane fusion. Conversely, the “provocateur” model postulates that G undergoes conformational changes after engaging with its receptor, which then support association with F and transduction of an allosteric

signal that triggers F conformational changes, resulting in fusion (3; 135). Paramyxovirus HN glycoproteins that bind sialic acid to gain cell entry, such as Newcastle NDV, human parainfluenza virus 3 (hPIV3) and parainfluenza virus 5 (PIV5), seem to use a “provocateur” mechanism (65; 137; 197), while protein-binding paramyxoviruses such as measles (MeV) or henipaviruses are thought to follow the “clamp” model of fusion (4; 6; 7; 26; 67).

HENIPAVIRUS RECEPTORS

Ephrin ligands and receptors

Ephrin ligands (ephrin) and ephrin receptors (Eph receptors) constitute one of the largest receptor tyrosine kinases families (245). Eph receptors are classified into two groups, nine EphA receptors (EphA1-8, Eph10) and five EphB (EphB1-4, EphB6) receptors, based on sequence homologies and binding characteristics (54). Ephrin ligands have been categorized into two subclasses and include five A-class (ephrin-A1-5) and three B-class (ephrin-B1-3) ephrin ligands according to their structural attachment at the cell surface (193). A-class ephrin ligands are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, but can be released as soluble ectodomains to interact in a paracrine manner with Eph receptors as was demonstrated to be the case for ephrin-A1 and EphA2 (19; 267). B-class ephrin ligands, however, are expressed as type I single transmembrane domain proteins with a short cytoplasmic tail (193). Menzel et al. (157) have reported that an additional ephrin ligand expressed in chicken embryonic retina, ephrin-A6, can activate the EphA4 receptor in nasal retinal cells. Nevertheless, only eight ephrin ligands have been identified in mammals.

Eph receptors tend to interact with ligands of their corresponding subclass; however, promiscuous interactions between Eph receptors of one subclass and ephrin ligands of the other has been reported (192). For instance, EphB2 can bind all five ephrin-A ligands (69; 144; 193; 194). The ectodomain of ephrin ligands is composed of an eight-stranded β -barrel structure and includes a solvent exposed 16 residue flexible region responsible for receptor binding termed G-H loop (106-108; 210; 244). Distinct regions of the G-H loop domain of ephrin ligands are involved in interactions with Eph receptors and ligand-ligand interactions that promote the oligomerization of ligands and receptors, which is required for activation of the signaling pathway (244).

Roles of ephrin signaling

Ephrin ligands and their receptors have been shown to engage in bidirectional signaling that directs a number of processes involved in cytoskeletal dynamics and adhesion/repulsion mechanisms (109; 173). During development, ephrin ligand/Eph receptor communication plays a critical role in cell segregation, positioning and migration, tissue segmentation, angiogenesis and axon guidance (125; 144; 170; 193). Ephrin signaling remains necessary to tissue homeostasis in adults, and regulates bone and synapse remodeling, as well as intestinal cells migration during the renewal of the epithelial monolayer (144; 194). In addition, Eph receptor activation has been implicated in several immune cell functions (69). Specifically, ephrin signaling is believed to impact adhesion and extravasation of monocytes and macrophages (204) and trafficking of dendritic cells (71), but also B cell activation and antibody production (281) and T cell proliferation and activation (69; 121; 259; 280).

Ephrins are highly conserved in mammals

Ephrin ligands and their receptors are expressed in a wide range of tissues (<https://www.proteinatlas.org>) (96) and are highly conserved across vertebrate and mammalian species (**Table 1-1**) (13; 92; 156; 244).

Role of Eph receptors and ephrin ligands in infectious diseases

Eph receptors

Various studies have demonstrated that infectious pathogens can utilize ephrin ligands and their receptors to gain cell entry and establish infections. EphA2 receptor was identified as a viral entry receptor for Kaposi's sarcoma-associated herpesvirus (KSHV) (97) and Epstein-Barr virus (EBV) (50; 283), but is also utilized by *Cryptococcus neoformans* to cross the blood-brain barrier (2) and *Plasmodium yoelii* sporozoites to invade hepatocytes (120).

Ephrin ligands: entry receptors to henipaviruses

Ephrin ligands act as viral receptors to allow henipaviruses entry into host cells. A six-residue subdomain of the G-H loop of ephrin ligands has been shown to interact with henipavirus G glycoproteins by insertion into the hydrophobic central cleft of the globular head of the ectodomain of G glycoproteins (38; 131; 238; 268). Within this core subdomain, four residue positions have been identified as critical to establish successful interactions with four preformed hydrophobic pockets of the central cavity of henipavirus G glycoproteins (38; 268): F/Y, P/A, I/T/S/Y/L, L/M/W (**Figure 1-6**). Outside of hydrophobic interface created by the four G-H loop residues and the four G binding pockets, interactions taking place between the ephrin G-H loop and the G glycoprotein that are based on hydrogen and ionic bonds involving charged and polar residues further strengthen the binding of the G-H loop to the henipavirus G glycoprotein (268).

Table 1-1: Shared percent identity of nucleotide and amino acid sequences of bat, rat and mouse compared to human.

Sequence alignments were obtained and analyzed with Clone Manager Suite9 software (Scientific & Educational Software, Cary, NC).

Ephrin ligand	Sequence	<i>Homo sapiens</i>	<i>Pteropus Alecto</i>	<i>Rattus norvegicus</i>	<i>Mus musculus</i>
EFNA1	DNA	100	88	86	86
	Amino acid	100	88	86	85
EFNA2	DNA	100	66	83	84
	Amino acid	100	62	89	89
EFNA3	DNA	100	92	90	91
	Amino acid	100	93	93	93
EFNA4	DNA	100	81	83	72
	Amino acid	100	79	83	63
EFNA5	DNA	100	97	96	96
	Amino acid	100	99	98	99
EFNB1	DNA	100	91	90	90
	Amino acid	100	95	95	95
EFNB2	DNA	100	94	92	92
	Amino acid	100	95	96	95
EFNB3	DNA	100	94	93	91
	Amino acid	100	97	96	95

	★ ★ ★★		
106	EKFQRFTPFI LGKEFK	121	mouse ephrin-A1
106	EKFQRFTPFT LGKEFK	121	human ephrin-A1
128	EKFQLFTPFS LGFEFR	143	human ephrin-A2
139	EKFQRYSAFSLG YEFH	154	human ephrin-A3
125	EKIQRFTPFS LGFEFL	140	human ephrin-A4
116	EKFQLFTPFS LGFEFR	131	human ephrin-A5
112	IKFQEFSPNY MGLEFK	127	human ephrin-B1
112	IKFQEFSPNL WGLEFQ	127	human ephrin-B2
115	IKFQEYSPNL WGHEFR	130	human ephrin-B3

Figure 1-6: Sequence alignment of critical residues of the G-H loop of human and mouse ephrin ligands.

Sequence alignments of the G-H loop amino acids of human and mouse ephrin ligands were generated and analyzed with Clone Manager Suite9 software (Scientific & Educational Software, Cary, NC). The 6-residue core subdomain of the G-H loop is shaded in grey. The positions of critical residues required for interaction with henipavirus G glycoproteins are denoted by stars.

Ephrin-B2 and -B3 are known to interact with HeV and NiV G (27; 181; 182; 268; 270), whereas GhV recognizes ephrin-B2 alone (136; 256). The G-H loop amino acids F₁₁₇/Y₁₂₀, P_{119/122}, L_{121/124} and W_{122/125} of ephrin-B2/B3 are believed to contact residues in the binding pocket of HeV and NiV G glycoproteins in an induced-fit lock and key mechanism where a conformational shift at W_{122/125} acts as a latch to stabilize the G-H loop/G cleft interaction (238; 268). Crystal structures of GhV G in complex with ephrin-B2 reveal that, similarly to what is observed in NiV G/ephrin-B2 interaction, GhV G accommodates deep burying of residues F₁₁₇ and W₁₂₂ into their respective binding pockets (136). However, Lee et al. (136) noted that reduced burying interface surface between GhV G and ephrin-B2 due to the lack of a secondary binding site to contact ephrin-B2 residues K₁₀₆, D₁₀₈, and Q₁₀₉ could result in weakened interaction between GhV G and ephrin-B2.

In contrast, CedV G is able to interact with both transmembrane and GPI-linked ephrin ligands, and displays a much broader ligand tropism in binding human ephrin-A2, -A5, -B1 and -B2, yet not -B3 (129; 131; 149). It has been suggested that the lack of a fourth hydrophobic binding pocket in CedV G results in the enlargement of the third binding pocket and helps accommodate interactions with G-H loop residues that bear larger side chains (131). Two major residue substitutions in the sequence of CedV G, when compared to HeV and GhV G glycoproteins, result in the abrogation of the fourth binding pocket and extension of the third pocket: W₅₀₄ in HeV G and W₅₁₄ in GhV G are replaced by Y₅₂₅ in CedV G, and L₃₀₅ in HeV G and Y₃₂₁ in GhV G are replaced by D₃₂₈ in CedV G (131). Crystal structure analysis of the CedV G/ephrin-B1 complex shows that the larger third binding pocket of CedV G facilitates the insertion of the G-H loop residue

Y₁₂₁ of ephrin-B1 which would have been prohibited due to steric hindrance in a four pocket topography (131). Although CedV G lacks a fourth binding pocket, it is still able to bind ephrin-B2 by stabilizing the G-H loop residue W₁₂₂ into place in its larger third pocket rather than a fourth pocket thanks to interactions with CedV G P₄₂₃ and T₄₂₄ residues (131). The M₁₂₂ residue of ephrin-B1 is similarly fitted and stabilized into the third binding pocket of CedV G (131). Interestingly, CedV G also recognizes the murine variant of ephrin-A1 as an entry receptor. This can be structurally explained by the fact that I₁₁₅ in the G-H loop of murine ephrin-A1 is larger and more hydrophobic than the human T₁₁₅ residue and can therefore support more energetically and spatially favorable interactions within the third binding pocket of CedV G (131).

Surprisingly, MojV G is not only unable to bind ephrin-B3, but also unable to bind the canonical henipavirus receptor ephrin-B2 (215). Comparative studies of NiV G and MojV G crystal structures have demonstrated that the structure of the central cavity of the globular head of MojV G diverges greatly from the organization of the binding pockets that accommodates interactions between NiV G and ephrin-B2 (215). Indeed, the spatial arrangement of the first and fifth blades of the MojV G β -propeller preclude any interaction with the G-H loop of ephrin-B2 or -B3 ligands due to steric hinderance (**Figure 1-1**) (215). However, it is not known if MojV G can interact with ephrin ligands other than ephrin-B2 and -B3.

Ephrin ligands: entry receptor tropism and pathogenesis of henipaviral disease

The receptor tropism of henipaviruses bears important consequences on viral dissemination and pathogenesis during infection. The expression of ephrin-B2 in kidney, lung and spleen tissues as well as endothelial cells, supports the characteristic vasculitis

and systemic dissemination of HeV and NiV infections (89) (<https://www.proteinatlas.org>). In addition, the neuro-pathobiology observed in henipaviral disease is consistent with the fact that ephrin-B3 is more abundantly expressed in the brain and throughout the CNS (142; 209). The suspected pathobiology of GhV infections and the possible role of ephrin-B2 in the course of a natural infection has not been confirmed due to the lack of GhV viral isolate. Receptor tropism can act as a barrier to cross-species transmission. The implications of the broad ephrin ligand tropism observed for the non-pathogenic CedV in terms of route of transmission and establishment of infection remain unclear but suggests an increased inter-species transmission potential and possible circulation of henipaviruses and henipa-like viruses in rodent species. In the absence of studies positively identifying the receptor(s) of MojV, host and tissue tropisms and their significance in pathobiology and transmission of the first henipavirus ever detected in a rodent still have to be elucidated.

HYPOTHESES

Based on the genomic sequence analysis that allowed for its identification (266), MojV seems to be closely related to the highly pathogenic zoonotic henipaviruses HeV and NiV. These viruses can cause severe and often fatal disease in natural infections of horses, pigs, dogs, cats and humans, and have also been shown to be pathogenic in experimental infections of African green monkeys, Syrian golden hamster and ferrets. Additionally, HeV, NiV, CedV and GhV usage of ligands widely conserved across mammalian species, ephrin ligands, as viral entry receptors, further suggests a high potential for cross-species transmission of viruses of the *henipavirus* genus.

Current knowledge about MojV is quite limited. Important questions regarding the possible pathogenicity of MojV, its host and reservoir range, cellular and receptor tropism, as well as the extent of its circulation in wildlife, livestock and human populations and its route of transmission still remain unanswered. My research focused on exploring foundational issues of MojV entry mechanism, species and receptor tropism, and means of detection for viral glycoproteins and exposure to the virus in the absence of a viral isolate.

Overall hypotheses of this research project

Mojiang virus, like other henipaviruses, gains host cell entry via ephrin(s)-mediated mechanisms that require its attachment (G) and fusion (F) glycoproteins.

Mojiang virus displays a species-specific tropism that suggests that different henipaviruses may be found in reservoirs other than bats.

CHAPTER 2: Materials and Methods

Disclaimer: All work was performed by Sofia Cheliout Da Silva unless otherwise indicated.

CELL LINES AND CULTURE CONDITIONS

Baby hamster kidney-derived BSR T7/5 cells, which constitutively express T7 polymerase, were obtained from Tzanko Stantchev (U.S. Food and Drug Administration, Silver Spring, MD). BHK-21 cells were provided by Norman Cooper (National Institute of Health, Bethesda, MD). HeLa-ATCC derived HeLa-USU cells were characterized in previous work by microarray analysis, which determined that these cells lack the HeV and NiV receptors ephrin-B2 and -B3 (27). HEK293T cells were provided by Brian Schaefer (Uniformed Services University, Bethesda, MD). Chou-Zen Giam and Regina Day (Uniformed Services University, Bethesda, MD) gifted Rat2 cells and A549, respectively, for this study. African green monkey (Vero) cells (ATCC CCL 81), CHO-K1 *Cricetulus griseus* (Chinese hamster) ovarian cells (ATCC CCL-61), C6 *Rattus norvegicus* brain cells (ATCC CCL-107) and L2 *Rattus norvegicus* lung cells (ATCC CCL-149) were obtained from the American Type Culture Collection (Manassas, VA).

BSR T7/5, HeLa-USU, HEK293T, Vero and BHK-21 cell monolayers were maintained in Dulbecco's Modified Eagle's Medium (Quality Biologicals, Gaithersburg, MD) supplemented with 2mM L-glutamine (Quality Biologicals, Gaithersburg, MD), 1% penicillin-streptomycin (Quality Biologicals, Gaithersburg, MD) and 10% cosmic calf serum (HyClone, Logan, UT) (DMEM-10) at 37 °C in 5% CO₂ humidified atmosphere. A549, CHO-K1 and L2 cell monolayers were maintained in Ham's F-12K (Kaighn's) Medium (Gibco, Gaithersburg, MD) supplemented with 2mM L-glutamine (Quality

Biologicals, Gaithersburg, MD), 1% penicillin-streptomycin (Quality Biologicals, Gaithersburg, MD) and 10% cosmic calf serum (HyClone, Logan, UT) (F-12K-10) at 37 °C in 5% CO₂ humidified atmosphere. C6 cells were grown in Ham's F-12K (Kaighn's) Medium (Gibco, Gaithersburg, MD) supplemented with 2mM L-glutamine (Quality Biologicals, Gaithersburg, MD), 1% penicillin-streptomycin (Quality Biologicals, Gaithersburg, MD), 2.5% cosmic calf serum (HyClone, Logan, UT) and 15% horse serum (ATCC 30-2040, Manassas, VA) (F-12K-2.5/15) at 37 °C in 5% CO₂ humidified atmosphere.

PLASMIDS AND EXPRESSION VECTORS

Synthetic HeV F and G (Genbank NC 001906.3), GhV F and G (Genbank NC_025256.1) and MojV F and G (Genbank NC 025352.1) open reading frames were codon optimized for mammalian cell expression in pUC57 plasmids and obtained from GenScript®, Piscataway, NJ. The recombinant F and G glycoproteins genes were subcloned into the mammalian expression vector pcDNA3.1 Hygro CMV. To allow for protein precipitation and detection a 15 amino acid oligopeptide termed S-tag derived from pancreatic ribonuclease A (KETAAAKFERQHMDS) was added to the end of the cytoplasmic domain of the glycoproteins, at the C-terminus of the F proteins and at the N-terminus of the G proteins, by site-directed mutagenesis using the QuickChange II XL Site-directed Mutagenesis kit (Agilent technologies, Santa Clara, CA), (**Figure 3-1a**). Codon optimized S-tagged CedV F and G open reading frames (ORFs) (Genbank KP271122.1) were obtained from Stephanie Petzing. Sequencing of the S-tagged CedV F and G constructs revealed mutations in the ORFs, which were corrected by PCR

mutagenesis. Tag-less constructs of CedV F and G plasmids were also generated by PCR mutagenesis.

A soluble construct of MojV F (MojV sF) was designed by deleting the predicted cytoplasmic and transmembrane domains of MojV F and replacing them with a GCN4 trimeric motif (MKQIEDKIEEILSKIYHIENEIARIKKLIGE) (48; 49) to promote the trimeric oligomerization of MojV sF glycoproteins followed by a S-tag (**Figure 4-2a**). An Igk leader sequence (METDTLLLWVLLLWVPGSTGD) was also included to allow secretion (88) (**Figure 4-2a**). A soluble construct of CedV F (CedV sF) was provided by Lianying Yan. A soluble construct of MojV G (MojV sG) was generated following a similar design with the exception of the GCN4 trimeric motif being replaced by a GCN4 tetrameric motif (MKQIEDKLEEIESKLKKIENELARIKK) to facilitate tetramerization of henipavirus sG (30; 88) (**Figure 3-4a**). A likewise designed CedV G (CedV sG) construct was obtained from Deborah Fusco. Preliminary work by Stephanie Petzing on a GhV G (GhV sG) construct was completed by confirming clones by sequencing and testing expression of the construct by western blot analysis.

Genes encoding human ephrin-A2, -A3, -A4, -B1 and -B2 in the expression vector pCMV6-XL4, human ephrin-A1 in the expression vector pCMV6-AC and human ephrin-A5 and -B3 in the expression vector pCMV6-XL5 were purchased from Origene™ (Rockville, MD). Open reading frames encoding mouse ephrin-A1 and rat ephrin-A4 were obtained in expression vector pCMV6-Kan/Neo and pCMV6-Entry, respectively (Origene™, Rockville, MD).

Human ephrin-A4 S119P and rat ephrin-A4 P122S mutants were generated by PCR-site directed mutagenesis with a QuickChange II XL Site-directed Mutagenesis kit (Agilent technologies, Santa Clara, CA).

A soluble version of rat ephrin-A4 was created by amplifying the sequence encoding L26-G171 by PCR. This region was subcloned into a BNN-CFC-pcDNA3.1 expression plasmid that includes a Fc tag, and was provided by Kai Xu (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

The NLuc gene (GenBank JQ513379.1), which encodes the Nano luciferase, was codon optimized, synthesized and obtained in pUC57 from GenScript®, Piscataway, NJ. This gene was then subcloned into the expression plasmid pTM1 kindly provided by Chad Mire (University of Texas Medical Branch at Galveston, Galveston, TX), to ensure T7 promoter driven expression of the luciferase gene.

WESTERN BLOTS ANALYSIS

BSR T7/5 cell monolayers were transfected with 2 µg of DNA for 4.2 µl of Lipofectamine™ LTX (Invitrogen - Thermo Fischer Scientific, Carlsbad, CA) (1:2.1 ratio; µg DNA: µl LTX) per well of 60% confluent cells in 6 well tissue culture plates pre-coated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich, St Louis, MO). The cells were collected and lysed in RIPA buffer (Thermo Fischer Scientific, Carlsbad, CA) with 1X cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland) 48h post-transfection. Lysates were incubated with S protein agarose (EMD Biosciences Inc., Madison, WI) at 4°C overnight. The agarose beads were washed three times in RIPA buffer before being boiled in sample buffer (2X LDS NuPage® sample buffer (Invitrogen

- Thermo Fischer Scientific, Carlsbad, CA), 25% β -mercaptoethanol (Sigma-Aldrich, St Louis, MO)). The proteins were resolved by NuPage® 4-12% Bis-Tris SDS PAGE (Invitrogen - Thermo Fischer Scientific, Carlsbad, CA). Wet transfer onto nitrocellulose membranes was performed with Invitrogen™ XCell II™ Blot Module (Thermo Fischer Scientific, Carlsbad, CA). Following blocking in 5% milk 1XPBST, the blots were probed with rabbit anti-S polyclonal antibody conjugated to horseradish peroxidase at a 1:12,500 dilution (Southern Biotechnology, Birmingham, AL) in 5% milk 1XPBST.

PRODUCTION OF RECOMBINANT PROTEINS

CedV and MojV sF

The production and purification of CedV and MojV sF proteins were adapted from methods previously designed to produce and purify HeV and NiV sF proteins (48; 49). Briefly, FreeStyle™ 293 cells (Thermo Fischer Scientific, Carlsbad, CA) grown in Dulbecco's Modified Eagle's Medium (Quality Biologicals, Gaithersburg, MD) supplemented with 2mM L-glutamine (Quality Biologicals, Gaithersburg, MD), 1% penicillin-streptomycin (Quality Biologicals, Gaithersburg, MD) and 5% cosmic calf serum (HyClone, Logan, UT) (DMEM-5) at 37 °C in 5% CO₂ humidified atmosphere were transfected with the corresponding expression plasmid with Xtreme Eugene9® (Roche, Basel, Switzerland). The culture supernatant collected 48h post-transfection was incubated with S protein agarose overnight at 4°C to precipitate soluble S-tagged glycoproteins. The samples were analyzed by denaturing SDS PAGE under reducing conditions followed by western blot as described above to confirm proper transient expression of the soluble glycoproteins. Cell lines stably expressing the soluble proteins were generated by replacing DMEM-5 medium with DMEM-5 containing 50 μ g/ml

Hygromycin B (Thermo Fischer Scientific, Carlsbad, CA) (DMEM-5+). Drug resistant cells were subjected to two rounds of limiting dilution cloning. Suitable candidate clones were identified by SDS PAGE and western blot analysis and expanded in FreeStyle™ 293 Expression Medium (Thermo Fischer Scientific, Carlsbad, CA). Cell suspensions were collected and centrifuged at 3700 rpm for 1h at 4°C. The supernatant was filtered through a 0.22 µm PES filter unit (Corning, Inc., Corning, NY) pre-treated with 1X PBS 1% Triton-100. Affinity purification of the supernatant on a S protein agarose affinity column (EMD Biosciences) was performed after addition of 0.3% of cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland) to the supernatant. The column was washed with 1X PBS, 0.1% Triton X-100, 0.1M arginine. CedV and MojV sF proteins were eluted by addition of 0.2M citrate, 0.2M L-arginine, pH2, followed by application of 1M HEPES, pH9. After concentration of the eluate with Amicon Ultra centrifugal filter units (Millipore, Burlington, MA), proteins were further purified by size exclusion chromatography with a HiLoad 16/60 Superdex 200 prep grade gel filtration column (GE Healthcare, Pittsburg, PA). Candidate fractions were pooled after 3-12% native PAGE (Invitrogen - Thermo Fischer Scientific, Carlsbad, CA) followed by western blot analysis confirmed the presence of oligomers. Large scale protein production and purification was performed by Lianying Yan.

CedV, GhV and MojV sG

Similarly, cell lines for the large scale production of CedV, GhV and MojV sG glycoproteins were prepared, and the proteins were produced and purified following the protocol for CedV and MojV sF (described above) with the following exceptions. The column was washed with 1X PBS alone, rather than 1X PBS, 0.1% Triton X-100, 0.1M

arginine, before elution. Additionally, the stably expressing MojV sG cell line was established in Neuro-2a cells (ATCC® CCL-131™) grown in roller bottles (Corning - Sigma-Aldrich; St. Louis, MO). Large scale protein production and purification was performed by Lianying Yan.

Rat ephrin-A4-Fc

A FreeStyle™ 293 cell line stably expressing soluble rat ephrin-A4-Fc was created as per the protocol applied to obtain CedV and MojV sF producing cell lines (see above) by Lianying Yan with one modification; the supernatant was purified through a protein G-agarose affinity column (EMD Biosciences) and eluted with a 0.1M Glycine pH 2.5 buffer.

PROTEIN CROSS-LINKING ASSAY

The oligomerization status of recombinant henipavirus F and G glycoproteins was investigated by performing the following cross-linking assays. For cell surface expressed proteins analysis, BSR T7/5 cells were transfected as described above, harvested by trypsinization 72h post-transfection, and allowed to recover overnight in suspension in DMEM-10 in 50 ml tubes placed at a 45° angle at 37°C in a humidified 5% CO₂ atmosphere. The cells were then washed three times in 1X PBS pH 7.4 (Quality Biologicals, Gaithersburg, MD) and re-suspended in 1X PBS pH 7.4 at 25x10⁶ cells/ml. Aliquots of 5x10⁶ cells in 200 µl 1X PBS pH 7.4 were incubated with 0, 1, 3 or 5mM (bis(sulfosuccinimidyl)suberate) (BS3) membrane impermeable cross-linking reagent for 45 minutes at 4°C. The reactions were quenched by addition of 20mM Tris and incubated for 15 minutes at room temperature. The cells were lysed as described above. The lysates

were processed for western blot analysis as described above. Transfer and detection were carried out as described above.

Soluble, purified, CedV and MojV sF and CedV, GhV and MojV sG proteins (1 µg per sample) were incubated with 0, 0.8, 1.0 and 1.2 mM BS3 for 30 minutes at room temperature. The reactions were quenched as described above, and 20 ng of cross-linked soluble proteins were resolved by SDS PAGE NuPage® 4-12% Bis-Tris gels for sF oligomers and 3-8% Tris-Acetate gels for sG oligomers (Invitrogen - Thermo Fischer Scientific, Carlsbad, CA), and analyzed by western blot as described above.

HEPTAD-REPEAT CAPTURE ASSAY

Thirty-six-residue biotinylated peptides mimicking a section of the heptad repeat region 2 (HR2) of CedV F (CedV FC2, LCBiot-KVDLSNEINKMNQSLKDSIFYLREAKRILDSVNISL-amide) and MojV F (MojV FC2, LCBiot-KIDIGNQLAGINQTLQEAEDYIEKSEEFLKGVNPSI-amide) were designed based on secondary structure predictions from the SABLE protein structure prediction server (<http://sable.cchmc.org/>), University of Cincinnati, OH, and analysis of sequence alignments of HeV F, NiV F, CedV F and MojV F generated with Clone Manager 9.0 (Sci-Ed Software). These 36 amino acid peptides sequences were synthesized by New England Peptide™, Inc. (Gardner, MA).

The heptad-repeat capture assay was performed based on the method previously described by Chan et al. and adapted for this study (3). Briefly, 2 µg of CedV sF or MojV sF peptide was treated by a combination of heat (50°C for 15 minutes), 80 ng of trypsin (6h digestion at 4°C) and addition of 12 µg of the corresponding biotinylated heptad-repeat peptide in different order of treatment in a 10 µl volume. The treatment

combinations tested were the following: no treatment, heptad-repeat peptide addition alone, heptad-repeat peptide addition first then heat treatment, trypsin digestion first then heptad-repeat peptide addition, heat treatment first followed by trypsin digestion then heptad-repeat peptide addition, trypsin treatment first followed by heptad-repeat peptide addition then heat treatment, and finally trypsin digestion first followed by heat treatment then addition of heptad-repeat peptide. The sF/ heptad-repeat peptide complexes were precipitated by addition of 30 µl 50% avidin agarose slurry (Vector laboratories, Burlingame, CA) in 160 µl of RIPA buffer 1X cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and incubated for 1h at 4°C. The avidin agarose beads were washed three times in RIPA buffer and boiled in 50 µl of reducing sample buffer as described above. Twenty-two microliters of each sample was resolved by NuPage® 4-12% Bis-Tris SDS PAGE and analyzed by western blot as described above. Primary mouse monoclonal 4G5 antibody (1:2,500) and mouse polyclonal serum (1:1,000) against MojV and CedV F, respectively, followed by secondary horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) antibody (1:12,500) (Pierce #31430 - Thermo Fischer Scientific, Carlsbad, CA) were used for detection.

SYNCYTIIUM FORMATION ASSAY

BSR T7/5 and BHK-21 cells were grown to 60% confluency in 6 well tissue culture plates pre-coated with 0.1 mg/ml poly-D-lysine. The cells were transfected with 2 µg of pcDNA 3.1 Hygro CMV untagged HeV, CedV or MojV F or G alone, or co-transfected with 1 µg of F and 1 µg of G HeV, CedV or MojV plasmid DNA, or with 2 µg of pcDNA 3.1 Hygro CMV with 4.2 µl of Lipofectamine™ LTX. Upon observation of giant multinucleated cell formation (syncytial events) the cells were fixed in methanol

and stained with crystal violet. Photographs were taken under brightfield conditions with a Zeiss Axio Observer A1 inverted microscope set to the 20X objective. White scale bars (bottom right corner of each picture) represents 50 μm .

CELL-CELL FUSION NANOLUC® LUCIFERASE REPORTER-GENE ASSAY

Effector BSR T7/5 cells plated at 0.6×10^5 cells per well in 24 well tissue culture plates pre-coated with 0.1 mg/ml poly-D-lysine were transfected 24h post plating with 0.8 μg untagged pcDNA 3.1 Hygro CMV HeV, CedV or MojV F or G alone, or co-transfected with 0.4 μg of F and 0.4 μg of G HeV, CedV or MojV plasmid DNA, or with 0.8 μg of pcDNA 3.1 Hygro CMV with 1.68 μl of Lipofectamine™ LTX per well. To assess species tropism of henipavirus F and G mediated cell-cell fusion, target HeLa-USU, A549, Vero, BHK-21, CHO-K1, L2, C6 and Rat2 cells grown in T-162 flasks to 60% confluency were transfected with 28.8 μg pTM1-NLuc using 28.8 μl of Plus™ Reagent and 86.4 μl of Lipofectamine™ LTX, while HEK293T cells were transfected with 28.8 μg pTM1-NLuc using 86.4 μl of X-tremeGENE™ 9 DNA Transfection Reagent (Roche, Basel, Switzerland). To investigate a possible ephrin tropism of MojV, target CHO-K1 cells were also transfected with single pCMV-human, mouse or rat ephrin plasmid DNA in addition to pTM1-NLuc. Target cells were harvested by trypsinization 24h post-transfection, re-suspended in DMEM-10 for HeLa-USU, HEK293T, Vero, BHK-21, Rat2 cells, in F-12K-10 for A549, CHO-K1 and L2 cells, or in F-12K-2.5/15 for C6 cells, and allowed to recover overnight in 50 ml tubes placed at a 45° angle at 37°C in a humidified 5% CO₂ atmosphere. The next day, 2.0×10^5 target cells per well were applied to the BSR T7/5 cell monolayers. Cell mixtures of effector and target cells were collected at times 0h, 10h, 24h, 48h, 72h, 96h, 120h, day 6 and day 7

post-application and re-suspended in 130 μ l 1X PBS per well-mixture. Triplicate aliquots of 40 μ l from each cell mixtures were assayed for luciferase activity with the Nano-Glo® Luciferase Assay System (Promega, Madison, WI). Normalized maximum luminescence values (see data analysis section) reached by each effector/target cell co-cultures are reported and corresponding collection times are indicated in each specific figure.

SEQUENCE ANALYSIS

Alignments of human, mouse and rat ephrin ligands, as well as henipavirus F and G, sequences were generated and analyzed with Clone Manager Suite9 software (Scientific & Educational Software, Cary, NC).

CO-PRECIPITATION ASSAY

Purified CedV, GhV and MojV sG proteins were incubated with a panel of soluble Fc-conjugated A-class and B-class ephrins including human ephrin-A1 Fc (transiently expressed in 293 cells and purified and provided by Kai Xu (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, 46), mouse ephrin-A1, mouse ephrin-A2, human ephrin-A3, human ephrin-A4, human ephrin-A5, mouse ephrin-B1, mouse ephrin-B2 and human ephrin-B3 (R&D Systems; Minneapolis, MN) and rat ephrin-A4 (see above) in a 1:2 ratio (1.5 μ g sG : 3 μ g ephrin) in a total reaction volume of 100 μ l in the presence of 1X complete protease inhibitor (Roche, Basel, Switzerland) in RIPA buffer at 4°C overnight. The henipavirus sG-ephrin ligand complexes were precipitated for 2.5h at 4°C using 50 μ l of a 50% protein G agarose slurry (for samples) or 40 μ l of a 50% S agarose slurry (for controls), washed three times with RIPA buffer, boiled in 2X NuPage® LDS sample buffer (Life Technologies; Carlsbad, CA) containing 5% 2-mercaptoethanol (Sigma-

Aldrich; St. Louis, MO) and run on a 4-12% NuPage® Bis-Tris gel. Co-precipitated henipavirus sG proteins were detected by western blot as described above using horseradish peroxidase-conjugated rabbit anti-S-tag polyclonal antibody (Southern Biotechnology, Birmingham, AL).

MOUSE IMMUNIZATION: MOJV sF, MOJV sG, GHV sF, GHV sG

Mice studies were conducted in accordance to protocol MIC 16-262 approved by the Uniformed Service University Animal Care and Use Committee. Seven-week-old BALB/cByJ mice (stock#001026) purchased from Jackson Labs (Bar Harbor, ME) were immunized by injection of 9 µg of purified soluble henipavirus glycoprotein in 100 µl of 1:1 v/v sterile 1XPBS:MPL-TDM adjuvant (48). The composition of the stable oil-in-water emulsion adjuvant included 0.5mg of monophosphoryl lipid A from Salmonella Minnesota monophosphoryl lipid A (detoxified endotoxin), and 0.5 mg synthetic trehalose dicorynomycolate in 2% squalene, 0.2% Tween⁸⁰ in water emulsion (Sigma, Saint-Louis, MO) to which was added 1 ml of sterile 1XPBS. Antigens were administered as a set of two 100 µl injections (subcutaneous and intraperitoneal) per mouse per immunization. Each henipavirus glycoprotein was used to immunize two mice. Pre-immunization samples were collected by tail bleed (~50 µl of blood per mouse) on day 0. The first immunization set was injected on day 14. A total of 3 immunogen boosts were administered every 28 days (on days 42, 70 and 98). Tail bleed samples were collected every 28 days, starting 14 days after the first immunization (on days 56, 84 and 112) to monitor rising antibody titers following re-exposure to immunogens. Tail bleed samples were processed to harvest serum immediately after blood collection by centrifugation for 5 minutes at 845xg, and transfer of the supernatant to a 1.5 ml tube.

Further clarification of the serum from the erythrocyte fraction was attained by repeating this step two more times and pooling the supernatants recovered. Serum was stored at -80°C until tested for antigen reactivity by ELISA (see section below). A fifth and final injection set was administered on day 126. Mice were sacrificed and exsanguinated a week later; at which time spleens were harvested. Mice immunizations using MojV sG and GhV sF glycoproteins were performed by Lianying Yan.

RABBIT IMMUNIZATION

Noble Life Sciences (Gaithersburg, MD) was contracted to raise quantities of polyclonal antibodies larger than those that could be obtained from our mouse studies. The standard protocol selected was performed as follows. A total of 750 mg of soluble glycoprotein was used per animal over the course of 4 immunizations. A pre-immunization bleed was obtained on day 0 of the protocol (~4.5 ml) before the primary immunization was conducted. Immunogen boosts were administered on days 21, 42 and 63. A 15 ml bleed was collected on day 52. Final exsanguination allowed for collection of ~50 ml of blood on day 73. Serum samples were stored at -80°C upon reception.

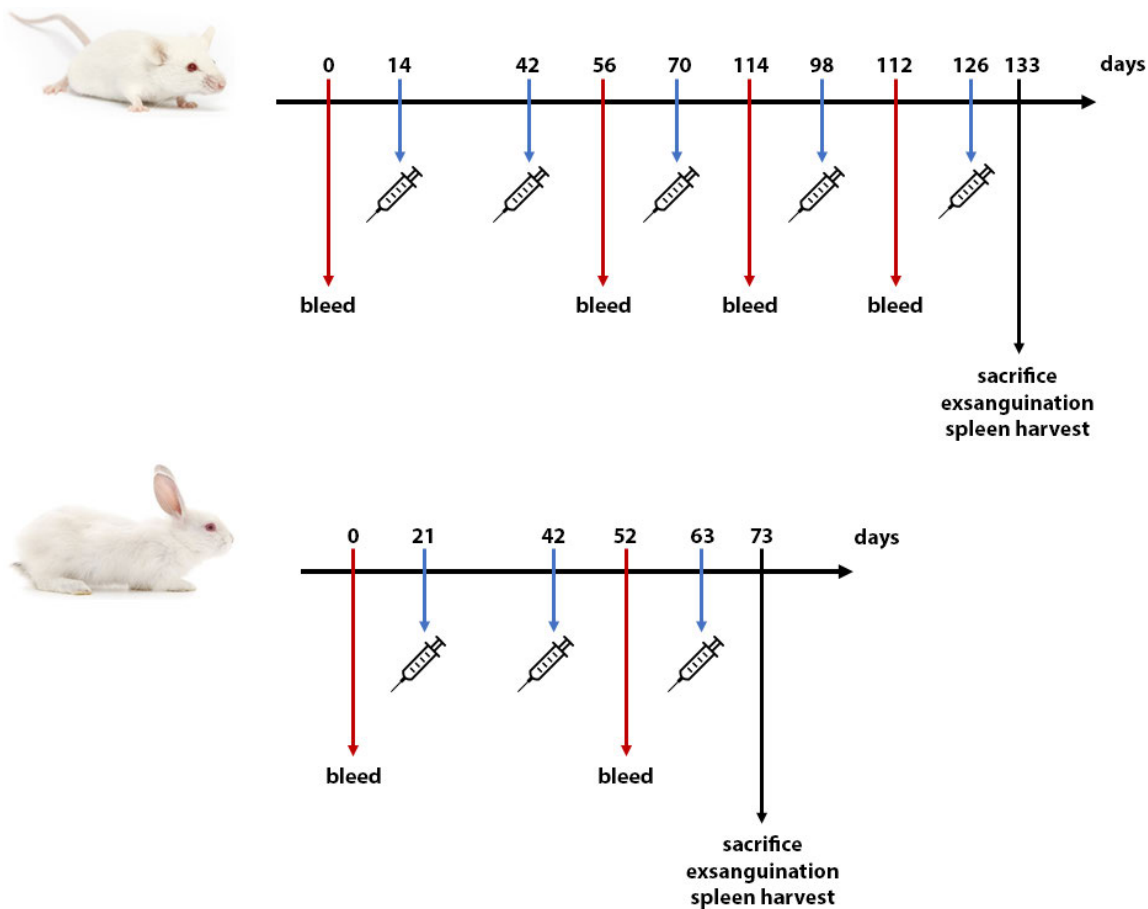


Figure 2-1: Mice and rabbits immunization schedule.

Serum was obtained from bleed samples and final exsanguination to assess rising antibody titers throughout the immunization studies.

**SANDWICH AND INDIRECT ELISA ASSESSMENT OF RISING ANTIBODY TITERS AFTER
MOUSE IMMUNIZATION WITH HENIPAVIRUS GLYCOPROTEINS: MOJV sF, MOJV sG,
GHV sF, GHV sG**

Serum reactivity to henipavirus F glycoproteins was evaluated by sandwich ELISA. Wells of a 96 well Immulon 2HB flat bottom plate (ThermoScientific, Waltham, MA) were coated with 50 µl of 0.3 µg/ml of goat anti-S tag antibody (Bethyl Laboratories, Inc, Montgomery, TX) per well and incubated overnight at 4°C. Coated wells were decanted and incubated with 50 ng of purified henipavirus F glycoprotein in 0.05% tween²⁰ 1XPBS per well (50 µl). Wells were washed three times with 0.05% tween²⁰ 1XPBS (ELx405 plate washer, BioTek Instruments, Inc., Winooski, VT) before 100 µl of blocking buffer (filtered 0.2% gelatin in 1XPBS w/v) was added to each well to reduce non-specific binding. After 1 hour incubation at 37°C, the wells were washed as described above and 100 µl of sera serial dilutions in 0.05% tween²⁰ 1XPBS was applied to the appropriate wells. The plates were incubated for another hour at 37°C, then washed as previously described. Secondary HRP-conjugated goat anti-mouse IgG (H+L) antibody (Bethyl Laboratories, Inc, Montgomery, TX) was added at a 1:5,000 dilution (50 µl of 0.2 µg/ml antibody per well) in 0.05% tween²⁰ 1XPBS for another hour at 37°C. Washed wells were incubated in of 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (80 µl per well) for a minimum of 30 minutes at room temperature with shaking. The optical density at 405 nm (O.D. 405 nm) was measured under the endpoint ELISA ABTS SDS program of a Versa max microplate reader (Molecular Devices, Sunnyvale, CA) supported by SoftMax® Pro 4.8 software (Molecular Devices) for data acquisition.

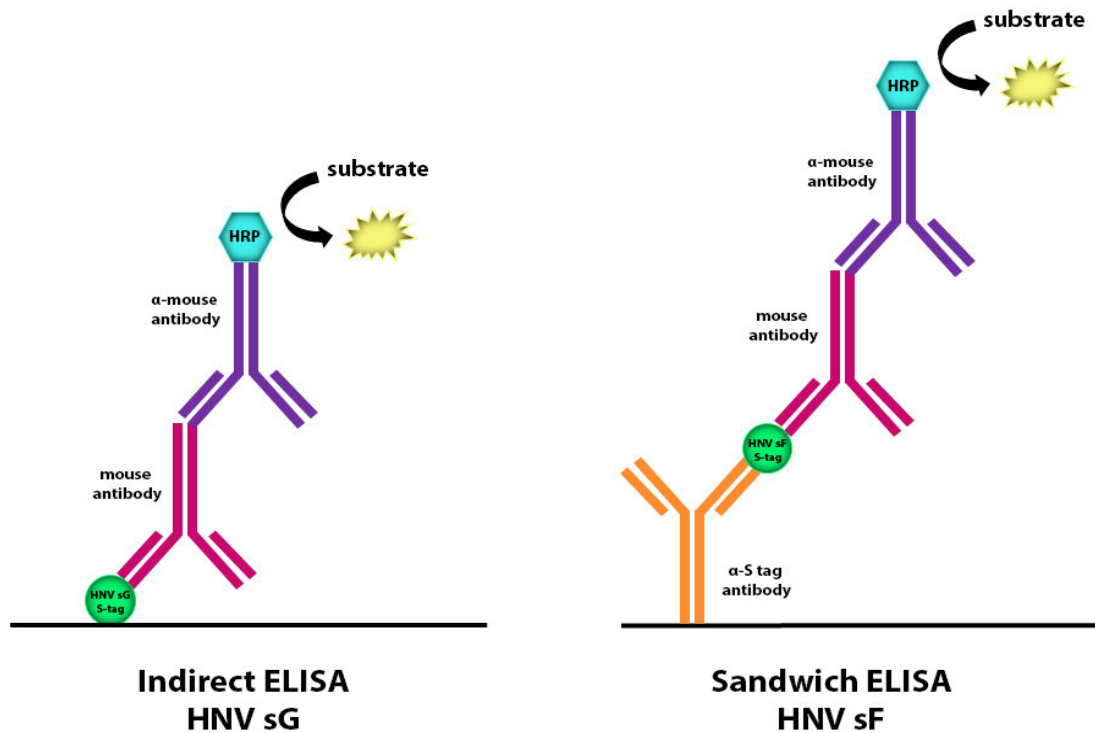


Figure 2-2: ELISA assessment of rising antibody titers in mice immunized with henipavirus sF and sG glycoproteins.
 Schematic highlighting the experimental design differences between ELISA performed to assess serum reactivity of animals immunized with henipavirus (HNV) sG or sF purified glycoproteins.

Assessment of serum reactivity to henipavirus G glycoproteins, however, was performed by indirect ELISA, rather than sandwich ELISA. The protocol described above was applied without alteration, but for omission of the pre-coating of wells with goat anti-S tag antibody, addition of 50 ng of purified henipavirus G instead of F glycoprotein per well and use of 5% BSA, 0.05% Tween²⁰ instead of 0.2% gelatin 1XPBS as blocking buffer. ELISAs testing reactivity to MojV sG and GhV sF glycoproteins were performed by Lianying Yan.

HYBRIDOMA PRODUCTION AND SCREENING

Splenocytes were isolated according to standard protocol and mixed with Sp2/0 myeloma cells at a 2:1 ratio of splenocytes to Sp2/0 cell in Gibco™ Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, ThermoFischer Scientific, Waltham, MA). Hybridomas were generated following a polyethylene glycol (PEG) splenic lymphocyte/myeloma fusion protocol previously described, submitted to plating at a limiting dilution and cultured in IMDM supplemented with Gibco® hypoxanthine-aminopterin-thymidine (HAT) (Invitrogen, ThermoFischer Scientific, Waltham, MA), 10% v/v HyClone™ Cosmic Calf™ Serum (GE Healthcare Life Sciences, Marlborough, MA), 1% v/v 50 mg/ml gentamicin (Quality Biological Inc, Gaithersburg, MD) and 100 units/ml of mouse recombinant Il-6 (R&D Systems, Minneapolis, MN) (IMDM-10-HAT-Il-6) (78). Hybridoma supernatants were screened for antigenic reactivity to henipavirus sG and henipavirus sF glycoproteins by indirect and sandwich ELISA, respectively. Positive cultures were subjected to 2 rounds of limiting dilutions to ensure clonality. Clonal hybridomas were further confirmed by ELISA which included controls to exclude

hybridomas reactive to GCN4 trimerization and GCN4 tetramerization motifs and S-tag epitopes. Hybridoma production and screening was performed by Lianying Yan.

PREPARATION OF MONOCLONAL ANTIBODIES

As described previously (78), supernatants from hybridoma cultures adapted to growth in HyClone SFM4MAb-utility (ThermoFischer Scientific, Waltham, MA) 1X HAT with 100 units/ml recombinant mouse IL-6 were clarified by centrifugation and applied to a 1L 0.22 μ m PES low protein-binding filter (Corning Inc., Corning, NY) (1). Filtrates were purified by protein G sepharose bead affinity chromatography (GE Healthcare Life Sciences, Marlborough, MA). Purified monoclonal antibodies were concentrated using a 50 ml format 30kD Amicon® Ultra-15 Centrifugal Filter Unit (Millipore Sigma, Burlington, MA). Purified concentrated IgG concentrations were determined with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFischer Scientific, Waltham, MA). Lianying Yan prepared the monoclonal antibodies used in this work.

CHARACTERIZATION OF MONOCLONAL ANTIBODIES

Western blot

BSRT7/5 cells plated in T-25 cell culture flasks (NEST Scientific, San Diego, CA) were transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA) at a 1 μ g DNA: 2.1 μ l LTX ratio with 3 μ g of each full-length S-tagged henipavirus F, G or empty expression vector pcDNA3.1 Hygro CMV when cells reached 70% confluency. Cells were collected by scraping and lysed for 30 minutes at 4°C in RIPA buffer (Thermo Fischer Scientific, Carlsbad, CA) supplemented with protease inhibitors (Roche, Basel, Switzerland) 48h post-transfection. Lysates were clarified by centrifugation and

precipitated with 40 μ l of a 50% S agarose slurry overnight rotating end over end at 4°C. Washed beads were boiled in 25 μ l of reducing sample buffer. The samples were resolved by SDS PAGE and proteins were transferred onto nitrocellulose membranes as described above. Blots were probed with anti-MojV sG or anti-MojV sF monoclonal or polyclonal antibodies. Monoclonal anti-MojV sG antibodies 2B2, 10G2 and 6E5 were tested at 3, 5 and 20 μ g/ml, whereas monoclonal anti-MojV sF were tested at 1.6 and 16 μ g/ml. Rabbit anti-MojV sG polyclonal antibodies were used at 1:10,000, and mouse anti-MojV sF polyclonal antibodies at 1:1,200 dilutions. Successful transfection and protein expression were confirmed in control blots with horseradish peroxidase-conjugated rabbit anti-S-tag polyclonal antibody (Southern Biotechnology, Birmingham, AL) at a 1:12,500 dilution.

Immunoprecipitation followed by western blot assay

Cell lysates from transfected BSRT7/5 cells were processed as described above. Lysates were quantified by Pierce™ BCA assay (Thermo Fischer Scientific, Carlsbad, CA). Anti-MojV sG monoclonal antibodies 2B2 (1 μ g), 6E5 (2 μ g) and 10G2 (2 μ g) and anti-MojV sF monoclonal antibodies 3C4 (2 μ g) and 4G5 (2 μ g) were incubated with ~200 μ g of the corresponding total protein cell lysate on a rotator at 4°C overnight. Glycoprotein/mAb complexes were precipitated with 50 μ l of a 50% protein G agarose slurry (rotating end over end for 1h at 4°C). Protein expression controls were incubated with 40 μ l of a 50% S agarose slurry (rotating end over end overnight at 4°C). Beads were washed three times and boiled in 25 μ l of reducing sample buffer, and samples were analyzed by western blot as described above. Membranes were probed with horseradish

peroxidase-conjugated rabbit anti-S-tag polyclonal antibody (Southern Biotechnology, Birmingham, AL) at a 1:12,500 dilution.

Flow cytometry

Seventy percent-confluent BSRT7/5 cells plated in T-150 cell culture flasks (NEST Scientific, San Diego, CA) were transfected with 45 µg of pcDNA3.1 Hygro CMV plasmid encoding each full-length henipavirus F, or G, or with the empty vector using Lipofectamine LTX transfection reagent (Invitrogen, Carlsbad, CA) without Plus reagent at a 1:2.1 ratio (µg DNA: µl LTX). Cells were collected 48 hours post-transfection by incubation at 37°C for 2 to 3 minutes in 5 ml of Versene (Gibco, Gaithersburg, MD) quenched with 15 ml of sterile 1XPBS (Quality Biological Inc., Gaithersburg, MD). The flasks were washed with an additional 20 ml of sterile 1XPBS to maximize cell collection. The cell suspensions were pelleted at 500xg (Sorvall Legend XTR, ThermoFischer Scientific, Waltham, MA) for 5 minutes at 4°C. Cell pellets were washed in a fresh 20 ml of sterile 1XPBS and centrifuged as described above. Cell density was measured using trypan blue dye to assess viability after the pellets were re-suspended in another 20 ml 1XPBS. Counted cells were centrifuged as described above and re-suspended to a density of 6.67×10^6 cells/ml in FACS buffer (1% BSA (Sigma-Aldrich, St. Louis, MO) in sterile 1XPBS). Cells were transferred to a Costar® round-bottom 96-well plate (Corning, Tewksbury, MA) by dispensing 150 µl (1×10^6 cells) of each cell type suspension per well, then centrifuged as described above. Supernatants were decanted, and cells re-suspended in 120 µl of each different antibody tested per well. Rabbit polyclonal sera raised against each henipavirus F or G or rabbit naïve serum were applied as a 1:50 dilution in FACS buffer. Mouse monoclonal antibodies raised

against MojV sG (2B2, 6E5, 10G2) and MojV sF (3C4, 4G5) were applied at 0.5 µg/well in FACS buffer. Following a 30-minute incubation in primary antibodies at 4°C, 100 µl of FACS buffer was added to each well, and cells were centrifuged as described above. Cells were then washed twice with 250 µl of FACS buffer. Secondary antibodies were applied to each well at a 1:200 dilution in 120 µl per well for 30 minutes at 4°C in the dark. Mouse monoclonal antibodies were detected with goat anti-mouse IgG(H+L)::AlexaFluor488® (Invitrogen, Carlsbad, CA, ThermoFischer cat#A-11029); rabbit polyclonal antibodies were detected with goat anti-rabbit IgG(H+L)::AlexaFluor647® (Invitrogen, Carlsbad, CA, ThermoFischer cat#A-21245). After incubation, 100 µl of FACS buffer was added to each well, and cells were pelleted as described above. Two additional washes in 250 µl of FACS buffer per well were performed before cells were re-suspended in 250 µl of FACS buffer and kept on ice until they were analyzed with a BD LSRII flow cytometer with High Throughput Sampler (HTS) (BD Bioscience, San Jose, CA). Data was analyzed by FlowJo3 software (FlowJo LLC, Ashland, OR).

ANTIBODY HARVEST FROM MOUSE FECAL PELLETS

Fecal pellets were collected from naïve BALB/cByJ mice (Jackson Labs, Bar Harbor, ME) and from mice 2 weeks after each purified MojV sG and GhV sF glycoprotein injection (see section on mouse immunization section, **Figure 2-1**). Additional fecal pellets were collected from naïve BALB/cByJ mice enrolled in ongoing experiments in the Mitre and Jerse labs (Uniformed Services University of Health Sciences, Bethesda, MD). Two mice were injected with each type of glycoprotein (see mouse immunization section). Pellets were pooled per type of protein per number of

injections. The fecal matter was weighted and re-suspended in 1XPBS supplemented with cOmplete EDTA-free protease inhibitor cocktail (Millipore Sigma, Darmstadt, Germany / Roche, Basel, Switzerland) at a ratio of 0.1 g fecal matter to 1 ml 1XPBS+protease inhibitor. Pellets were incubated at room temperature for 15-20 minutes to allow softening, then triturated. The fecal matter suspension was then incubated at room temperature for another 15-20 minutes before debris were removed by centrifugation at 4122xg (Sorvall Legend XTR, ThermoFischer Scientific, Waltham, MA) for 10 minutes. The supernatant was subjected to 2 rounds of filtration, and was first applied to a 1XPBS pre-wetted Puradisc™ 25AS 0.45 µm filter (Whatman plc, Maidstone, United Kingdom), then to an Acrodisc® 0.22 µm filter (Pall Corporation, New York, NY). The fecal filtrate was aliquoted in 200 µl fractions and stored at -20°C until use.

IGG PURIFICATION FROM MOUSE SERUM

IgG purification

A Poly-Prep® Chromatography Column (Bio-Rad, Hercules, CA) was wetted with 2 ml of 1XPBS allowed to flow through the column before 500 µl of protein G bead 1:1 slurry (Sigma, Saint-Louis, MO) was added to the capped column. The beads were washed with 5x500 µl of 1XPBS (5 times the bed volume of beads applied). Fifty to seventy microliters of murine polyclonal serum diluted to a total volume of 500 µl in 1XPBS was transferred to the column and allowed to flow through the uncapped column. The beads were washed with 500 µl of 1XPBS, then with 5x500 µl of 1XPBS 5 more times. The column was capped, and the beads were incubated in 500 µl of elution buffer (0.1M glycine pH2.5) standing for 15 minutes at room temperature. The cap was removed, 25 µl of flow through was collected directly from the tip of the column and

mixed with 6.5 µl of Bradford reagent (Bio-Rad, Hercules, CA) to confirm the presence of protein in the eluate. Four hundred microliters of eluate were collected and transferred to 100 µl of neutralization buffer (1M Tris-HCl pH8). Purified IgG fractions were further collected in additional increments of 500 µl of elution buffer repeating the incubation/protein confirmation/elution steps described above as long as the Bradford test turned positive (blue). Collection was halted when the Bradford reagent/eluate mixture remained brown (negative for protein content). Neutralization of the eluate was confirmed by litmus strip test (Sigma, Saint-Louis, MO). A 30kD Amicon® Ultra-15 Centrifugal Filter Unit (Millipore Sigma, Burlington, MA) was centrifuged 5 minutes at 3500xg at 4°C after application of 2 ml of 1XPBS. Purified IgG fractions were combined into the Amicon® filter unit and centrifuged as described above for a total of 20 minutes in 5-10 minutes increments until less than 100 µl of purified IgG remained in the filter (~80 µl). Concentrated purified IgG was transferred from the filter to a 1.5 ml tube, and 1 ml of 1XPBS was pipetted up and down into the filter to wash the unit. After another 15 minutes centrifugation, the volume remaining in the filter was combined with the ~80 µl collected previously and centrifuged as described above for 20 minutes. Concentrated purified IgG was again transferred into a 1.5 ml tube. The filter was washed with 25 µl of 1XPBS and centrifuged as described above. The volume remaining in the unit after centrifugation was added to the volume collected in the 1.5 ml tube, and concentrated purified IgG concentration was determined with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFischer Scientific, Waltham, MA).

ADSORPTION OF ANTI-S-TAG AND ANTI-GCN4 TRIMERIZATION MOTIF ANTIBODIES FROM MURINE SERUM AND FECAL SAMPLES

S and GCN4 trimerization peptides (1 mg each) were immobilized on SulfoLink agarose resin (2 ml each) as per guidelines from the SulfoLink™ Immobilization Kit manufacturer (ThermoFischer Scientific, Waltham, MA). Parafilmed peptide-coupled resin columns were stored in 2 ml of degassed buffer (BupH™ Phosphate Buffered Saline Pack, 0.1M phosphate, 0.15M sodium chloride, pH7.2, ThermoFischer Scientific, Waltham, MA) at 4°C until resin aliquots could be used to adsorb anti-S-tag and anti-GCN4 trimerization motif antibodies from serum and fecal samples.

Three volumes of S peptide-coupled resin or GCN4 trimerization peptide-coupled resin were added to one volume of murine anti-MojVsG and anti-GhV sF purified serum IgG or fecal filtrate, respectively. The samples were incubated at 4°C on a rotator overnight. The resin was then pelleted by centrifugation for 1 minute at 9300xg. The concentration of adsorbed purified serum IgG was measured with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFischer Scientific, Waltham, MA). Fecal IgG and IgA levels in adsorbed samples were determined by ELISA quantification as detailed below.

ELISA QUANTIFICATION OF MURINE IGG AND IGA

IgG and IgA levels in murine serum and fecal samples were quantified with mouse IgA ELISA development kit 3865-1AD-6 and mouse IgG ELISA development kit 3825-1AD-6 from Mabtech, Inc. (Cincinnati, OH). Briefly, Immulon 2HB ELISA flat bottom plates (ThermoScientific, Waltham, MA) were coated overnight at 4°C with 100 µl of 1 µg/ml proprietary anti-IgG antibody or 2 µg/ml anti-IgA monoclonal antibody MT45A in 1XPBS pH7.4. The plates were then washed twice with 200 µl 1XPBS per well and blocked with 200 µl of incubation buffer (0.1% BSA 0.05% tween²⁰ 1XPBS)

per well for 1 hour at room temperature. After 6 washes in 0.05% tween²⁰ 1XPBS, 100 µl of diluted serum and fecal filtrate samples or of standards prepared by serial dilution of 50 µg/ml reconstituted stock solution of Mabtech IgG or IgA to 500, 100, 50, 10, 5, 1, 0.5, 0.1 ng/ml and additional 0.05 ng/ml of IgA were applied to the appropriate wells. The plates were incubated for 2 hours at room temperature. Wells were washed as described above before 100 µl of a 1:1,000 dilution of alkaline phosphatase (ALP)-conjugated anti-IgA MT39A or proprietary anti-IgG was added to each well and the plates incubated for another hour at room temperature. The plates were again washed and incubated in 100 µl of p-Nitrophenyl Phosphate in diethanolamine buffer (pNPP) alkaline phosphatase substrate (Mabtech cat#3652-P10) per well for 1 hour at room temperature while shaking. O.D. 405nm was measured with a Versa max microplate reader (Molecular Devices, Sunnyvale, CA). Data was acquired with SoftMax® Pro 4.8 software (Molecular Devices). Calibration curves were generated with GraphPad Prism7 (GraphPad Software, San Diego, CA) and sample concentrations were interpolated from the calibration curves. Neat concentrations were back calculated based on the dilution factors applied to ensure the solutions tested would fall within the standard dilution range as well as to account for the processing of dry fecal matter into filtrate solutions. Serum IgG concentrations were reported in µg/ml; fecal IgG and IgA concentrations were reported in µg/mg feces as per field literature practices (202; 229; 274).

BIO-PLEX BEAD-BASED MULTIPLEX ASSAY

Coupling of purified henipavirus sF and sG glycoproteins to Bio-Plex Pro™

Magnetic COOH Beads

Henipavirus sF and sG glycoproteins were coupled to Bio-Plex Pro™ Magnetic COOH Beads according to a two-step amine coupling procedure based on the coupling kit instruction manual from Bio-Rad (Hercules, CA). Briefly, uncoupled bead stocks (1.25×10^7 beads/ml) of each bead number were thoroughly vortexed and sonicated before 100 µl of each bead stock were transferred to 1.5 ml tubes, washed 3 times with 100 µl of 0.05% Tween®²⁰ (Millipore Sigma, Darmstadt, Germany) in calcium-free magnesium-free 1XPBS (Corning, NY) (PBSA-T). A magnetic separator rack was used to facilitate removal of the washes by pipetting without substantial loss of beads. Beads were then washed twice in 200 µl of activation buffer (10% PBS pH6.2) per tube for 1 minute while shaking at 900rpm on a Labnet Orbit P2 shaker (Corning Inc, Corning, NY) before being incubated in 80 µl of activation buffer per tube for 3 minutes as described above. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) and Sulfo-NHS (N-hydroxysulfosuccinimide) powders (ThermoScientific, Waltham, MA) were re-suspended to one-time use 50 mg/ml solutions in activation buffer. Beads were activated by addition of 10 µl of each EDC and Sulfo-NHS 50 mg/ml solutions and incubation for 20 minutes, shaking as described above. The activation mixture was removed as tubes were placed on the magnetic separator. Beads were incubated for 2 hours while shaking in 100 µl of 0.3 µg of the corresponding glycoprotein in PBSA (30 µg protein/ 1.25×10^6 beads/tube). The supernatant was removed from the coupled beads on the magnetic separator. The coupled beads were washed twice in 200 µl PBSA-T as described above. Beads were collected in a total of 300 µl of storage buffer (1%BSA 1XPBSA 0.05% sodium azide 1X protease inhibitor) applied 100 µl at the time, shaken for 1 minute as described above before collection. The final bead concentration is expected to approach

~4x10⁶ beads/ml. Coupled beads were stored at 4°C and protected from light. Each polystyrene bead type, which is assigned an identifying number, is infused with two different fluorescent dyes present at different concentrations in the bead, resulting in one hundred bead types available for purchase from Bio-Rad (Hercules, CA). Each bead type, therefore, has its own specific emission profile signature. The different bead numbers assigned to the glycoproteins used in this work are listed in **table 2-1**. Successful glycoprotein-bead coupling was confirmed by testing each bead for detection of its specific coupled glycoprotein by 50 and 500 ng/well of purified rabbit polyclonal IgG antibody raised against the corresponding glycoprotein of interest (see Bio-Plex200 serology assay protocol below). Beads were considered to have passed quality control when saturation levels were reached at 29,000 to 30,000 MFI (data not shown).

Table 2-1: Bio-Plex Pro™ Magnetic COOH Beads number assignment to henipavirus sF and sG glycoproteins.

Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein		Mag Bead No.	
Marburgviruses						
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	sGP _(1,2)		37	
Henipaviruses						
					sF	sG
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sF	sG	36	46
Nipah virus/H. sapiens/MYS/2000	NiV	NC_002728.1	sF	sG	26	43
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sF	sG	55	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sF	sG	27	29
Ghana virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sF	sG	63	35
Rubulaviruses						
Menangle virus/S. domesticus/AUS/2001	MenV	NC_007620.1	sHN		28	
No virus glycoprotein, background control (mock-prep)						
					62	

Multiplex microsphere immunoassay (Figure 2-3)

Bio-Plex Pro™ Magnetic COOH Beads (Bio-Rad, Hercules, CA) coupled to recombinant glycoproteins (**Table 2-1**) were vortexed for 30 seconds at speed 7, then sonicated for 1 minute before being vortexed for another 30 seconds. A master bead mixture was prepared by combining 1 µl per well ($\sim 4 \times 10^4$ beads) of each coupled bead stock numbers tested in a given panel experiment in PBSA; 100 µl of bead master mix per well was dispensed in a black Bio-Plex Pro™ flat bottom plate (Bio-Rad, Hercules, CA). The beads were washed with PBSA-T with a Bio-Plex Pro™ wash station (Bio-Rad, Hercules, CA), and 100 µl of murine antibodies of various dilutions in PBSA were applied to each well. For quality control testing of freshly coupled beads, glycoprotein-specific purified rabbit polyclonal IgG antibody was applied at 50 and 500 ng/well in PBSA. A PBSA-T alone plate negative control was included in each experiment. Plates wrapped in aluminum foil were incubated with antibodies for 1 hour at room temperature shaking at 900rpm on a Labnet Orbit P2 shaker (Corning Inc, Corning, NY), then washed with a Bio-Plex Pro™ wash station. Biotin conjugated mouse polyclonal anti-IgG (H+L) (ThermoFischer Scientific, cat#31800, Waltham, MA) or anti-IgA (H) (SouthernBiotech, cat#1040-08, Birmingham, AL) were applied at 1:500 and 1:250 dilutions in PBSA-T (100 µl/well), respectively. A 1:1 mixture of Pierce™ recombinant biotinylated protein A/G (ThermoFischer Scientific, Waltham, MA) at a 1:500 dilution (200 ng G/, 200 ng A/well) was added to bead quality control wells instead. Plates were incubated for 45 minutes as described above. After being washed, the beads were shaken as described above in 100 µl of a 1:1,000 dilution in PBSA (100 ng/well) of streptavidin-R-phycoerythrin (SA-PE) (Qiagen, Hilden, Germany) for 30 minutes. Plates were again washed, and the beads were re-suspended in 100 µl of PBSA. Plates were shaken for at

least 5 minutes before they were analyzed by a Bio-Plex® 200 array reader with microplate platform for 96 well plate automated reading (Bio-Rad, Hercules, CA). Data was acquired with Bio-Plex Manager™ software (Bio-Rad, Hercules, CA). Separate duplicate experiments were performed in technical duplicates or triplicates.

DATA ANALYSIS

Each cell-cell fusion NanoLuc® Luciferase reporter-gene assay was performed at least three times and in technical triplicates. Relative Luminescence Units (RLUs) from the various cell mixtures tested for cell-cell fusion were normalized against background defined as RLUs from mixtures of each target cell type applied to BSR T7/5 cells transfected with pcDNA 3.1 Hygro CMV alone. Normalized values were compared to background (value of 1) for significance by applying one sample Welch's t-test.

ILLUSTRATIONS

Figures were prepared using Graphpad Prism, Adobe Illustrator and Adobe Photoshop.

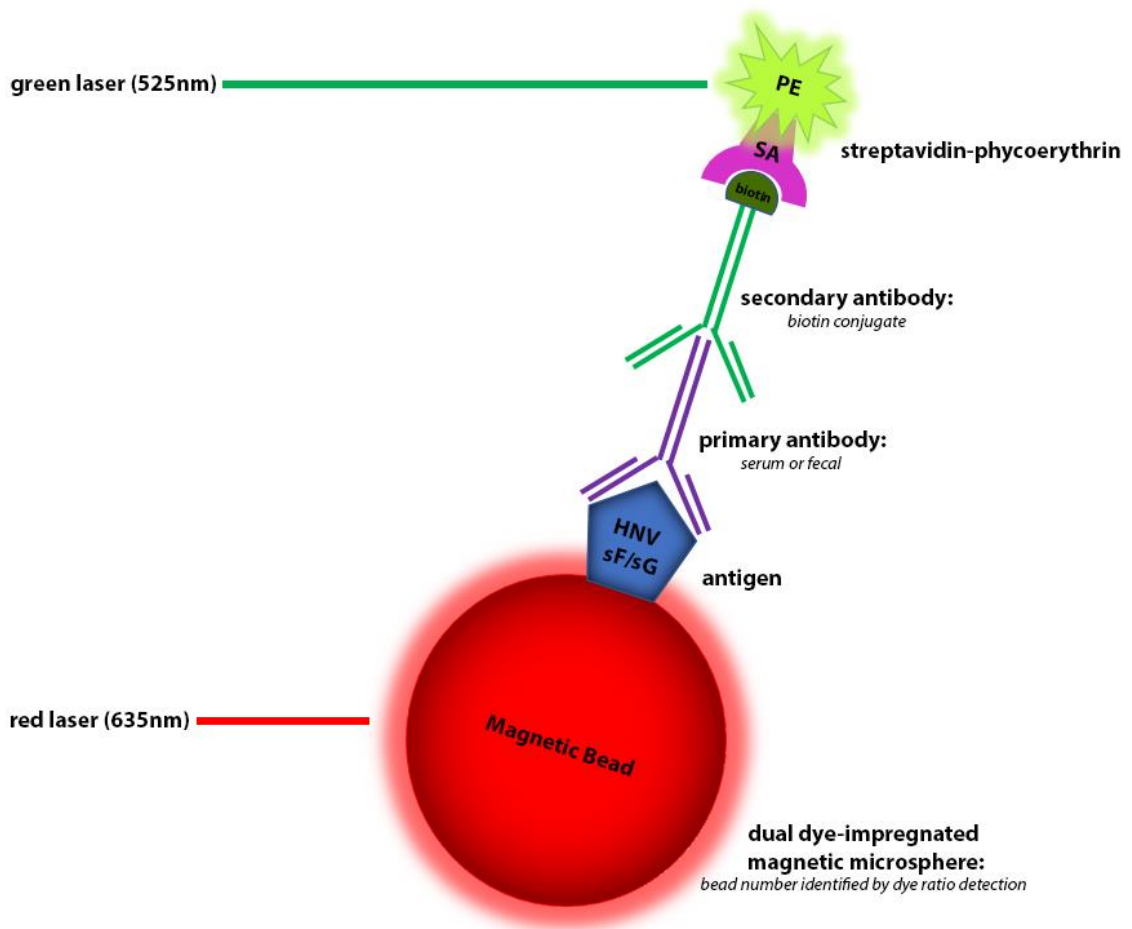


Figure 2-3: Multiplex microsphere immunoassay based on Luminex's xMAP® Technology.

Key elements are represented in the schematic to illustrate the mechanism of this assay.

CHAPTER 3: Functionality of recombinant MojV F and G glycoproteins and assessment of species and ephrin ligand tropism

INTRODUCTION

In 2012, a novel paramyxovirus, Mojiang virus (MojV) (148; 214), was detected in three out of nine rectal swabs from *Rattus flavipectus* specimens collected in an abandoned mine in the Mojiang Hani autonomous county of the Yunnan province, China (266). Additional samples were obtained from five musk shrews (*Crocidura dracula*) and 20 bats (*Rhinolophus ferrumequinum*), but tested negative for the presence of MojV (266). Wu et al. (266) performed raw read alignments using the BLASTx program and protein sequence database from the National Center for Biotechnology Information (NCBI) site, and applied MEGAN4 MetaGenome Analyzer software to conduct taxonomic parsing of the aligned reads. Their analysis led to the classification of MojV as a henipavirus based on its genomic organization (3'-N-P/V/W/C-M-F-G-L-5'), conserved henipavirus intergenic regions (CTT), close similarities of its gene start and stop sequences with henipavirus consensus sequences and a genomic length (18,404 nucleotides) within the range of what is seen in other henipaviruses (CedV: 18,162 nucleotides, HeV: 18,234 nucleotides, NiV: 18,246 nucleotides, GhV 18,530 nucleotides) (59; 76; 149; 253; 266). Phylogenetic analysis relying on the N, L, F and G gene sequences supported this classification as MojV clustered with henipaviruses (266; 282). Furthermore, MojV seem to be ancestral to the four other members of this genus and more closely related to GhV than CedV, HeV or NiV (266).

Although three miners who worked in the cave where the MojV positive samples were collected succumbed to pneumonia six months before the study took place, it is

unknown whether MojV infection was the direct cause of these unexplained cases of fatal pneumonia (266). No MojV viral isolate was recovered from the 2012 samples and the pathogenic potential of MojV remains unknown. However, sequence analysis of the MojV genome established the presence of RNA editing sites necessary for the expression of the V and W accessory proteins, which, as antagonists to the type I IFN response, are known to play a critical role in the pathogenicity of NiV and HeV infection (20; 219; 220; 266). Rescue of a recombinant MojV is therefore precluded outside of BSL-4 settings. Nevertheless, investigation of the molecular biology of MojV can still be conducted in the context of BSL-2 regulations when using isolated recombinant viral glycoproteins in experimental designs absent of infectious or replication-competent reagents. Such tools can help confirm that the sequences generated by Wu et al. (266) in their original identification of MojV encode functional viral proteins that are expressed and behave similarly to henipavirus proteins, corroborating the classification of MojV as an emerging henipavirus.

Moreover, the *henipavirus* genus includes NiV and HeV, which are highly pathogenic zoonotic viruses that can also infect a wide range of non-human mammalian species (53; 55; 57; 111; 175; 191; 206; 216; 263). Studies using recombinant MojV glycoproteins F and G, which are involved in viral entry, can shed light on the zoonotic potential and range of possible natural hosts of MojV through the investigation of its species tropism in vitro. Furthermore, host cell receptor tropism can restrict species and tissue tropism, limiting the range of natural hosts susceptible to infection to a given virus and determining the clinical presentation, and oftentimes severity, of the infection. Ephrin-B2 and -B3 ligands have been identified as entry receptors for both HeV and NiV

(27; 181; 182; 268). CedV however is characterized by a uniquely broad ephrin tropism and recognizes mouse ephrin-A1, as well as human ephrin-A2, -A5, -B1 and -B2 as entry receptors (129; 131). Though CedV G displays 29% and 30% homology with HeV G and NiV G (**Table 3-1**), respectively, it only shares ephrin-B2 as an entry receptor with HeV and NiV (131). Interestingly, G sequences from HeV and NiV are 25% and 26% homologous to the G sequence of GhV (76; 256); nevertheless, GhV G recognizes ephrin-B2 as its receptor (128; 136; 256). Sequence alignment analyses show that the amino acid percent homology of MojV G to HeV G, and NiV G is 22% and 23%, respectively (**Table 3-1**). Interestingly, MojV G is unable to mediate cell-cell fusion by utilizing the canonical ephrin-B2 and -B3 henipavirus receptors; also, MojV does not bind the morbillivirus receptor CD150 (SLAM), nor does it bear sialic acid binding residues (215). However, the ability of MojV to interact with other ephrin ligands has not been examined.

It is also possible that MojV species tropism may be defined, at least in part, by receptor restrictions. Pteropid bats have been identified as the reservoir of HeV, NiV and CedV (100; 103; 140; 149; 276), and as of now, no other natural host has been reported as a henipavirus reservoir. Importantly, the ability of CedV G to functionally interact with murine ephrin-A1 (131), the establishment of the golden or Syrian hamster (*Mesocricetus auratus*) as an experimental model for acute human HeV and NiV infection (95; 262), and the fact that MojV is the first henipavirus identified in a rodent (266), suggest the possibility that henipaviruses, as a genus, may naturally infect mammalian hosts other than bats, pigs, dogs, cats, horses and humans. Furthermore, MojV G may be able to interact with rodent-specific receptors. An examination of

alternative ephrin ligand use, as one or more entry receptors of MojV, and of a possible selective affinity for ephrin ligands of specific species, is thus warranted.

Table 3-1. Shared percent identity of F and G glycoprotein sequences of henipaviruses.

Sequence alignments were obtained and analyzed with Clone Manager Suite9 software (Scientific & Educational Software, Cary, NC).

Fusion glycoproteins

HeV-F					
HeV-F	-	NiV-F			
NiV-F	87	-	CedV-F		
CedV-F	42	43	-	GhV-F	
GhV-F	44	44	32	-	MojV-F
MojV-F	41	40	33	36	-

Attachment glycoproteins

HeV-G					
HeV-G	-	NiV-G			
NiV-G	78	-	CedV-G		
CedV-G	29	30	-	GhV-G	
GhV-G	26	25	25	-	MojV-G
MojV-G	22	23	18	19	-

AIMS AND HYPOTHESES

Aim 1: Determine MojV receptor and species tropism.

Subaim 1a: Demonstrate that the sequence data by which MojV was identified encodes functionally competent MojV F and G glycoproteins.

Underlying hypotheses:

- Recombinant MojV fusion (F) and attachment (G) glycoproteins can be expressed in mammalian cells.
- Co-expression of MojV F and G glycoproteins is required to mediate cell-cell fusion.
- MojV F and G are able to mediate cell-cell fusion in a range of mammalian cell lines.

Subaim 1b: Investigate if MojV F and G mediate cell-cell fusion in an ephrin ligand-dependent manner.

Underlying hypotheses:

- MojV G glycoprotein is able to engage in functional interactions with ephrin ligands.
- MojV G glycoprotein interactions with its cognate receptor(s) include species specific use of ephrin ligands.

RESULTS

Design and expression of recombinant Mojiang virus attachment and fusion glycoproteins

In absence of a MojV isolate and reagents (266), recombinant constructs of MojV F and G are an important tool to study the species and receptor tropism as well as the entry mechanism of this emerging novel henipavirus. Here, F and G constructs of codon optimized ORFs for expression in mammalian cells were designed and included a S-tag at the cytoplasmic end of each construct to facilitate detection (**Figure 3-1a**). S-tagged MojV F and G glycoproteins were transiently expressed in BSR-T7/5 cells to gauge similarities and differences in expression and migration patterns of these glycoproteins when compared to control S-tagged HeV and CedV F and G glycoproteins (**Figure 3-1b**). Western blot analysis of MojV F and G displayed migration patterns similar to that of HeV and CedV glycoproteins (**Figure 3-1b**) and were congruent with previous observations in the field (215). Furthermore, henipavirus F glycoproteins are known to be first expressed as a F₀ inactive precursor at the cell surface, then recycled and cleaved by the endosomal protease cathepsin L to generate a biologically active protein composed of two subunits, F₁ and F₂, linked by disulfide bonds and subsequently trafficked back to the cell surface (158; 188; 247). Indeed, MojV, HeV and CedV F display a F₀/F₁ band pattern that suggests proper cellular proteolytic processing of the F₀ precursor into the mature F₁/F₂ form of the glycoprotein (**Figure 3-1b**). Densitometry analysis established that ~50% of MojV F₀, similarly to HeV and CedV F₀, is being processed into F₁/F₂. This experimental design allowed for the detection of proteins bearing an S-tag (**Figure 3-1a**). Because the S-tag was added at the carboxyl-terminus of the F constructs, only the

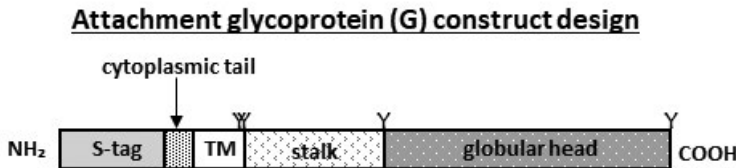
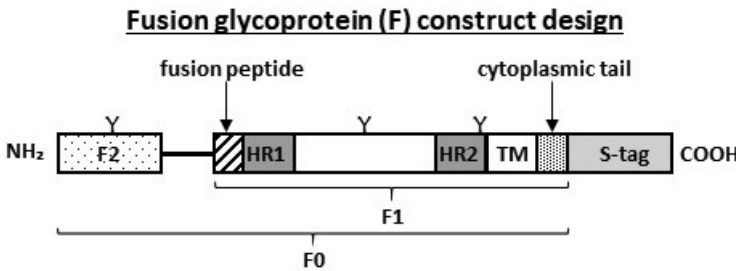
unprocessed F₀ precursor and the F₁ subunit, which retained the S-tag under reducing conditions during analysis by SDS PAGE and western blot, could be visualized in **figure 3-1b**. A marked difference in the size of monomeric MojV G compared to HeV and CedV G was observed; when expressed in BSR-T7/5 cells, MojV G seems to be ~10kDa smaller than HeV and CedV G (**Figure 3-1b**). This may be explained by the fact that MojV G bears no N-linked glycosylation sites in the β propeller domain of its globular head, whereas HeV and CedV G β propeller domains carry five and eight N-linked glycosylation modifications, respectively (131; 215; 238). N-glycosylation sites were predicted (NetNGlyc 1.0 Server, Technical University of Denmark) at positions 61, 64, 189 and 619 in MojV G. However, the recent analysis of the 166-625 region of the crystalized MojV G ectodomain by Rissanen et al. (215) did not detect any N-acetylglucosamine at N189, and a disordered C-terminus did not allow for the determination of the N-glycosylation status of N619.

Figure 3-1. Design and expression of recombinant MojV F and G glycoprotein

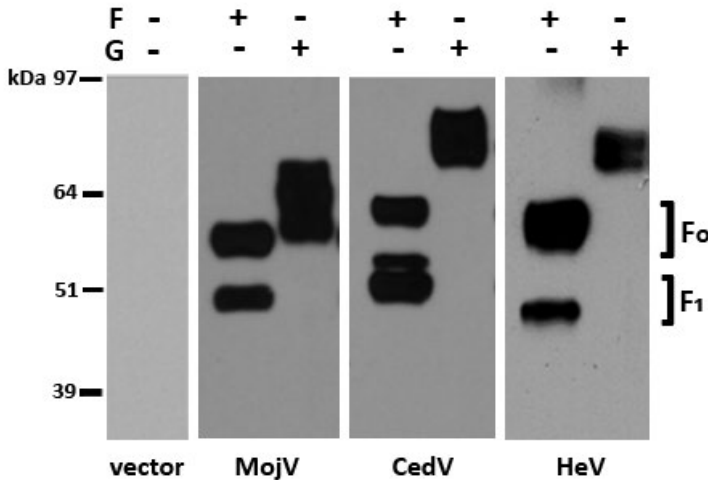
constructs.

(a) The open reading frames of MojV attachment (G) and fusion (F) glycoproteins were subcloned into a pcDNA3.1 Hygro CMV expression vector. The constructs were designed to express a S-peptide fragment at the cytoplasmic tail end of each glycoprotein, at the C-terminus of F and at the N-terminus of G. Critical functional domains of the attachment and fusion glycoproteins are featured in the schematics. Fo: fusion glycoprotein precursor. F₁, F₂: subunits of the mature fusion glycoprotein. TM: transmembrane domain. HR1: heptad repeat 1. HR2: heptad repeat 2. Y: N-glycosylation sites predicted by NetNGlyc 1.0 Server (Technical University of Denmark) at positions 69, 283 and 484 for MojV F and 61, 64, 189 and 619 for MojV G. **(b)** Transient expression of S-tagged MojV, CedV and HeV attachment and fusion glycoproteins in BSR-T7/5 cells. Cell lysates were precipitated with anti-S agarose beads 48h post transfection. Western blot analysis was performed, and the membranes were probed with rabbit anti-S:HRP antibodies (1:12,500).

(a)



(b)



Functional assessment of recombinant MojV F and G glycoproteins

Next, functional similarities that MojV F and G are expected to share with HeV and CedV F and G in mediating virion-cell membrane fusion and target cell entry were explored. A hallmark of cytopathic effects (CPE) caused by paramyxovirus infections is the formation of multi-nucleated giant cells, syncytia (reviewed in (87; 263)). This phenomenon is the result of membrane fusion between infected cells that express viral attachment and fusion glycoproteins at their surface and neighbor cells that express cognate receptors of the attachment glycoprotein (87; 263). Evidence of syncytia formation can be qualitatively evaluated by direct visualization. The ability of MojV F and G glycoproteins to cause syncytia formation was assessed by co-transfecting untagged MojV F and G expression vectors in subconfluent BSR-T7/5 cell monolayers. Cells were co-transfected with untagged HeV or CedV F and G expression vectors as a positive control, while cells transfected with pcDNA3.1 Hygro CMV were used as a negative control (**Figure 3-2a**). Untagged rather than S-tagged constructs were chosen for this experiment to avoid possible tag interactions that would interfere with the results obtained, and to create experimental conditions recapitulating accurate cell-cell fusion events as much as possible, despite the necessity of using recombinant constructs. To confirm that co-expression of F and G glycoproteins is required for the successful completion of fusion events, each glycoprotein construct was also transfected by itself with the expectation that no fusion event would occur in these cells, and therefore no syncytia would be observed (**Figure 3-2a**). Interestingly, while syncytia rapidly developed and spread through cultures co-expressing HeV F and G or CedV F and G within 24h post-transfection, syncytia in cells co-expressing MojV F and G developed at a much slower pace, were less extensive and were only observed 5 days post-transfection

(**Figure 3-2a**). The functionality of MojV F and G was confirmed by repeating this experiment in BHK-21 cells (**Figure 3-2b**). The kinetics of syncytia formation in BHK-21 cells co-transfected with MojV F and G differed from those observed in BSRT7/5; syncytia were detected 3 days post-co-transfection in BHK-21 cells (**Figure 3-2b**). MojV F and G mediated cell-cell fusion was observed under pH-independent conditions, as seen for the HeV and CedV F and G controls here (**Figure 3-2**) and shown for other henipavirus F and G glycoproteins in previous work (5; 35; 36).

Additionally, when this experiment was conducted using cell lines such as HEK293T or Vero cells (data not shown), which are known to be susceptible to HeV, NiV (35) and CedV (unpublished data) F and G mediated cell-cell fusion, no syncytia formation mediated by MojV F and G was detected. It was surprising that co-expression of MojV F and G did not result in readily visible syncytia in HEK293T and Vero cells. To further confirm that MojV F and G mediated cell-cell fusion did not depend on acidic pH, and that pH dependence was not the reason why syncytia was not visualized in these cells, pH pulse experiments using pH 5, 6 and 7.4 were conducted in Vero cells co-transfected with CedV or MojV F and G, or ABLV G (data not shown). The pH-independent control (CedV F and G) and pH-dependent control (ABLV G) behaved as anticipated, but no syncytia was reported in Vero cells co-expressing MojV F and G at any of the three pH conditions tested (data not shown). Overall, these data establish that the sequences reported by Wu et al. (266) encode functional MojV F and G, and that the recombinant constructs designed here can mediate cell-cell fusion with rodent cells.

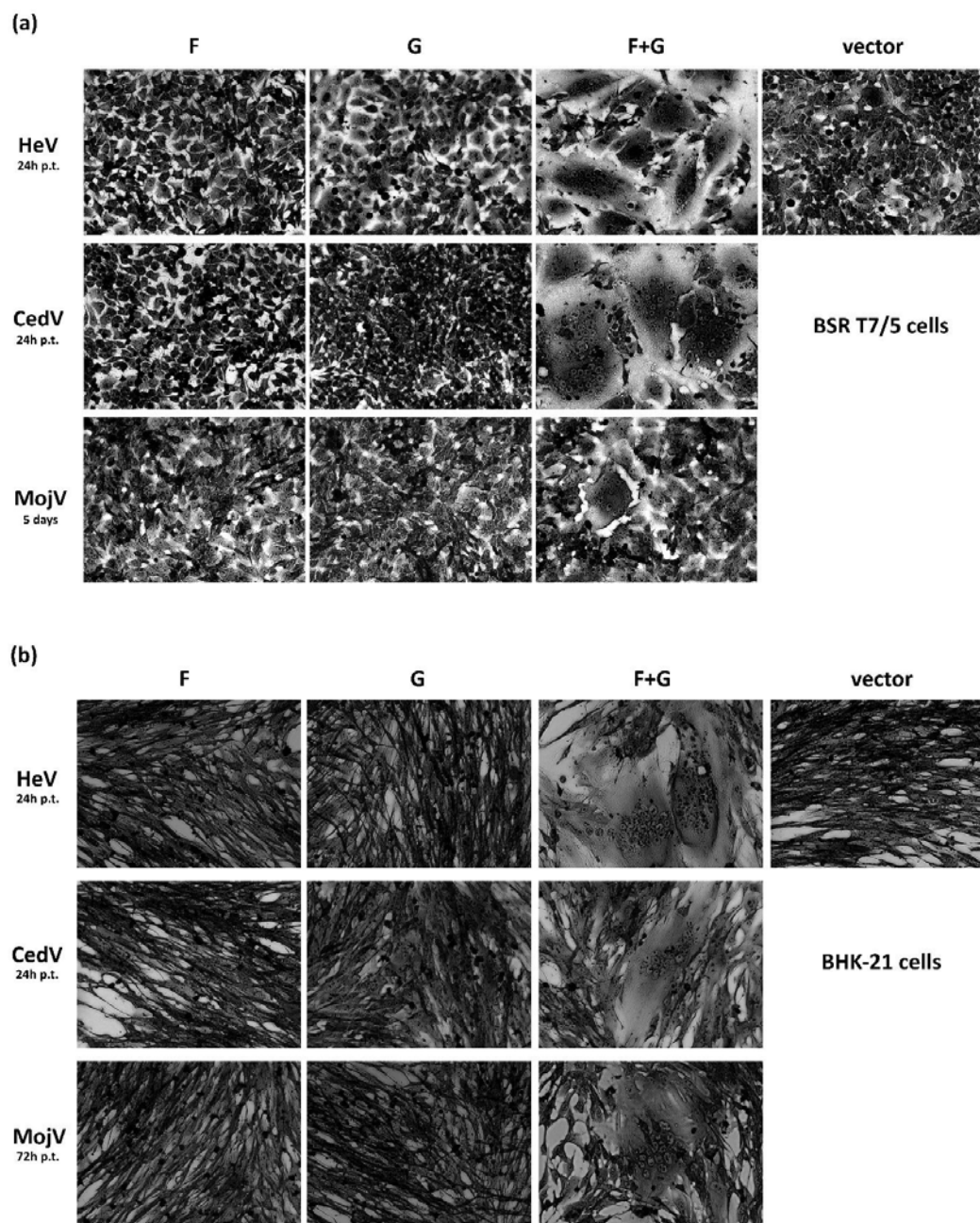


Figure 3-2. MojV F and G are functional and mediate cell-cell fusion as evidenced by syncytial formation.

(a) BSR T7/5 and (b) BHK-21 cells were transfected with pcDNA 3.1 Hygro CMV henipavirus F or G alone, or co-transfected with henipavirus F and G, or with empty vector. Upon observation of giant multinucleated cell formations (syncytia) the cells were fixed in methanol and stained with crystal violet. Images were obtained with a Zeiss Axio Observer A1 inverted microscope with a 20X objective. p.t.: post-transfection.

Species susceptibility to MojV F and G mediated membrane fusion

To assess low levels of cell-cell fusion that may not be detectable by microscopy and explore the species tropism of MojV F and G mediated fusion, an alternative strategy was explored using reporter gene systems. First, a vaccinia-based T7 polymerase/*lacZ* reporter-gene cell-cell fusion assay that has been applied to numerous studies on henipaviral glycoproteins in our lab in the past (29) was employed to quantify cell-cell fusion. However, the experimental design inherent to this assay did not appropriately address the slower kinetics of MojV F and G mediated cell-cell fusion compared to that of other henipavirus F and G, and the background levels generated by the T7/ β -galactosidase system masked the low levels of fusion from MojV F and G interaction with target cells (data not shown).

Next, in order to improve sensitivity, a quantitative cell-cell fusion Nano luciferase reporter-gene assay was developed. Nano luciferase was engineered from the subunit of an enzyme found in *Oplophorus gracilirostris*, a deep-sea shrimp by Hall et al., who also co-optimized its substrate (furimazine) (98). At 19 kDa, this novel luciferase is similar in size to the 20 kDa *Gaussia* luciferase, but smaller than the 36 kDa *Renilla* and 61 kDa firefly luciferases, making it a good candidate as a reporter gene (98). Nano luciferase has been shown to be more stable, remaining active in 37°C cultures for over 15h, and to generate 150-fold increased luminescence compared to firefly and *Renilla* luciferases. This luciferase thus provided a highly sensitive alternative applicable to the challenges posed by the unusual kinetics and lack of robustness in MojV F and G mediated membrane fusion (81; 98). The NLuc gene was codon optimized for expression in mammalian cells and cloned into a pTM1 vector to ensure the expression of Nano luciferase would be under the control of a T7 promoter. Effector BSR-T7/5 cells

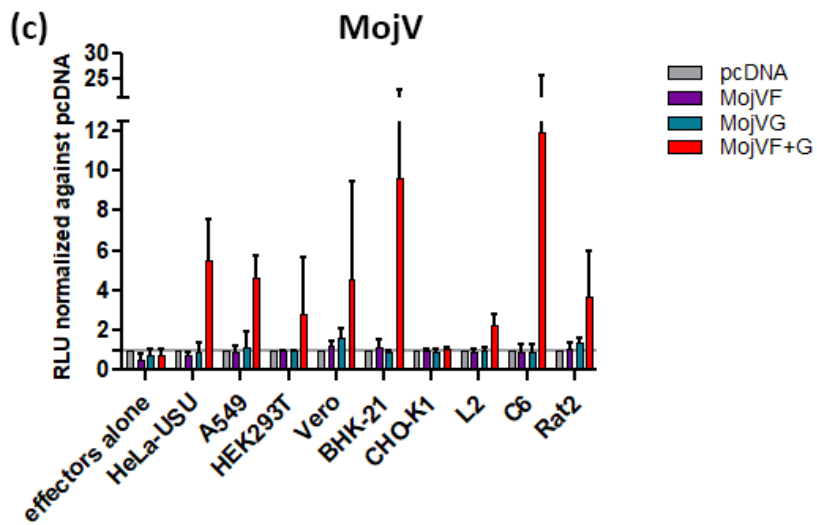
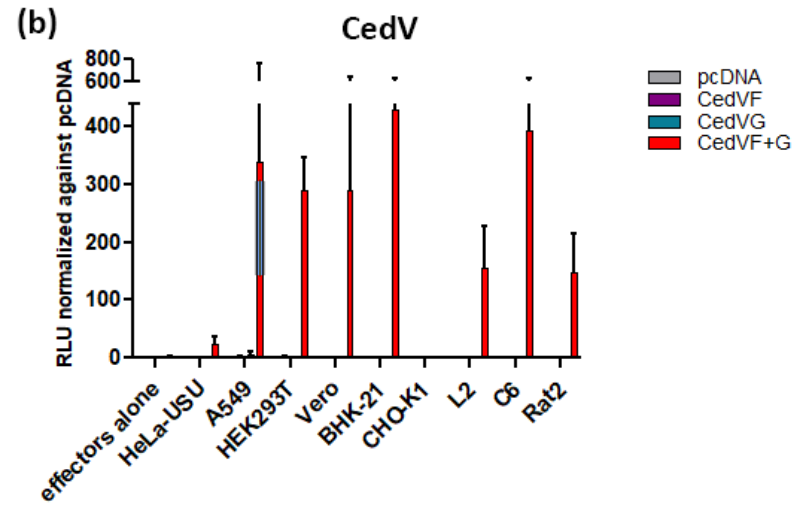
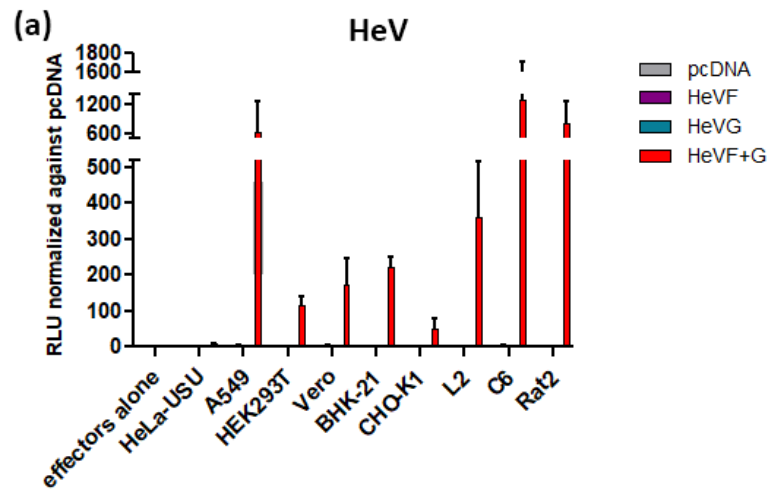
constitutively expressing T7 polymerase and transiently expressing untagged HeV, CedV or MojV F or G glycoproteins alone, or F and G glycoproteins together, were incubated with target cells transfected with the reporter pTM1-NLuc plasmid. Upon cell-cell fusion between effector and target cells the T7 polymerase can gain access to the T7 promoter in the target cells, and initiate expression of the Nano luciferase. The various effector/target cells co-cultures tested were collected at different timepoints; substrate was applied to cell lysates and the maximum luminescence was reported for each mixture combination.

First, the susceptibility of mammalian cells to MojV F and G mediated cell fusion was investigated by testing human (HeLa-USU, A549, HEK293T), non-human primate (Vero) and rodent (BHK-21, CHO-K1, L2 rat, C6 rat, Rat2) cells as targets (**Figure 3-3**). Human lung epithelial (A549), human embryonic kidney (HEK293T), African green monkey kidney (Vero), baby hamster kidney (BHK-21), rat lung epithelial (L2), and rat glioma (C6) cells, as well as rat fibroblasts (Rat2), were permissive to both HeV and CedV F and G mediated cell-cell fusion (**Figure 3-3a, b**). In agreement with findings by Rissanen et al. (215), human (A549, HEK293T) and rodent (BHK-21) cells were found to be susceptible to MojV F and G mediated cell-cell fusion. The positive results seen in this assay with BHK-21 cells were also congruent with the syncytia visualized by microscopy (**Figure 3-2**). Further, the Nano luciferase assay established that MojV F and G can mediate varying levels of cell-cell fusion with additional human (HeLa-USU), non-human primate (Vero) and rat cells (L2, C6, Rat2) (**Figure 3-3c**). Interestingly, the highest levels of fusion recorded were for C6 rat brain cells (**Figure 3-3c**). However, similarly to what was observed in the qualitative investigation of MojV F and G functionality (**Figure 3-2**), the Nano luciferase reporter-gene assay revealed that

maximum luminescence levels were reached much faster in cultures containing HeV or CedV F and G expressing effector cells (within 48h) compared to cultures containing effectors expressing MojV F and G (within 4 to 6 days depending on the type of cells) (**Figure 3-3**). Additionally, levels of MojV F and G mediated fusion were less robust than those obtained with HeV or CedV F and G (**Figure 3-3**), which correlated with the less extensive syncytial formations observed by microscopy (**Figure 3-2**).

Figure 3-3. MojV F and G can mediate cell-cell fusion with human, non-human primate and rodent cells in a quantitative Nano luciferase reporter-gene cell-cell fusion assay.

BSRT7/5 effector cells that constitutively express T7 polymerase were transfected with empty vector, henipavirus F alone, henipavirus G alone, or co-transfected with henipavirus F and G. Human, non-human primate and rodent target cells transfected with the Nano luciferase gene under the control of the T7 promoter were incubated with HeV **(a)**, CedV **(b)**, or MojV **(c)** effector cells. Cell-cell fusion events were indirectly measured by recording maximum relative luminescence unit (RLU) upon application of Nano-Glo® substrate. Maximum RLUs were recorded 48h post-application of target cells to effector monolayers co-expressing HeV or CedV F and G constructs. Effector cells co-transfected with MojV F and G constructs yielded maximum RLUs on day 4 with C6 and Rat2 cells, day 5 with HeLa-USU, A549, HEK293T and L2 cells, and day 6 with Vero and BHK-21 cells. Maximum RLUs normalized against values obtained from target cells co-incubated with effector cells transfected with empty vector are reported here. The grey line represents the normalized reference level of 1 (background). Experiments were repeated at least three times in technical triplicates.



Investigation of protein/protein interactions between MojV G and ephrin ligands

HeV and NiV G recognize ephrin-B2 and -B3 ligands as their entry receptors, whereas GhV G is restricted to ephrin-B2 alone. Conversely, CedV G displays a broad ephrin ligand tropism, which includes mouse ephrin-A1, as well as human ephrin-A2, -A5, -B1 and B2 (129; 131); yet, MojV G does not recognize ephrin-B2 nor -B3 as its receptor (215). Considering that MojV was identified through the sequencing of *Rattus flavipectus* samples (266), and that CedV G can utilize a rodent A-class ephrin ligand as a receptor (131), the possibility that MojV G may recognize rodent and human ephrin ligands other than ephrin-B2 and -B3 was investigated next. First, co-precipitation assays were performed to assess protein/protein interactions between MojV G and a panel of A- and B-class ephrin ligands. To do so, a soluble form of MojV G (MojV sG) was generated to bear a S-tag for detection and include a GCN4 tetramerization motif (MKQIEDKLEEEIESKLKKIENELARIKK) to promote the oligomerization of the secreted protein into the predicted tetrameric structure that recapitulates the native form of the protein as it would be expressed at the surface of an infected cell (**Figure 3-4a, b**). To generate a cell line stably expressing MojV sG, transient expression of the protein was first evaluated in HEK293FS cells, which are routinely used in our lab to stably generate high yield production of other henipaviral sG proteins, by western blot analysis. MojV sG expression in these cells resulted in a cleaved product (data not shown). Additional cell lines including HEK293T, HeLa-ATCC, HeLa-USU, L2 mouse fibroblasts and Vero cells yielded similar products (data not shown). However, BHK-21, L2 rat lung, NIH3T3 and Neuro2a cells were identified as adequate candidates and produced intact MojV sG proteins. Nevertheless, only Neuro2a cells were successfully isolated as single clone

colonies during the limiting dilution process by Lianying Yan, who then established this cell line as a stable expressor of MojV sG under drug selection and carried out large scale protein production and purification. Next, the oligomerization status of MojV sG was confirmed in a BS3 cross-linking assay (**Figure 3-4c**). Soluble CedV G (CedV sG) and GhV G (GhV sG) glycoproteins were included as controls in the cross-linking assays and subsequent co-precipitation assays (**Figure 3-4c**). Similar to what was observed with the expression of full-length G constructs (**Figure 3-1b**), the smaller size of MojV sG in its monomeric and consequently in its tetrameric forms (**Figure 3-4c**) may be explained by the lack of confirmed N-linked glycosylation modifications present in its β propeller domain compared to CedV sG and GhV sG, which bear eight and four N-linked glycosylation sites, respectively.

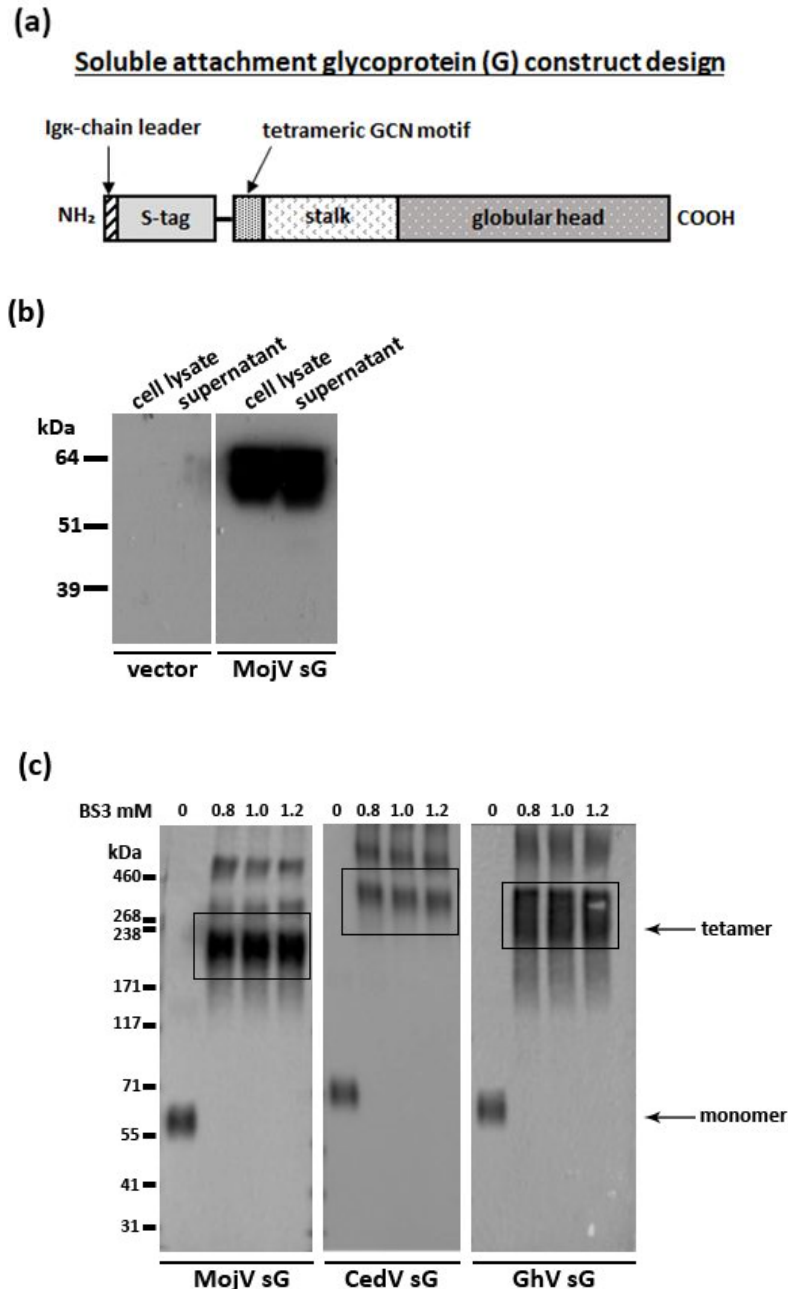


Figure 3-4. Design, expression and oligomerization status of MojV sG.

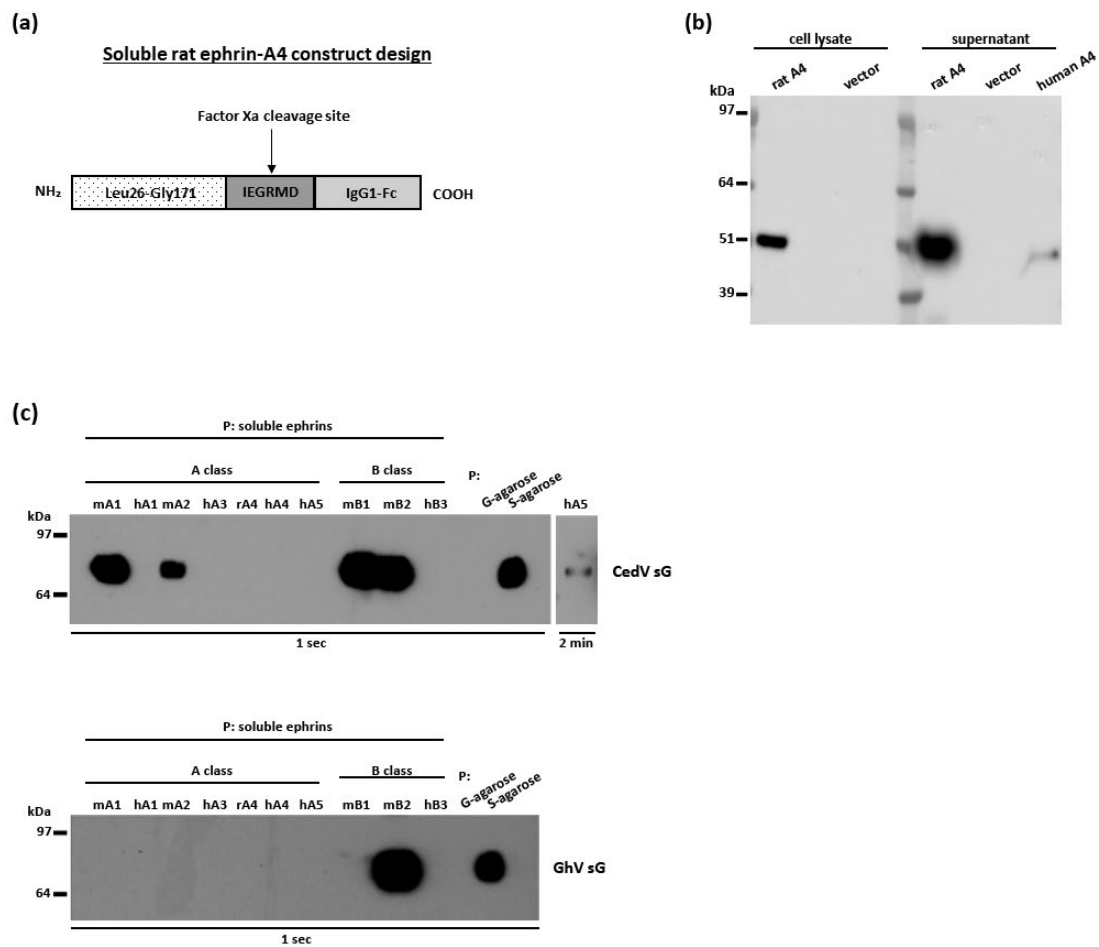
(a) Constructs of recombinant MojV, CedV and GhV soluble attachment (sG) glycoproteins were designed to include a GCN4 motif to allow tetramerization of the proteins. (b) Transient expression of recombinant MojV sG glycoprotein was confirmed in L2 rat cells. Cell lysate were precipitated 48h post transfection with S agarose beads and analyzed by SDS PAGE and western blot. (c) Tetrameric oligomerization of MojV, CedV and GhV sG glycoproteins was tested by treating purified MojV, CedV and GhV sG glycoproteins with cross-linking reagent BS3. The oligomers (50ng) were analyzed by SDS PAGE followed by western blot under reducing conditions.

Protein/protein interactions between ephrin ligands and henipavirus sG glycoproteins were evaluated in co-precipitation experiments. The panel of purified soluble Fc-tagged ephrin ligands tested here was commercially obtained from R&D systems (Minneapolis, MN) and included mouse ephrin-A1, mouse ephrin-A2, human ephrin-A3, human ephrin-A4, human ephrin-A5, mouse ephrin-B1, mouse ephrin-B2 and human ephrin-B3, in addition to human ephrin-A1 (provided by Kai Xu, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Sequence analysis of the G-H loop of human and rat ephrin ligands revealed a critical difference in a key G-binding residue in ephrin-A4 (further discussed below), but no Fc-tagged rat ephrin-A4 ligand was commercially available. Therefore, a soluble construct comprising the ectodomain of rat ephrin-A4 was designed and its expression was confirmed by western blot analysis (**Figure 3-5a, b**). The cell culture supernatant containing the product was then purified by Lianying Yan, and the protein was integrated in the panel of Fc-tagged soluble ephrin ligands tested. As expected, results from the co-precipitation assay (**Figure 3-5c**) correlated with the formerly observed robust cell-cell fusion mediated by CedV F and G with mouse ephrin-A1, human ephrin-A2, -A5, -B1 and -B2 (**Figure 3-6a**) (131). GhV sG co-precipitated with ephrin-B2 alone (**Figure 3-5c**) in agreement with previous work identifying ephrin-B2 as the cognate receptor of GhV G (128; 136; 256). However, no interactions between MojV sG and any of the ephrin ligands examined here were captured in the context of this co-precipitation assay. In addition, during the preparation of this manuscript, similar results were obtained by Ha Dang (Veesler lab, University of Washington, Seattle, WA) using Bio-Layer Interferometry (BLI) technology, which offers a more sensitive

assessment of protein/protein interactions, and could not detect any binding of MojV sG to mouse ephrin-A1, rat ephrin-A4, human ephrin-A4, -A5, or -B2 (data not shown).

Figure 3-5. Soluble rat ephrin-A4-Fc construct design and expression and henipavirus soluble G/ephrin ligand panel co-precipitation assay.

(a) A construct to express recombinant rat soluble ephrin-A4 protein was designed with a Fc tag at its carboxylic end. Clones were confirmed by sequencing (GeneWiz, Frederick, MD). (b) Transient expression of recombinant rat soluble ephrin-A4-Fc protein was tested in BSRT7/5 cells. Human soluble ephrin-A4-Fc (R&D systems, Minneapolis, MN) was included as a control. Cell lysate were precipitated 48h post transfection with G agarose beads and analyzed by SDS PAGE and western blot using HRP conjugated-anti-human IgG (1:12,500). (c) A panel of soluble Fc-tagged A-class and B-class ephrin ligands including mouse ephrin-A1, human ephrin-A1, mouse ephrin-A2, human ephrin-A3, rat ephrin-A4, human ephrin A4, human ephrin-A5, mouse ephrin-B1, mouse ephrin-B2 and human ephrin-B3, was co-precipitated with CedV, GhV or MojV sG glycoproteins and G-agarose beads. MojV, CedV or GhV sG incubated with G-agarose beads or S-agarose beads in absence of ephrins were included as controls. Western blot analysis was performed to assess the interaction of MojV, CedV and GhV sG with each ephrin. The blots were probed with polyclonal anti-S:HRP antibody. As previously shown by Laing et al. (129; 131), CedV sG was found to bind mouse ephrin-A1 and -A2, human ephrin-A5, as well as mouse ephrin-B1 and -B2. GhV sG only interacted with ephrin-B2 in agreement with the current literature (128; 136; 256). However, no MojV sG/ephrin-Fc interactions were detected in this experiment (negative data not shown).



MojV F and G mediate detectable cell-cell fusion with A-class ephrin ligands.

Because such low levels of MojV F and G mediated fusion were recorded in the syncytia (**Figure 3-2**) and Nano luciferase assays (**Figure 3-3c**), one cannot exclude the possibility that weak or transient protein/protein interactions between MojV G and ephrin ligands may not have been captured in the co-precipitation assay, but may still occur and result in successful fusion events. Therefore, we pursued a more sensitive method to detect such interactions in Nano luciferase cell-cell fusion assays with BSR-T7/5 effectors co-expressing untagged MojV F and G and target cells expressing one ephrin ligand at the time (**Figure 3-6**). CHO-K1 cells were chosen as target cells because they proved to be non-susceptible to both MojV and CedV F and G mediated cell-cell fusion in this study (**Figure 3-3b, c**). Sequence alignment analyses of the G-H loop of all mouse and human ephrin ligands revealed differences only between mouse and human ephrin-A1 (131), and ephrin-A4 (**Figure 3-7a**). Previous work showed that a single substitution of T₁₁₅ in human ephrin-A1, a nucleophilic residue at a position critical for CedV G binding, with I₁₁₅ in mouse ephrin-A1, a hydrophobic residue, was responsible for the fusion phenotype observed for CedV G with mouse ephrin-A1 (131). Differences in residues occupying two G-H loop positions important for binding to henipavirus G glycoproteins were identified when comparing mouse to human ephrin-A4 sequences (**Figure 3-7a**). On the one hand, human ephrin-A4 F₁₁₅ is replaced by Y₁₁₈ in mouse ephrin-A4. However, both are aromatic residues; consequently, this substitution would presumably not significantly impact binding affinity with henipavirus G glycoproteins. On the other hand, nucleophilic residue S₁₂₂ in human ephrin-A4 is replaced by hydrophobic residue P₁₁₉ in mouse ephrin-A4. Importantly, the only difference noted

between critical residues of human and rat G-H loop sequences across all eight ephrin ligands was for the same P residue. Therefore, mouse ephrin-A1 and rat ephrin-A4 were included in the otherwise all human ephrin ligand panel tested in the Nano luciferase cell-cell fusion assays (**Figure 3-6**). CedV F and G were used as controls for this experiment (**Figure 3-6a**), and yielded data congruent with our earlier findings (131). Unexpectedly, MojV F and G mediated cell-cell fusion was detected for target cells expressing rat ephrin-A4, human ephrin-A5 and mouse ephrin-A1, albeit at much lower levels (**Figure 3-6b**). Nevertheless, MojV F and G mediated fusion was again overall less robust and slower to develop than what was observed with CedV F and G (**Figure 3-6**).

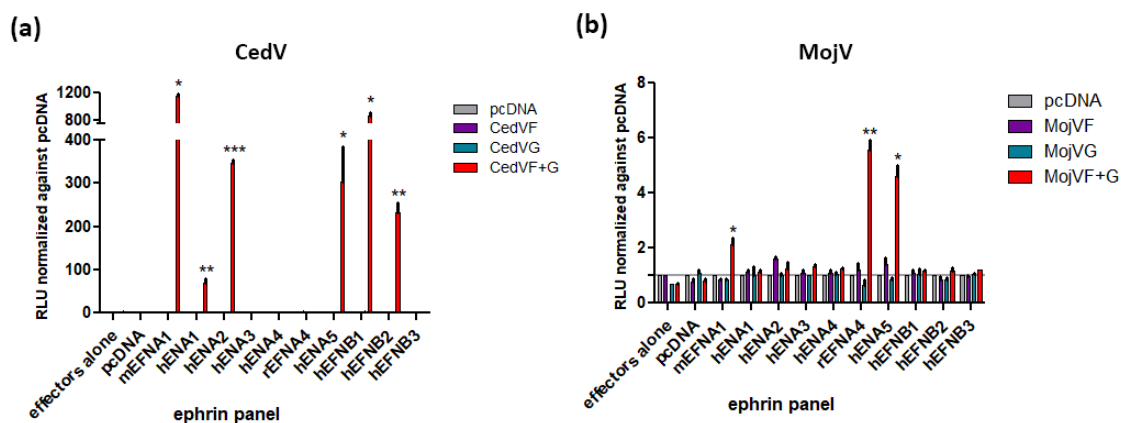


Figure 3-6. MojV F and G mediate weak cell-cell fusion with human and rodent A-class ephrin ligands.

Cell-cell fusion reporter-gene assays were performed as described in figure 3-3 with the following modifications. CHO-K1 target cells were co-transfected with pTM1-Nluc and one ephrin-expressing plasmid at the time or an empty vector. Cell mixtures of effector and target cells were assayed for Nano luciferase activity. **(a)** CedV F and G or **(b)** MojV F and G effector cells were tested for their ability to mediate cell-cell fusion against a panel of target cells transiently expressing human and rodent ephrin ligands. Maximum RLUs were recorded 48h post-application of target cells to effector monolayers co-expressing CedV F and G constructs. Co-cultures containing effector cells co-transfected with MojV F and G constructs yielded maximum RLUs on day 6. The grey line represents the normalized reference level of 1 (background). The experiments were performed at least 3 times in technical triplicates.

The contribution of rat ephrin-A4 to MojV F and G mediated fusion is not determined by the G-H loop residue P₁₂₂.

To further investigate the putative contribution of rat ephrin-A4 residue P₁₂₂ to functional interactions with MojV G, site-directed PCR mutagenesis was used to generate a mutant human ephrin-A4 mimicking the rat ephrin-A4 G-H loop (hEFNA4 S119P) expression plasmid, and the converse (rEFNA4 P122S) (**Figure 3-7b**). MojV F and G mediated fusion was assessed with cells expressing rat ephrin-A4 G-H loop residue P122S and human ephrin-A4 G-H loop residue S119P in Nano luciferase reporter-gene cell-cell fusion experiments (**Figure 3-7b**). Interestingly, mimicking the human G-H loop in the rat ephrin-A4 background did not abrogate fusion, and introducing the rat G-H loop in the human ephrin-A4 sequence did not restore fusion (**Figure 3-7b**). Moreover, no statistically significant difference was recorded in MojV F and G mediated fusion levels with target cells expressing rat ephrin-A4 and rat ephrin-A4 P122S (**Figure 3-7b**). Yet, attempts to co-precipitate MojV sG with soluble rat ephrin-A4-Fc purified protein (**Figure 3-5c**) were unsuccessful (data not shown).

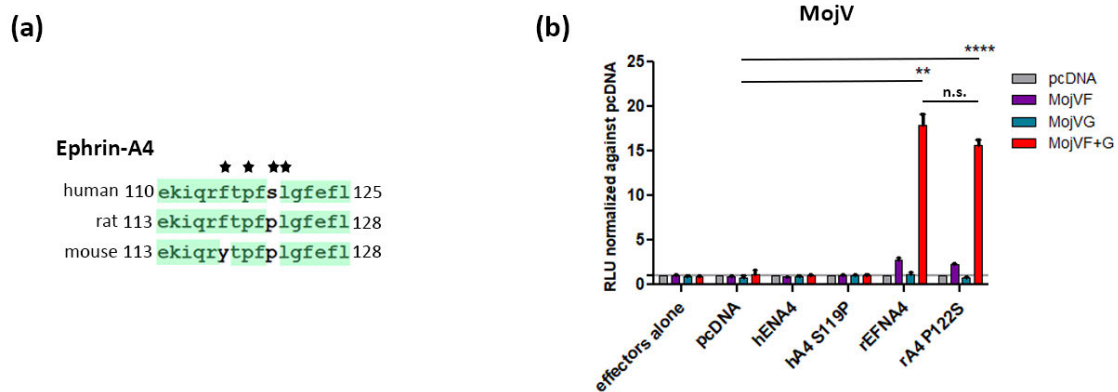


Figure 3-7. Assessment of the contribution of rat ephrin-A4 G-H loop residue P122 in MojV F and G mediated cell-cell fusion.

(a) Sequence alignment of critical residues (*) of the G-H loop of human, rat and mouse ephrin-A4 ligand. (b) Cell-cell fusion Nano luciferase assay assessing MojV F and G mediated fusion with CHO-K1 target cells transiently expressing wild type or mutant human or rat ephrin ligand A4. m: mouse ephrin ligand; h: human ephrin ligand; r: rat ephrin ligand. Maximum RLUs were recorded six days post-application of target cells to effector monolayers. The experiments were performed at least 3 times in technical triplicates. The assay background level is represented by a grey line and set to one after normalization of the data to RLU values for co-cultures of empty vector transfected targets incubated with effector cells. A representative experiment is shown here. Data was analyzed by Welch's t test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

DISCUSSION

In the absence of MojV infectious isolate, studies investigating viral entry mechanism and tropism of MojV required generating recombinant synthetic molecular tools. Here, due to a lack of means of detection for this novel henipavirus, recombinant MojV F and G glycoprotein constructs were designed to bare a S-tag along with control HeV and CedV F and G glycoprotein control constructs. Successful expression of these recombinant glycoproteins in mammalian cells revealed shared similarities in their expression and migration patterns, suggesting proper structure and folding and supporting the classification of MojV as a henipavirus (**Figure 3-1**). Yet, monomeric MojV G glycoprotein migrated at a size ~10kDa smaller than that observed for HeV and CedV G monomers by western blot analysis (**Figure 3-1**). The reason for this difference may be the number of N-linked glycosylation sites present on HeV, CedV and MojV G glycoproteins. Contrary to HeV and CedV G glycoproteins, which count five and eight N-linked glycosylation sites, in the β propeller domain of their globular head, respectively (131; 215; 238), MojV G glycoprotein bears no confirmed sites (215). Moreover, the total number of predicted N-linked glycosylation sites in the entire MojV G glycoprotein is limited to four, two of which lay at the border of the transmembrane domain whereas the other two flank each ends of the β propeller domain of MojV G globular head (215). Conversely, eight sites were observed in CedV G (131) and six of eight predicted sites are glycosylated in the HeV G glycoprotein (40).

The functionality of recombinant MojV F and G glycoproteins was assessed by syncytia experiments in rodent cell lines. To conduct these experiments tag-less constructs were generated to limit artificial interactions that may interfere with the results obtained. The data established that co-expression of MojV F and G glycoproteins is

required for cell-cell fusion to occur (**Figure 3-2**). In agreement with findings of Rissanen et al. (215), syncytia experiments also showed that BSR-T7/5 cells are susceptible to MojV F and G mediated cell-cell fusion (**Figure 3-2a**). Further, qualitative evidence of MojV F and G cell-cell fusion in BHK-21 cells by direct observation of syncytial formations (**Figure 3-2b**) was obtained and supports quantitative reporter-gene assay data recently published (215). Less extensive and fewer syncytia formed in wells containing cells co-transfected with MojV F and G, compared to cells co-transfected with HeV or CedV F and G, revealed less robust MojV F and G mediated fusion. MojV F and G fusion, in addition, became visible over three (BHK-21 cells) to five (BSRT7/5 cells) days compared to 24h for HeV and CedV F and G mediated fusion. Lower cell surface expression and/or transfection efficiency of MojV F and G compared to HeV or CedV F and G expression plasmids is most likely not a factor explaining these results, as expression assessed by western blot seems similar across all three henipavirus species (**Figure 3-1 and 4-1**). The atypical weakness of fusion and slow kinetics of MojV F and G mediated fusion, compared to HeV and CedV F and G mediated fusion, may instead be due to low cell surface density of MojV G cognate receptor(s). Defects in MojV F and/or G folding or structure which may affect their functionality, and may have resulted from inaccuracies in the MojV F and G reference sequences stemming from possible RT-PCR errors during viral nucleic acid sample processing, could also be hypothesized.

In order to investigate the species susceptibility to MojV F and G mediated membrane fusion, a highly sensitive cell-cell fusion Nano luciferase reporter-gene assay was designed to assess slow-to-develop and low levels of fusion mediated by MojV F and G that may not be detectable by microscopy. The data thus generated showed that despite

low fusion levels and slow kinetics of fusion, human (HeLa-USU, HEK293T, A549 cells), non-human primate (Vero cells) and rodent cells (BHK-21, CHO-K1, rat L2, Rat2 cells), particularly rat brain C6 cells, are susceptible to MojV F and G mediated cell-cell fusion (**Figure 3-3**). These results allude to the possibility that MojV may be able to gain entry into host cells of human, non-human primates and rodent species, suggesting a zoonotic and broader cross-species transmission potential for MojV (266).

Although HeV and NiV utilize ephrin ligands B2 and B3 as host cell entry receptors (27; 181; 182; 268), GhV G seems restricted to ephrin-B2 (128; 136; 256). Yet, CedV can recognize A-class ephrin ligands, including murine ephrin-A1, as entry receptors (131). Rissanen et al. (215) recently reported that MojV G cannot bind to ephrin ligands B2 and B3, but it was not known if interactions between other ephrin ligands and MojV G could occur. To evaluate this possibility, a S-tagged soluble tetrameric construct of MojV G was generated (**Figure 3-4**) and tested against a panel of soluble Fc-tagged ephrin ligands in co-precipitation assays. No direct lasting protein/protein interactions between MojV sG and the ephrin ligands tested were captured using this experimental design (**Figure 3-5**). This inability to detect any protein/protein binding between MojV sG and ephrin ligands was confirmed by Ha Dang during the preparation of this manuscript in an experiment using the more sensitive BLI technology. Nano luciferase reporter-gene assays were performed next to detect possible transient or weak interactions, and identified low levels of MojV F and G mediated cell-cell fusion with target cells expressing mouse ephrin-A1, rat ephrin-A4 and human ephrin-A5 (**Figure 3-6b**), while confirming data from Rissanen et al. (215) showing that MojV G does not recognize ephrin-B2 or -B3 as a ligand. MojV F and G mediated fusion was weaker and

developed through slower kinetics than what is observable in other henipavirus F and G mediated cell-cell fusion (**Figure 3-6**). These findings suggest that if ephrin ligands play a role in MojV F and G mediated cell-cell fusion, they most likely do not act as primary functional receptor(s) of MojV. The fact that MojV F and G can mediate cell-cell fusion with cells expressing rat ephrin-A4, but not human ephrin-A4, also seem to indicate a possible species-specific role of ephrin ligand in the fusion process, however limited that role may be.

Nucleophilic residue S₁₂₂ in human ephrin-A4 occupies a position in the G-H loop domain that is predicted to be critical to binding to henipavirus G glycoproteins. It is replaced by hydrophobic residue P₁₁₉ in the G-H loop of rat ephrin-A4. In order to assess the putative contribution of rat ephrin-A4 residue P₁₂₂ to a functional interaction with MojV G, expression plasmids for human ephrin-A4 mimicking the rat ephrin-A4 G-H loop (hEFNA4 S119P), and rat ephrin-A4 mimicking the human ephrin-A4 G-H loop (rEFNA4 P122S) were generated, and the ability of these mutated ephrin-A4 ligands to support MojV F and G mediated cell-cell fusion was tested in Nano luciferase assays (**Figure 3-7**). Based on the data obtained from these experiments, it can be inferred that the domains of rat ephrin-A4 that support MojV F and G mediated cell-cell fusion are most likely located outside of the rat ephrin-A4 G-H loop. The identification of such domains would require additional studies including, for example, cell-cell fusion assays testing chimeric human/rat ephrin-A4 constructs and structural analyses of possible docking sites.

Though the interaction of effector cells expressing MojV F and G with target cells expressing some A-class ephrin ligands resulted in fusion, the nature and extent of the

contribution of these ephrin ligands to the process of MojV F and G mediated fusion remains unknown. Alternative ligand usage that supports entry mechanisms and allows for enhanced productive viral infections have been reported in enteroviruses. For instance, human enterovirus coxsackievirus A21 (CAV21) utilizes intercellular adhesion molecule 1 (ICAM-1) as its receptor, but undergoes limited replication in absence of decay-accelerating factor (DAF), which closely associates with ICAM-1 at the cell surface (233). Although CAV21 is also able to bind to DAF, cell infection cannot be initiated without ICAM-1 (233). Shafren et al. (233) have thus proposed that DAF may promote higher local ICAM-1 cell surface density, allowing for consecutive high-affinity binding to ICAM-1, or may, as a low-affinity receptor, improve viral presentation. Perhaps similarly to the cooperation of ICAM-1 and DAF in the viral binding of CAV21, the co-expression of an unknown MojV G primary receptor and A-class ephrin ligands may be implicated in the viral entry mechanism of MojV. Nevertheless, taken together, the findings of the present study do not permit the clear identification of (a) primary cognate receptor(s) for MojV G; further investigations are warranted to determine what binding partner(s) expressed at the host cell surface can act as entry receptor(s) of MojV.

CHAPTER 4: Structural characterization of MojV F and G and in vitro investigation of the mechanistic competence of MojV F

INTRODUCTION

To infect host cells, henipaviruses must successfully complete specific steps mediated by the membrane-anchored tetrameric attachment (G) and the trimeric fusion (F) glycoproteins (30; 36; 38; 238; 269). The membrane fusion events between the virion and host cell allowing henipavirus cell entry require that sequential interactions and conformational changes in these glycoproteins occur in a carefully choreographed mechanism. Engagement of G by a cognate host cell surface receptor triggers F to undergo an irreversible conformational cascade that ultimately leads to virion-cell membrane fusion and delivery of the nucleocapsid into the host cell cytoplasm (reviewed in (5; 133; 135; 238)). During this induced conformational change, F transitions from a metastable pre-fusion conformation to a lower energy intermediate structure (5; 133; 135; 238). This intermediate structure, the pre-hairpin intermediate (PHI), allows for the hydrophobic fusion peptide located at the N-terminus end of the F₁ subunit of the F glycoprotein to insert itself into the target cell membrane to establish a protein bridge between the viral and host membranes (5; 133; 135; 238). At this stage, the α -helical heptad repeat domains HR1 and HR2 of F₁ are free to interact to form a six-helix bundle (6 HB) structure (5; 238; 272). This structure promotes the subsequent merging of the membranes by pulling together the viral and cellular membranes to create a fusion pore as F completes its structural rearrangement and adopts a final and irreversible post-fusion conformation (5; 133; 135; 238).

Studies exploring the functionality of recombinant MojV F and G glycoproteins, species tropism and the possible use of ephrin ligands as receptors of MojV G (see chapter 3) revealed surprisingly slow kinetics and less robust cell-cell fusion compared to what is typically observed with F and G glycoproteins of a prototypical henipavirus such as HeV or of the non-pathogenic CedV (**Figures 3-2, 3-3 and 3-6**). These findings called for further investigation into the underlying causes of the atypical MojV fusion phenotype observed. Henipavirus F and G glycoproteins are naturally expressed as trimers and tetramers, respectively, at the host cell surface. This specific quaternary assembly may be critical to support the optimal function of these glycoproteins to efficiently mediate fusion. Are the recombinant MojV F and G glycoproteins defective in their expression at the cell surface? Are they folding into canonical trimeric fusion and tetrameric attachment glycoproteins structures? In addition, mechanistic inadequacy of MojV F may also affect its functionality and result in low fusion levels.

Short soluble peptides designed to mimic the HR1 or HR2 domain of fusion glycoproteins can act as potent inhibitors of fusion and live virus infections (36; 79; 272). Such a strategy was applied to develop the anti-HIV drug Enfuvirtide (Fuzeon® or T-20), which is based on a 36 residues region of the HR2 domain of the gp41 envelope glycoprotein of HIV-1 and prevents viral entry by blocking membrane fusion (51; 124). Synthetic peptides mimicking HR1 or HR2 regions of HeV, NiV and CedV F glycoproteins have proved to be potent inhibitors of fusion, and have been utilized to study conformational changes of HeV and NiV F as well as the fusion mechanism of henipaviruses (4; 6; 7; 33; 35; 36; 48; 203; 272). Further structural characterization of the recombinant MojV F and G glycoproteins and a mechanistic study of the aptitude of

MojV F to undergo the pre- to post-fusion conformational rearrangement are warranted. Such mimic peptides can constitute powerful tools to do so.

AIMS AND HYPOTHESES

Aim 2: Investigate structural and mechanistic causes of MojV F and G mediated low membrane fusion levels and atypical kinetics.

Subaim 2a: Demonstrate that MojV F and G assume canonical henipaviral structure and cell surface expression features.

Underlying hypothesis:

- Recombinant MojV F and G glycoproteins adopt the typical functional native conformation of henipavirus F and G glycoproteins and oligomerize as trimeric F and tetrameric G glycoproteins at the cell surface.

Subaim 2b: Establish that MojV F is mechanistically competent to mediate cell-cell fusion.

Underlying hypotheses:

- MojV F glycoprotein can be triggered to undergo the pre- to post-fusion conformational rearrangement required for the completion of a successful membrane fusion event.

- Low membrane fusion levels and slow kinetics of MojV F and G mediated cell-cell fusion are due to receptor restrictions rather than defects in structure or function of MojV F.

RESULTS

Oligomerization status of MojV F and MojV G

To investigate whether the kinetics and low fusion levels observed in cell-cell fusion assays were the result of structural defects, MojV F and G oligomeric expression in mammalian cells was assessed in protein cross-linking assays using the membrane impermeable reagent bis(sulfosuccinimidyl)suberate (BS3). BSR-T7/5 cells were transfected with HeV, CedV and MojV F or G constructs bearing a S-tag at their respective cytoplasmic tails (**Figure 3-1a**). Cells were collected 72h post-transfection and left to recover in suspension overnight before being washed and counted. Cell surface proteins were treated with increasing concentrations of BS3. S-tagged proteins were precipitated and analyzed under reducing conditions by SDS PAGE followed by western blot (**Figure 4-1**). As anticipated, HeV and CedV F controls were expressed as trimeric oligomers, whereas HeV and CedV G assembled as tetrameric oligomers at the cell surface (**Figure 4-1**). Similarly, MojV F and G formed trimers and tetramers, respectively at the cell surface (**Figure 4-1**).

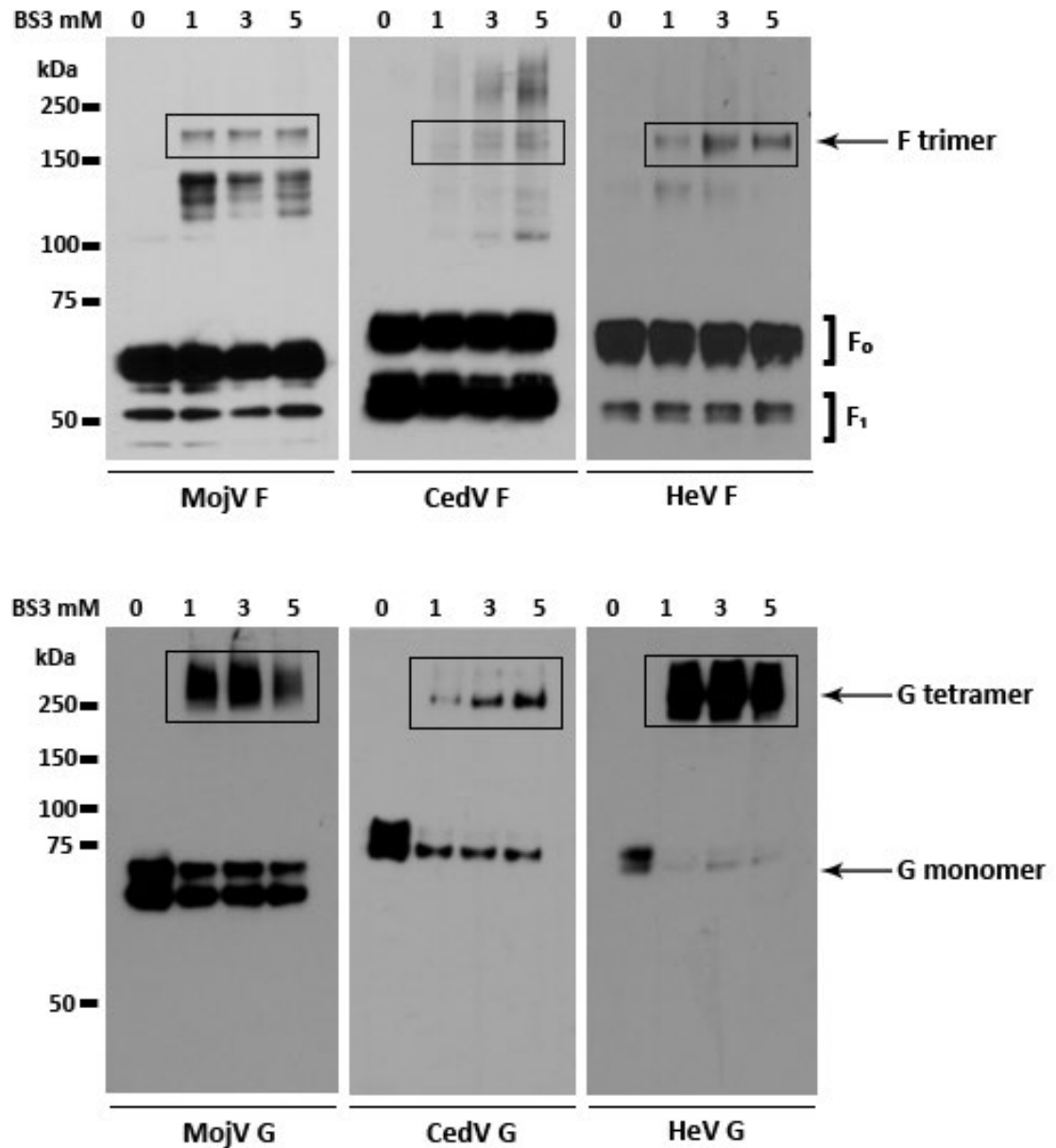


Figure 4-1. MojV F and G are expressed as trimers and tetramers, respectively, at the cell surface.

The oligomerization status of cell-surface expressed MojV F and G was assessed by treating cells transfected with each construct with the cell-surface protein cross-linking reagent BS3. Cross-linked S-tagged proteins were precipitated with S agarose beads. The precipitates were resolved by SDS PAGE and analyzed by western blot under reducing conditions. HeV and CedV F and G were included as controls.

Triggered MojV F undergoes pre- to post-fusion change of conformation.

Oligomerization defects in recombinant MojV F and G do not seem to be a cause for the slower and less robust MojV F and G mediated cell-cell fusion observed. Thus, the possibility that a mechanistic defect in MojV F would preclude the completion of the putative conformational change required to achieve a productive fusion event was investigated. A soluble construct of parainfluenza virus 5 (PIV5 F) bearing a GCN helix motif that promotes trimeric oligomerization to mimic the native state of PIV5 F was reported to spontaneously fold into its pre-fusion conformation (275). This pre-fusion conformation could be triggered to refold into a post-fusion conformation by application of heat and trypsin in vitro (66). Previous studies established similar observations regarding purified soluble trimeric constructs of HeV F (HeV sF) and NiV F (NiV sF) (48). Inhibitory heptad peptides derived from the HR2 domain of HeV and NiV F, have been designed to block HR1/HR2 interactions, thus preventing the formation of the 6HB structure necessary for the virion and host cell membranes to be brought together during fusion events (35; 36). Furthermore, biotinylated heptad-repeat peptides specific to HeV and NiV F can be used to capture NiV sF and HeV sF in their PHI state, between pre- and post-fusion conformations, upon triggering the conformational change following trypsin and heat treatment (48).

A soluble construct of MojV F with a GCN4 trimerization domain as well as a S-tag (MojV sF) was designed to assess if MojV F, like HeV and NiV F, undergoes activation and triggering events involving a pre- to post-fusion conformation change that would presumably be required for a successful fusion event to occur between the virion and target cell membranes (**Figure 4-2a**). A soluble construct of CedV F (CedV sF)

(**Figure 4-2a**) was included as a control, and to further ascertain that the non-pathogenic CedV relies on a fusion mechanism based on a conformational rearrangement cascade similar to that observed in the prototypical HeV and NiV (203). The constructs were confirmed by sequencing, and expression of MojV and CedV sF was evaluated by western blot analysis (**Figure 4-2b**). FreeStyle™ 293 cell lines stably expressing MojV and CedV sF were generated to allow for large scale production of the glycoproteins by Lianying Yan. An in vitro BS3 protein cross-linking assay was performed to confirm that MojV and CedV sF glycoproteins, like full-length cell-surface expressed MojV and CedV F, oligomerize as trimers (**Figure 4-2c**).

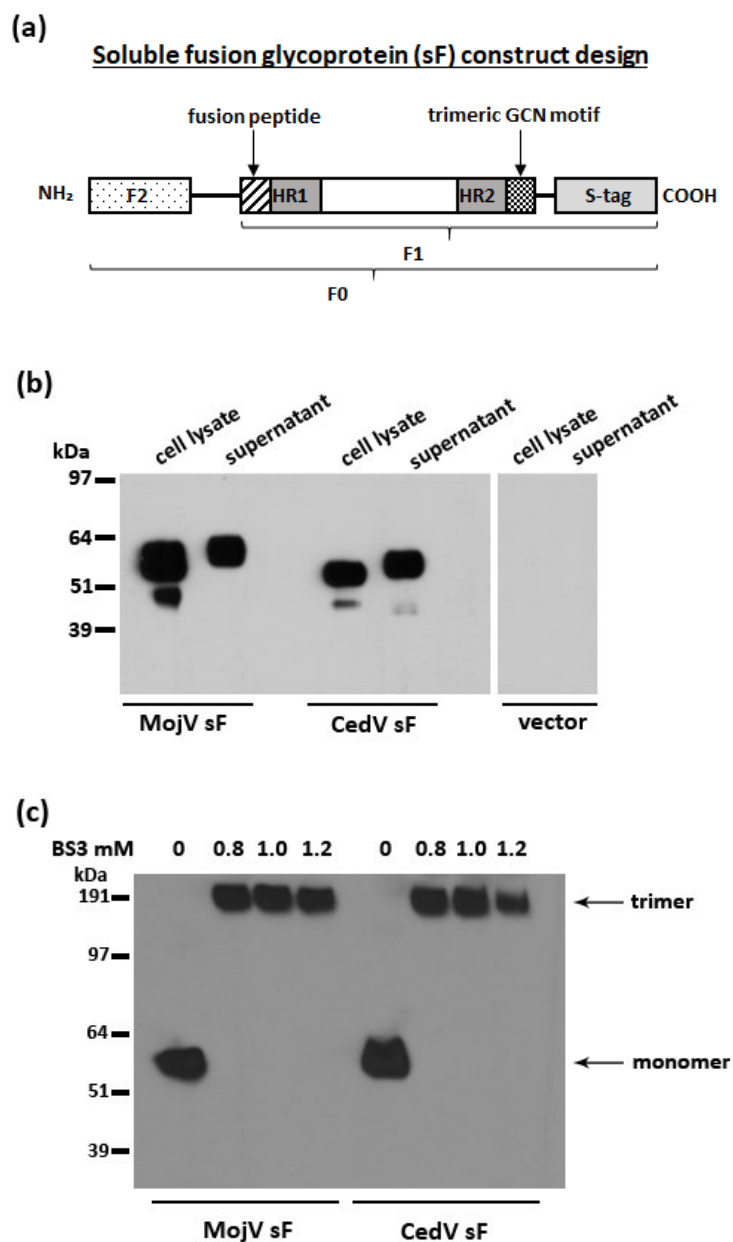


Figure 4-2. Design, expression and oligomerization of MojV sF.

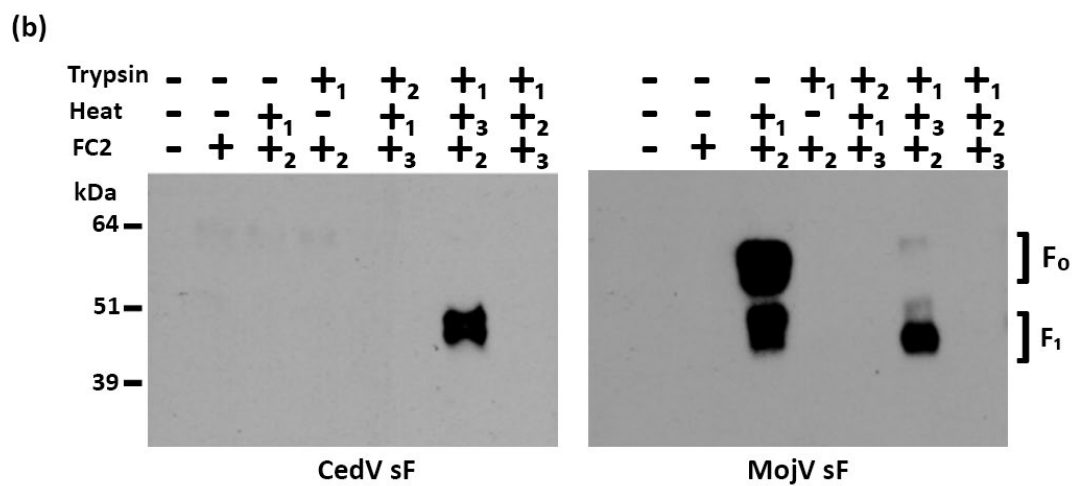
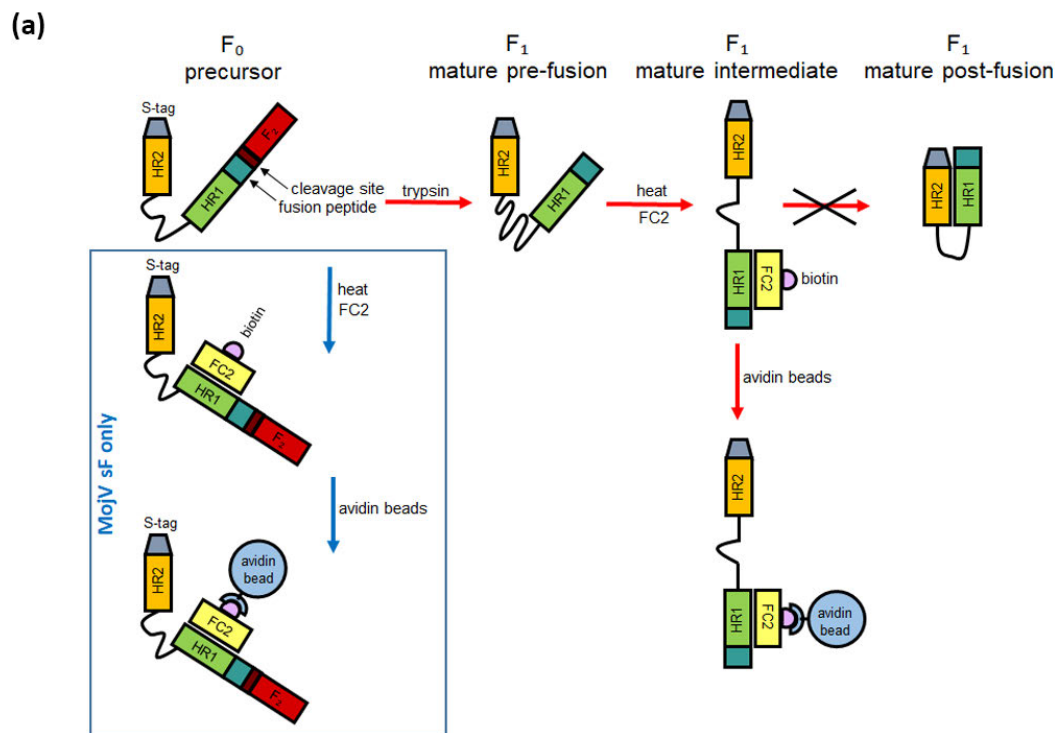
(a) The constructs of recombinant MojV and CedV soluble fusion glycoproteins (sF) were designed to include a GCN4 motif to promote trimerization of sF glycoproteins into the native conformational state adopted by cell-surface expressed henipavirus F glycoproteins. (b) Transient expression of recombinant MojV sF and CedV sF glycoproteins was confirmed in HEK293T cells. Cell lysates were precipitated 48h post transfection with S agarose beads and analyzed by SDS PAGE and western blot. (c) To assess their oligomerization status, purified MojV and CedV sF glycoproteins (50ng) were treated with cross-linking reagent BS3. The oligomers were subjected to western blot analysis under non-reducing conditions.

Heptad-repeat capture assays were carried out to evaluate if MojV and CedV sF transition from a pre-fusion conformation to a post-fusion conformation through a PHI structure that could be trapped with an heptad-repeat peptide mimicking their respective HR2 domain (**Figure 4-3a**). This synthetic heptad-repeat peptide would presumably be able to interact with its cognate HR1 domain preventing the folding of HR1 and HR2 into the 6HB structure necessary to allow the subsequent completion of the fusion mechanism, in effect trapping sF in its PHI stage (**Figure 4-3a**). MojV and CedV sF are expressed as trimeric F₀ precursors (**Figure 4-2a, b**). To mimic the cellular proteolytic processing of the F₀ precursor and generate a functional F₁ subunit, MojV and CedV sF were digested with trypsin (**Figure 4-3a**), whereas the transition from pre- to post-fusion conformation was triggered by applying heat to the digested soluble F glycoproteins (**Figure 4-3a**). MojV and CedV sF were treated with various combinations of heat, trypsin and respective biotinylated heptad-repeat peptides in different orders of application, precipitated with avidin agarose beads and analyzed by SDS PAGE followed by western blot (**Figure 4-3b**). The results indicate that similarly to what was previously observed for HeV and NiV sF (48), a combination of trypsin followed by addition of heptad-repeat peptide and heat application can indeed capture an intermediate stage of the conformational transition of MojV and CedV sF from a pre-fusion to a post-fusion state. This suggests that MojV and CedV F are functional and can fulfill a critical part of a fusion mechanism comparable to that of other henipaviruses (reviewed in (64)). As shown for NiV and HeV sF (48), only mature trypsin-processed CedV sF₁ is triggered into a conformational transition by heat treatment and trapped in this state in the presence of the heptad-repeat peptide (**Figure 4-3b**). Similarly, MojV sF₁ can be captured after

heat application in the presence of heptad-repeat peptide; however, applying heat to MojV sF₀ without trypsin treatment also seems to allow enough flexibility in the protein to make the HR1 domain available for interaction with the heptad-repeat peptide (**Figure 4-3**). These data indicate that MojV F expressed as a precursor and in its pre-fusion conformation may be less stable than other henipavirus F. Nevertheless, these results confirm close similarities between the F glycoproteins of prototypical henipavirus HeV and NiV, and MojV and CedV in structural characteristics required for competent fusion function.

Figure 4-3. MojV F is mechanistically competent and, upon triggering, undergoes the pre- to post-fusion conformation change required for successful fusion.

(a) Schematic representation of the heptad-repeat capture assay mechanism. The henipavirus sF₀ is cleaved into its mature sF₁ subunit by trypsin digestion. The pre-fusion conformation of sF₁ is triggered into transitioning from pre- to post-fusion conformation by heat application. Upon triggering, pre-fusion sF₁ rearranges into an intermediate structure before reaching its post-fusion conformation. This intermediate conformation allows access to the HR1 domain of sF₁ by a biotinylated HR2 mimic peptide termed heptad-repeat peptide. The interaction between sF₁ in its intermediate stage and the heptad-repeat peptide prevents sF₁ from completing its transition into the post-fusion conformation by blocking HR1/HR2 interactions. The heptad-repeat peptide thus captures the intermediate stage of sF₁ as it transitions from pre- to post-fusion conformation. The sF₁/ heptad-repeat complex can then be precipitated by incubation with avidin beads. FC2: heptad-repeat peptide. **(b)** Soluble MojV F (MojV sF) and CedV F (CedV sF) proteins were treated with various combinations of trypsin, heat and addition of biotinylated heptad-repeat peptide. The order of treatment applied in each combination is numbered 1, 2, 3. Treated proteins complexes were precipitated with avidin agarose beads, resolved under reducing conditions by SDS PAGE and analyzed by western blot. CedV sF was detected with anti-CedV sF mouse polyclonal serum at a 1:1,000 dilution. MojV sF was detected with anti-MojV sF mouse monoclonal antibody 4G5 at a 1:2,500 dilution. FC2: heptad-repeat peptide



Low levels of MojV F and G mediated cell-cell fusion levels are not due to defective MojV F functionality.

The respective contribution of the F and G glycoproteins to the fusion process can be examined in cell-cell fusion assays that evaluate heterotypic F and G pairings. Here, such an experimental design can help further determine if possible inadequacies in MojV F are responsible for the particular fusion phenotype observed with MojV F and G, or if the role of MojV G and the absence of its cognate receptor are the main drivers of this phenotype. These complementation assays can, in addition, provide supportive evidence of close relatedness between the viruses from which the glycoproteins are tested. Heterotypic complementation assays are based on the co-expression of non-homologous fusion and attachment glycoprotein pairings. For example, Nussbaum et al. (185) have shown that the H (attachment) glycoprotein of measles virus (MeV) and F glycoprotein of canine distemper virus (CDV), which are both paramyxoviruses of the genus *Morbillivirus*, retain functionality when co-expressed in NIH 3T3 cells. Furthermore, various heterotypic combinations of HeV, NiV and GhV F and G were shown to mediate cell-cell fusion (36; 200). To confirm the functionality of MojV F despite the low levels of fusion observed and possible lesser stability of its pre-fusion conformation, Nano luciferase reporter-gene cell-cell fusion assays were conducted with BSR-T7/5 effectors co-transfected with heterotypic combinations of henipavirus F and G plasmids. C6 rat glial cells were chosen as target cells because they were strongly permissive to all henipavirus F and G mediated fusion tested in this project (**Figure 3-3**). Given the likelihood that CedV and MojV could both infect rodent hosts (131; 266), CedV F and G were also included in the heterotypic henipavirus F/G combinations assessed to

investigate the possibility of successful complementation of CedV F with MojV G and the converse (**Figure 4-4c, e**). As established by Bossart et al. (36), heterotypic combinations of HeV/NiV F and G were found to be functional. Findings by Rissanen et al. (215) showing that co-expression of GhV G with either HeV or NiV F was successful, but not the converse were also confirmed here (**Figure 4-4a, b and d**). In addition, HeV G and NiV G successfully triggered CedV F to cause cell-cell fusion; although CedV F/NiV G mediated fusion was less robust (**Figure 4-4a, b**) (203). Conversely, co-expression of NiV F with CedV G did not result in fusion, but weak fusion was observed with the HeV F/CedV G complementation (**Figure 4-4c**) (203). However, CedV G was unable to trigger MojV F, and the complementation of CedV F with MojV G (**Figure 4-4c**), likewise, did not result in cell-cell fusion (**Figure 4-4e**). Successful heterotypic complementations involving MojV F or G were limited to the MojV F/GhV G pairing alone (**Figure 4-4**) (215).

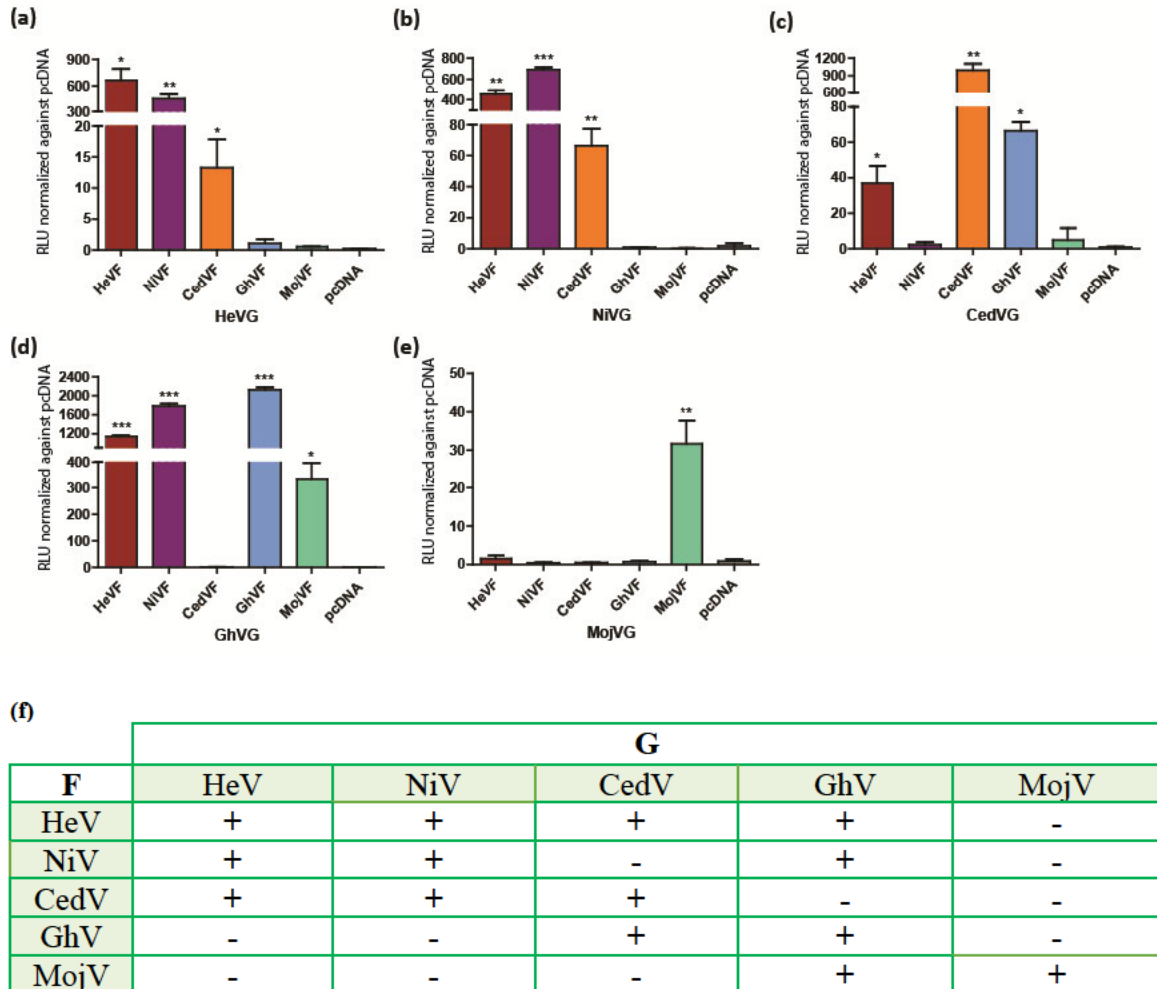


Figure 4-4. Heterotypic henipavirus F and G complementation assay confirms MojV F functionality.

BSR-T7/5 effector cells constitutively expressing T7 polymerase were co-transfected with an empty vector or expression plasmids encoding henipavirus F and either HeV G (a), NiV G (b), CedV G (c), GhV G (d), or MojV G (e). Effector cells were co-incubated with C6 rat glial cells transfected with a vector encoding the Nano luciferase gene under the control of the T7 promoter. Maximum RLUs were recorded 48h post-application of target cells to all effector monolayers, except for co-cultures with effector cells expressing MojV G, which reached maximum RLUs on day 4. Maximum RLUs were normalized against background defined as RLUs from mixtures of the target C6 cells applied to effector cells co-transfected with henipavirus G and empty vectors. The experiments were performed at least 3 times in technical triplicates. A representative experiment is shown here. Data was analyzed by Welch's t test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (f) Results of the heterotypic combination cell-cell fusion assays are summarized in a table format recapitulating positive and negative F and G complementation pairings.

DISCUSSION

MojV F and G mediated cell-cell fusion evidenced by formation of syncytia and quantified by Nano luciferase fusion assays (see chapter 3) presented characteristics specific to MojV compared to what can be observed with other henipavirus F and G mediated cell-cell fusion. The lower levels and slower kinetics of MojV F and G mediated membrane fusion warranted further investigation through structural characterization of MojV F and G glycoproteins as well as evaluation of the mechanistic competence of the MojV F glycoprotein.

Protein cross-linking experiments were performed to establish that recombinant MojV F and G glycoproteins expressed at the cell surface adopt canonical henipaviral structure and folding. The data obtained ruled out possible structural defects in MojV F and G glycoproteins by confirming that the constructs recapitulate the oligomerization status expected of other henipavirus F and G glycoproteins (**Figure 4-1**). Indeed, as previously shown for HeV and NiV F and G (30; 36; 38; 238; 269; 270), MojV and CedV F and G formed trimeric and tetrameric structures, respectively (**Figure 4-1**). These results support the classification of MojV as closely related to prototypical henipavirus such as HeV as well as the non-pathogenic CedV.

To evaluate the ability of MojV and CedV F to undergo the pre- to post-fusion conformational cascade required for the completion of a successful fusion event, soluble constructs were designed (**Figure 4-2a, b**). These constructs oligomerized into quaternary structures recapitulating the native cell surface trimeric conformation observed in the expression of full-length constructs of MojV and CedV F (**Figure 4-c**), in agreement with the previous work on HeV and NiV sF by Chan et al. (48). A heptad-repeat capture assay using soluble biotinylated 36 residues-long mimic peptides based on the HR2 domain

sequences of MojV and CedV F glycoproteins was conducted to demonstrate that MojV and CedV F glycoproteins are mechanistically competent. Heptad-repeat capture assay data showed that, like HeV and NiV sF (48), MojV and CedV sF can be triggered to transition through a PHI as they undergo the pre- to post-fusion conformational cascade (**Figure 4-3b**). Interestingly, MojV sF, in its pre-fusion conformation, also seems to be less stable than other henipavirus sF glycoproteins, suggesting that premature triggering of MojV F to irreversibly transition to its post-fusion conformation may be possible (**Figure 4-3b**). This could preclude successful fusion from occurring if MojV G were to recognize a host cell surface receptor, yet MojV F was already locked in a post-fusion conformation. Nevertheless, overall these results confirm close similarities between the F glycoproteins of prototypical henipavirus HeV and NiV, as well as CedV, and MojV in structural characteristics required for proper fusion function.

To evaluate the respective contributions of MojV F and G, a Nano luciferase reporter-gene cell-cell fusion assay was applied to test the functionality of heterotypic F and G complementation pairings across the full HeV, NiV, CedV, GhV and MojV F and G panel (**Figure 4-4**). The results confirmed previous findings involving heterotypic combinations of HeV and NiV F and G (36), GhV F and G (215) and CedV F and G (203), and further established that the combination of GhV F/CedV G, but not the converse, was successful (**Figure 4-4c**). Interestingly, the only functional heterotypic complementation involving MojV F or G was MojV F/GhV G. Nevertheless, this data attest that MojV F is indeed functional (**Figure 4-4d**). Moreover, these results suggest that the domains of MojV F that interact with GhV G to induce triggering of MojV F into undergoing the pre- to post-fusion conformational rearrangement are closely related to

those present in GhV F. The fact that the overall percent identity of protein sequence shared is the highest between MojV F and HeV F at 41%, and that of MojV F with GhV F only reaches 36% further underlines the importance of defined and specific conserved domains of the fusion glycoproteins involved in their functionality (**Figure 3-1**). Identification of such domains could be further pursued in studies based on chimeric GhV/MojV F constructs and comparative crystal structure analysis.

Additionally, the low levels and atypical kinetics of MojV F and G mediated cell-cell fusion may be explained by other factors. The experiments designed in this study rely on the co-expression of recombinant F and G viral glycoproteins, but it cannot be excluded that, perhaps, premature triggering of MojV F can occur as a result of artificial instability due to the lack of proper interactions between the cytoplasmic tail ends of MojV F and G and matrix (M) glycoproteins. Indeed, work by Cifuentes-Muñoz et al. suggests that HeV F and M proteins pre-assemble before they reach the plasma membrane (62). Without this potential stabilizing effect, MojV F may be spontaneously triggered into irreversibly transitioning from pre- to post-fusion conformation. Consequently, the MojV F glycoprotein already transitioned into a post-fusion conformation, or some intermediate conformational state, may no longer be able to mediate membrane fusion upon engagement of MojV G with its cognate receptor.

Finally, because the only sequence of MojV currently available was assembled from amplified viral nucleic acid obtained from *Rattus flavipectus* samples, without isolation of infectious viral particles (266), one cannot exclude the possibility that (a) RT-PCR error(s) may have occurred and resulted in the reporting of inaccurate reference sequences to the Genbank database. For example, Pernet et al. (200) reported that

correction of the sequence of GhV F originally published upon discovery of the virus by addition of one nucleotide resulted in increased cleavage efficiency of F₀ to generate F₁/F₂, incorporation in pseudotyped virions and fusogenicity. Perhaps sequence correction could likewise improve the stability and fusogenicity of MojV F. Taken together, the results presented here also suggest that, as supported by data from the heptad-repeat capture assay (**Figure 4-3b**), the syncytia formation assay (**Figure 3-2**) and the heterotypic F/G complementation cell-cell fusion experiments (**Figure 4-4d and e**), weaker MojV F and G mediated fusion (**Figures 3-2, 3-3 and 3-6**) might more so be due to receptor restrictions rather than faulty functionality from MojV F. The principal cognate receptor of MojV G may not have been expressed, or expressed at levels too low, on the panel of cells tested here, for robust cell-cell fusion to be detected.

CHAPTER 5: Serologic detection of MojV F and G glycoproteins, and application of henipavirus F and G multiplex microsphere immunoassays to serum and fecal rodent samples.

INTRODUCTION

A clear need exists for the extensive evaluation of levels of exposure to and geographic circulation and distribution of emerging pathogenic viruses such as henipaviruses. The zoonotic and cross-species transmission potential of henipaviruses, evidenced by NiV outbreaks that involved human, swine, horse, cat and dog cases in Southeast Asia, as well as human, horse and dog cases during HeV outbreaks in Australia is established (reviewed in (191)) (10; 53). Furthermore, evidence that various bat species, which have a wide geographic distribution and travel range, are natural carriers of HeV, NiV(100), CedV and GhV has been documented in reports including sampling conducted in Australia (279), peninsular Malaysia (276), Madagascar (45; 112), India (273) and West Africa (Ghana) (103; 198). The seropositivity of bats sampled in locations outside of the regions where known outbreaks have been listed suggests that such outbreaks may occur in these areas as well. Expanding human activities can lead to increasing encroachment of bats natural habitat, and the disturbance of climate patterns can further result in bat population migrations and adaptation to new localities in search of suitable resources (8; 118; 218; 237). In these circumstances, the probability of contact between bat and human populations, and consequently the occurrence of zoonotic spillover events, are bound to rise (116).

Some data on exposure to henipaviruses and evidence of current or past infection in wildlife populations are available, but remain limited and tend to focus more heavily on bat sampling. Such studies have mostly relied on exposure assessment by ELISA

blood serum analysis or identification of ongoing infection by viral nucleic acid detection in RT-PCR assays with confirmation by serum neutralization tests (SNT). Various studies have explored exposure of different bats species in South East Asia, in countries that have experienced outbreaks. For example, frugivorous (*Cynopterus brachyotis*, *Eonycteris spelaea*, *Pteropus hypomelanus*, *Pteropus vampyrus*) and insectivorous (*Scotophilus kuhlii*) bats in Peninsular Malaysia were found to be NiV seropositive, with confirmation by SNT, although RT-PCR for NiV N RNA detection was negative, and virus isolation was unsuccessful (276). ELISA and NiV N RT-PCR analyses of saliva and urine samples from frugivorous (*Pteropus hypomelanus*, *Pteropus lylei*, *Pteropus vampyrus*) and insectivorous (*Hipposideros larvatus*) bats collected in Thailand were positive for NiV infection (248; 249). Blood serum samples from Cambodian *Pteropus lylei* bats, including samples obtained from restaurants where bats are killed for their meat, tested positive for NiV exposure by ELISA, were confirmed by SNT, and provided infectious isolates (213). *Pteropus giganteus* blood sera from Haryana State in northern India was found to be reactive to HeV and NiV proteins by ELISA and confirmed by SNT (82), whereas samples of the same species collected in West Bengal were found positive by serum IgG ELISA and RT-PCR analysis of liver homogenate (273). Bats from neighboring countries where no henipavirus outbreaks have been reported to date were also evaluated. Blood serum from frugivorous *Cynopterus sphinx* and *Rousettus leschenaultia* specimens collected in Vietnam was assessed for reactivity to NiV N by ELISA and western blot, and was found positive, suggesting that NiV or NiV-like species may be circulating in these bat populations as well (102). In Indonesia NiV seropositive samples from *Pteropus vampyrus* were identified by ELISA (232), and three putative

novel henipaviruses were identified by RT-PCR detection of viral RNA in spleen samples from *Pteropus vampyrus*, *Pteropus hypomelanus*, and *Acerodon celebensis* flying fox bats (225). *Myotis* bat serum samples collected in ten provinces of mainland China were identified as seropositive for NiV N and G glycoproteins by ELISA (140).

Limited serologic studies have been extended to bat populations in Africa to assess the potential of these regions for circulation of henipaviruses or henipa-like viruses. Serum samples from Malagasy *Eidolon dupreanum* and *Pteropus rufus* bats were identified as seropositive for NiV and HeV proteins by ELISA and confirmed by neutralization assays (112). In addition, five henipavirus-related viruses were detected in splenic samples from Zambian *Eidolon helvum* bats by RT-PCR (171). Other species, which can act as spillover and amplifying hosts due to their close proximity to bat and human populations, have been included in recent African studies. Serological evidence of henipavirus or henipa-like virus exposure in horses and pigs in Nigeria was revealed by HeV sG based ELISA assays (187). Slaughterhouse pigs from Uganda were found to be seropositive by ELISA using NiV and HeV G glycoproteins and western blot against NiV N, suggesting exposure to henipaviruses or henipa-like viruses in this region (16). In 2014 work by Pernet et al. (201) relied on SNT using vesicular stomatitis virus (VSV)-based pseudoparticle to test exposure to GhV, NiV and HeV in sera collected in Cameroon, Africa. Interestingly, this group reported seropositivity to all three particle types in human samples from individuals that butchered bats for their meat, alluding to a possible spillover of henipavirus or henipa-like viruses from local bats to human populations (201).

As mentioned above, multiple techniques can be useful in assessing past exposure or current infection in wildlife, and domestic animals, as well as in human populations. Each technique of analysis carries advantages and short falls, underlying the fact that a coordinated use of different types of assays can provide a more complete picture of the geographic distribution and circulation of henipaviruses in spillover hosts and reservoir species. Viral nucleic acid detection by RT-PCR can be fruitful, and indicative of an infection that is active or in the process of being resolved. However, probing in bats is relevant only for a narrow window of time as these animals, in their capacity of natural reservoir hosts, can control henipaviral infections well and act as subclinical carriers (100). Therefore, if henipaviruses are circulating in a given bat population, it may be at lower levels and more challenging to detect. Furthermore, such data cannot inform on possible past exposure to these viruses. Instead, serologic assays, such as ELISA, can provide this information, and SNT will assess if the antibodies raised during these infections were neutralizing and would allow for the control of these infections. Nevertheless, recent technologic advances have supported the development of novel assays.

In particular, Luminex xMAP technology has provided the field of serologic surveillance of emerging viruses with interesting tools. It can be applied to multiplexed immunoassays formatted for microplate applications to combine principles of ELISA analysis and flow cytometry detection. The multiplex microsphere immunoassay (MMIA) is based on nonmagnetic or magnetic beads (microspheres) which are infused with two fluorescent dyes (one red and one infrared) in 100 unique dye concentration ratios. Each bead can thus be identified by its signature dye ratio upon interrogation by a

red laser (635nm), allowing the assay to be run in a multiplex format (**Figure 2-3**). Bead panels can be customized by coupling beads to specific antibodies or antigens of interest and indirectly providing each reactive target a specific spectral signature. Samples are screened for the presence of immunogens or reactive antibodies, respectively, by incubation with the conjugated beads (**Figure 2-3**). Addition of a biotinylated secondary antibody against all targets followed by streptavidin-phycoerythrin (PE) allows for the detection of targets bound to the antibodies or antigens coupled to each bead upon excitation of PE with a green laser (532nm) (**Figure 2-3**). Up to 100 tests can be multiplexed and run simultaneously in a microplate format with automated detection, reducing cost, labor and amount of sample consumed to perform the analysis. In addition, the spherical format of the assay provides an increased surface area compared to flat surface-based ELISA experiments, offering a wider range of detection (24).

Traditionally, the evaluation of exposure to henipaviruses by serologic screen has been conducted by ELISA experiments that relied on antigens from HeV or NiV infected cells lysed and gamma-irradiated for safe use outside of BSL-4 containment restrictions, resulting in batch variations in antigen amount and quality (32). Moreover, issues of cross-reactivity and lack of specificity can be associated with such an experimental design. Ambiguous positive results due to high levels of conservation in some of the antigens pooled in such assays can be resolved by using viral antigens more specific to a given viral species instead, namely proteins with more limited homology across henipaviruses (32). Also, the gold standard test remains the labor intensive SNT; this test can only be performed in BSL-4 settings and requires 3 to 4 days to judge if antibodies were successful in inhibiting cytopathic effects in cell cultures (32). MMIA have thus

become an interesting alternative that can prove more efficient and conservative, and easily applicable outside of BSL-4 settings.

A number of studies have used MMIA where nonmagnetic beads coupled to NiV and HeV sG glycoproteins were multiplexed to screen *Eidolon helvum* bat sera collected in Ghana, West Africa (103) and the Gulf of Guinea island of Annobon (198), as well as *Dobsonia magna*, *Pteropus Alecto* and *Pteropus conspicillatus* fruit bats sera from Papua New Guinea (43), providing evidence of the presence of henipavirus or henipa-like viruses in these regions. Work by Hayman et al. (104) identified seropositive sera from pigs in Ghana, West Africa using a similar experimental design, whereas Chowdhury et al. (56) similarly analyzed blood serum samples from cattle, goats, pigs collected in Bangladesh for reactivity to NiV and HeV sG glycoproteins. Lastly, a study taking place in Madagascar reported in 2019 the use of an extended multiplexed panel of soluble henipavirus glycoproteins including HeV sG/sF, NiV sG/sF and CedV sG/sF, as well as filovirus EBOV sGp and MARV sGp, and identified reactivity to all three henipaviruses and Ebola glycoproteins in samples collected from bats that are typically hunted and consumed during winter months on the island (45). Such studies highlight the zoonotic potential of these viruses, although no outbreak have been reported to date in these areas (45).

In order to facilitate and expand sampling opportunities, less invasive alternatives to blood serum collection, such as fecal matter sampling, can prove invaluable, particularly to gain insight into local emerging virus circulation in wildlife populations or in peridomestic animals and pests. Bat feces have been used as primary material in studies based on viral nucleic acid detection and identification by RT-PCR. For example,

Drexler et al. (115) applied RT-PCR detection to fecal pellets from a *Myotis myotis* bat colony in Germany, from which they were able to identify a novel bat mastadenovirus, a bat-alphacoronavirus and six bat-associated mamastoviruses. Begeman et al. (21) also recently compared fecal pellets and oral swab samples as materials for RT-PCR studies of the prevalence of European bat lyssavirus RNA (EBLV-1) in insectivorous *Eptesicus serotinus* bats from the Netherlands, and found that detection of viral RNA was nearly as sensitive in the fecal pellets as in the oral samples. Additionally, *Eidolon helvum* droppings collected in Ghana were analyzed by RT-PCR and revealed the presence of three novel putative henipaviruses (75). These findings suggest that fecal material collection may be an interesting non-invasive alternative to blood or swab sampling for epidemiologic and screening studies of wildlife populations.

Furthermore, fecal materials can be used for serologic screening to assess exposure to emerging viruses in addition to viral nucleic acid detection. Feces from wild gorillas and chimpanzees collected at night nests and feeding sites have been analyzed for the presence of antibodies reactive to simian immunodeficiency virus of Western lowland gorillas (SIVgor) in central Africa and Cameroon using western blot and Inno-LIA HIV I/II scoring, which is based on antibodies binding to lines of recombinant and synthetic HIV-1 and -2 proteins coated on a nylon strip (83; 180). RNA extracted from these fecal samples was also tested by RT-PCR (83; 180). Additionally, EBOV reactive antibodies in wild gorillas were detected by EBOV NP western blot analysis of IgG antibodies in fecal matter collected opportunistically in Odzala-Kokoua National Park, Republic of Congo (212). One can thus hypothesize that fecal samples can also be used as primary material for serologic studies of other zoonotic emerging viruses, such as henipaviruses.

MojV is the first henipavirus to be identified in a rodent (*Rattus flavipectus*), based on sequence data from viral nucleic acids present in rectal swab samples collected in Yunnan, China (266). This discovery raises new questions about natural hosts and the circulation of henipaviruses. Could rodents be natural spillover and amplifying hosts of henipaviruses, or possibly act as alternative reservoirs to bats? Sasaki et al. (224) have identified novel henipavirus-related paramyxoviruses in Zambian wild rodents and shrews. RT-PCR analysis of kidney samples collected from these specimens detected the presence of henipavirus-related paramyxoviruses in *Crocidura hirta* (lesser red musk shrew) and *Rattus rattus* (black rat) specimens; however, no virus was isolated (224).

Taken together, these elements emphasize the need for reagents to detect MojV in wildlife, peridomestic, particularly in rodents and shrews, and human populations. Exploring applications to evaluate exposure levels, extent of virus circulation and geographical distribution of this emerging virus is also warranted. Critical tools will include soluble forms of viral proteins known to be potent immunogens of other henipaviruses; recombinant henipaviral fusion and attachment glycoproteins that recapitulate native oligomerization and receptor binding are proven to be excellent candidates to fulfil this function (30; 48). Such reagents can be used, as noted above, in multiplexed serologic screening studies, but also in immunization studies to raise polyclonal and monoclonal antibodies, which can themselves be used to detect viral particles. Generating MojV sF and sG reagents could allow for the production of polyclonal and monoclonal antibodies. Moreover, MojV sF and sG could be integrated into a full henipavirus sF or sG serologic MMIA panel. Such an assay may be applied to

the detection of antibody reactivity in fecal samples, as well as blood serum samples in rodents.

AIMS AND HYPOTHESES

Aim 3: Develop tools applicable to serologic surveillance studies and multiplex microsphere immunoassays to screen rodent serum and fecal samples for exposure to henipavirus F and G glycoproteins.

Subaim 3a: Generate immunogenic soluble MojV F (MojV sF) and MojV G (MojV sG) glycoproteins to raise polyclonal and monoclonal antibodies.

Underlying hypotheses:

- Recombinant MojV sF and sG can be expressed to recapitulate the native oligomerization status of cell-surface expressed full length MojV F and G glycoproteins.

- Recombinant MojV sF and MojV sG can act as potent immunogens to raise murine monoclonal antibodies as well as murine and leporine polyclonal antibodies.

Subaim 3b: Apply a multiplex microsphere immunoassay to antibodies extracted from rodent fecal samples as a non-invasive alternative to blood serum samples to screen for reactivity against comprehensive HeV, NiV, CedV, GhV and MojV sF or sG panels.

Underlying hypotheses:

- Murine antibodies specific to henipavirus glycoproteins are detectable by multiplex microsphere immunoassay.

- Fecal rodent antibodies can be screened to evaluate exposure to henipaviral F and G glycoproteins as an alternative to blood serum antibodies.

RESULTS

Design and expression of immunogenic soluble MojV F and G glycoproteins

In assessing the levels of circulation and exposure to novel emerging viruses that have pathogenic and zoonotic potential, the elaboration of detection means is critical. Soluble recombinant viral glycoproteins can be utilized as immunogens to raise polyclonal antibodies and help produce monoclonal antibodies that can be applied to the detection of specific viral proteins. Alternatively, these soluble recombinant glycoproteins can also be integrated in assays to screen the reactivity of blood, urine, saliva or fecal samples and determine exposure to the corresponding virus in various human, livestock or wildlife populations. Bossart et al. (30) have established that a soluble form of HeV G glycoprotein that naturally assembled as dimers linked by disulfide bonds to form tetramers could generate neutralizing antibodies in experimentally immunized rabbits. Moreover, oligomers were shown to be better immunogens than monomers in raising anti-viral neutralizing antibodies by Bachman et al. (18). In addition, recent work on murine 5B3 and humanized h5B3 monoclonal antibodies demonstrated that these NiV neutralizing antibodies act by binding a discontinuous epitope composed of regions from two neighbor NiV F protomers accessible only in the pre-fusion conformation of the trimeric NiV sF (68). These studies emphasize the fact that the oligomerization status and conformational state of the glycoproteins can impact their quality as immunogens. Recombinant soluble MojV F and G constructs were designed to recapitulate the natural oligomerization state of these two glycoproteins. Clones were confirmed by sequencing; their expression was evaluated by western blot analysis of transfected cells and their oligomerization assessed by cross-

linking assays. Large scale production of S-tagged MojV sF was established in a HEK293FS stable cell line, whereas that of S-tagged MojV sG was obtained by generating a Neuro2a stable cell line. Details of the design and expression of these constructs were previously described in chapters 3 (**Figure 3-4**) and 4 (**Figure 4-2**).

Polyclonal and monoclonal antibodies against MojV sF and MojV sG glycoproteins

BALB/c mice were immunized by subcutaneous and intraperitoneal injections with a total of 4.5 µg of purified soluble F or G glycoprotein per injection according to the schedule depicted in **figure 2-1**. Rising titers of anti-sG antibodies were assessed throughout the immunization schedule by indirect ELISA analysis of serum obtained from tail bleeds (**Figure 2-1**), where wells of a 96 well plate are coated with S-tagged sG protein prior incubation with the serum sample tested, followed by incubation with a HRP-conjugated anti-mouse IgG and detection upon substrate application by measure of O.D.405nm (**Figure 2-2**). Previous work in our lab has revealed that henipavirus sF glycoproteins can become unstable in the context of indirect ELISA experiments. Therefore, an adapted experimental design was applied to monitor anti-sF antibody titers. Instead, sandwich ELISA were conducted by coating the plate wells with anti-S peptide antibodies first to capture S-tagged sF proteins, and was then incubated with the serum samples tested; the sandwich ELISA were completed following steps identical to that of the indirect ELISA described above (**Figure 2-2**). Final bleeds for serum collection and spleen harvests to perform hybridoma fusions were obtained by Lianying Yan who isolated monoclonal antibodies against MojV sF (3C4 and 4G5) and MojV sG (2B2, 10G2 and 6E5) by performing ELISA screens of hybridoma supernatants. Clones producing antibodies cross-reactive to the GCN4 trimerization motif for sF and the

GCN4 tetramerization motif for sG, as well as to the S-tag were excluded. In order to obtain larger quantities of serum polyclonal antibodies rabbit immunization schedules were contracted to Noble Life Sciences (Gaithersburg, MD) (**Figure 2-1**).

Anti-MojV sG monoclonal antibodies characterization by western blot, flow cytometry and immunoprecipitation analyses

Full-length S-tagged constructs of HeV, NiV, CedV, GhV and MojV G or an empty vector were transfected in BSR-T7/5 cells. Cell lysates were collected 48h after transfection, incubated with S beads and analyzed under denaturing reducing conditions by western blot. Blots were probed with anti-S antibodies (as a positive control of protein expression), anti-MojV sG rabbit polyclonal serum (as a positive detection control for MojV G) and each of the anti-MojV sG monoclonal antibodies. Although, all henipavirus G glycoproteins were successfully detected by anti-S antibodies, and rabbit polyclonal serum specifically detected MojV G, none of the three anti-MojV sG monoclonal antibodies (2B2, 10G2, 6E5) were able to detect MojV G or any other henipavirus G glycoproteins at any of the concentrations tested (3, 5, 20 µg/ml). These results suggest that 2B2, 10G2 and 6E5 monoclonal antibodies are confirmation dependent.

To confirm this hypothesis, 2B2, 10G2 and 6E5 were evaluated in their ability to bind cell-surface expressed MojV G in its native conformation in flow cytometry experiments (**Figure 5-1**). BSR-T7/5 cells transiently expressing one untagged henipavirus G or an empty vector were collected 48h post transfection, washed, counted and incubated with 0.5 µg/well of mouse monoclonal antibody (2B2, 10G2 or 6E5) diluted in FACS buffer. Detection of bound antibodies was done with secondary goat anti-mouse IgG(H+L)::AlexaFluor488® (Invitrogen, Carlsbad, CA). In addition, possible

cross-reactivity of polyclonal antibodies raised against MojV sG with other henipavirus G was assessed by performing a similar experiment using rabbit polyclonal antibodies at 1:50 dilution in FACS buffer. Polyclonal antibodies against each of the different henipavirus G glycoprotein (HeV, NiV, CedV and GhV G) were included as positive protein expression controls. Bound polyclonal antibodies were detected with goat anti-rabbit IgG(H+L)::AlexaFluor647® (Invitrogen). These experiments were performed in a 96 well microplate format for high through put analysis with an LSRII flow cytometer. The data showed that indeed each monoclonal anti-MojV sG antibody was able to bind cell-surface expressed full-length MojV G in its native conformation, and did so with high specificity to MojV G (**Figure 5-1b**). Polyclonal antibodies raised against MojV G, but also GhV G and CedV G were also highly specific to each G glycoprotein corresponding to their respective immunogen (**Figure 5-1a**). Congruent with other studies reporting cross-reactivity of anti-HeV G and anti-NiV G antibodies, polyclonal antibodies from rabbit immunized with HeV sG recognized both HeV and NiV G glycoproteins; the converse was true as well as (**Figure 5-1a**) (30; 34; 285) .

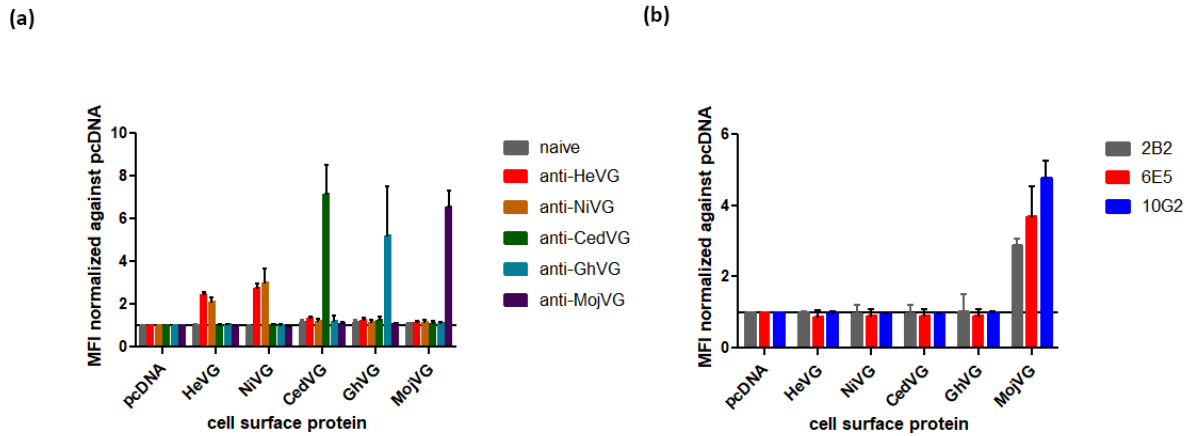


Figure 5-1: Assessment of anti-MojV G antibodies cross-reactivity to other henipavirus G glycoproteins.

BSR-T7/5 cells transiently expressing one henipavirus (HNV) G glycoprotein, or an empty pcDNA vector, were incubated with (a) rabbit naïve or polyclonal serum raised against each of the henipavirus glycoproteins or (b) one of the anti-MojV G monoclonal antibodies (2B2, 10G2, 6E5). Antibodies bound to cell surface glycoproteins were detected with secondary goat anti-rabbit IgG(H+L)::AlexaFluor647® or goat anti-mouse IgG (H+L)::AlexaFluor488®. Data is presented as median fluorescent intensity (MFI) normalized against the signal obtained from cells transfected with the empty vector. The grey line indicates the background level of 1. Experiments were performed in triplicates and data was analyzed by FlowJo3 software (FlowJo LLC, Ashland, OR).

Anti-MojV sG antibodies 2B2, 10G2 and 6E5 were further characterized by conducting immunoprecipitation experiments. Cell lysates from BSR-T7/5 cells transiently expressing one S-tagged henipavirus G glycoprotein or transfected with an empty vector were incubated with each monoclonal anti-MojV sG antibody or with S beads as a control. Glycoprotein/antibody complexes were precipitated with G agarose beads or S beads and analyzed by western blot. Membranes were probed for the detection of the S-tag born by the G glycoprotein. All three monoclonal antibodies successfully interacted with full-length MojV G glycoprotein and no cross-reactivity with other henipavirus G glycoproteins was observed (**Figure 5-2**). Of note, the amount of 10G2 and 6E5 antibodies used in this experiment was twice that of 2B2 antibody, demonstrating possible differences in binding strength amongst these antibodies (**Figure 5-2**).

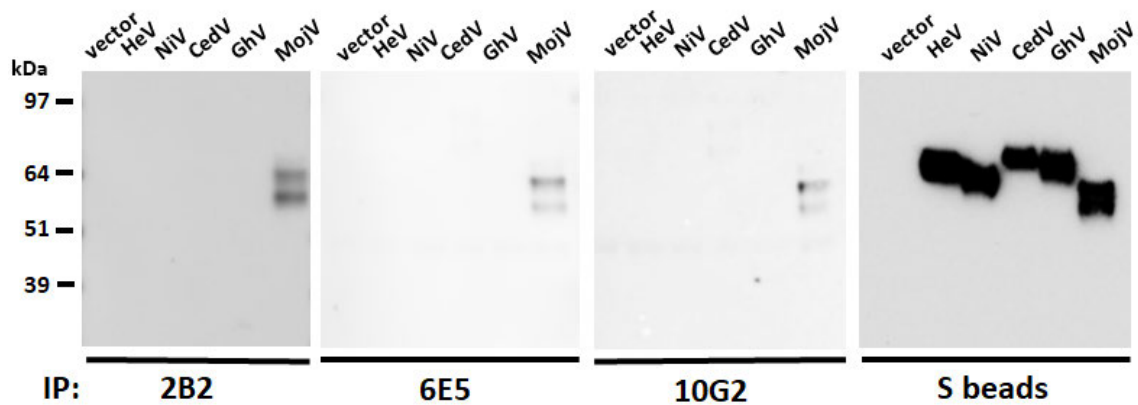


Figure 5-2: Immunoprecipitation of full-length MojV G glycoprotein by monoclonal anti-MojV sG antibodies 2B2, 10G2 and 6E5.

Equal aliquots of cell lysates from BSR-T7/5 cells transfected with one S-tagged henipavirus G glycoprotein or an empty vector were incubated with each monoclonal antibody. Antibody/G glycoprotein complexes were precipitated with G agarose beads or with S beads as a protein expression control.

Precipitates were resolved by SDS PAGE and transferred onto nitrocellulose membranes which were probed with HRP-conjugated rabbit anti-S peptide polyclonal antibody.

Anti-MojV sF monoclonal antibodies characterization by western blot, flow cytometry and immunoprecipitation analyses

Similarly to the characterization conducted for anti-MojV sG antibodies, the capacity of anti-MojV sF monoclonal antibodies 3C4 and 4G5 to detect denatured full-length MojV F in transfected cell lysates was assessed by western blot analysis as described above. Monoclonal antibody 4G5 was able to detect MojV F under these conditions (**Figure 5-3**), whereas 3C4 did not at either concentration tested (1.6 and 16 µg/ml). Furthermore, polyclonal anti-MojV sF serum, as anticipated, recognized MojV F (**Figure 5-3**). Polyclonal antibodies raised against MojV sF and monoclonal antibody 4G5 showed no cross-reactivity to other full-length henipavirus F glycoproteins, and control detection of each henipavirus F glycoprotein by probing of their S-tag demonstrated that each F glycoprotein was expressed in the cell lysates analyzed (**Figure 5-3**). These results suggest that 3C4 is conformation dependent.

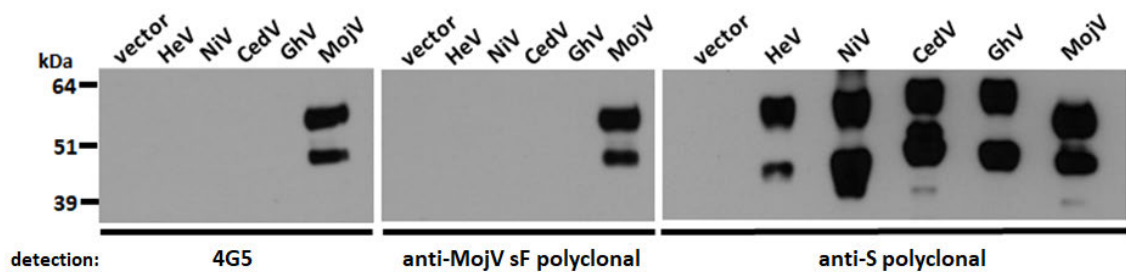
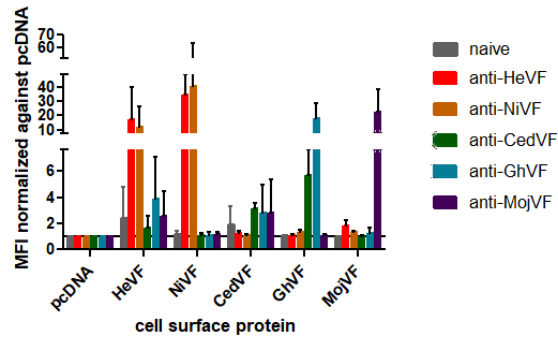


Figure 5-3: Characterization of monoclonal and polyclonal anti-MojV sF antibodies by western blot analysis.

Lysates from BSR-T7/5 cells transfected with one full-length S-tagged henipavirus F glycoprotein construct or an empty vector were collected 48h post-transfection and precipitated with S beads. Precipitates were resolved by SDS PAGE and protein blots were probed with anti-MojV sF monoclonal antibodies 4G5 and 3C4 (negative data not shown), as well as mouse anti-MojV sF polyclonal antibodies and, as a control of protein expression, HRP-conjugated rabbit anti-S peptide antibody.

To confirm this hypothesis, flow cytometry experiments were performed to examine the ability of anti-MojV sF monoclonal 4G5 and 3C4 and polyclonal antibodies to detect full-length cell surface expressed MojV F in its native conformation, as well as their possible cross-reactivity to other henipavirus F glycoproteins in their native form. In addition, anti-HeV and -NiV sF polyclonal antibodies served as controls for known detectable cross-reactivity to NiV and HeV F glycoproteins, respectively. Experiments run in triplicates were designed similarly to those conducted to characterize anti-MojV sG and described above. Monoclonal antibodies 4G5 and 3C4 were able to bind to the native form of MojV F with specificity (**Figure 5-4b**). Polyclonal antibodies for each henipavirus F detected their respective glycoproteins demonstrating that each construct was expressed; however, one can note that the normalized MFI levels for CedV F were much lower than those recorded for other henipavirus F, which may be due to lower levels of cell surface expression of this construct (**Figure 5-4a**). Full-length cell surface MojV F was recognized by anti-MojV sF polyclonal serum, and no cross-reactivity from other polyclonal anti-henipavirus F antibodies was seen toward MojV F glycoprotein (**Figure 5-4a**). Nevertheless, some limited cross-reactivity was observed between anti-MojV sF polyclonal antibodies and HeV F and CedV F glycoproteins, whereas anti-HeV sF polyclonal antibodies bound to NiV F, and the converse. Anti-GhV sF polyclonal antibodies also recognized HeV F and CedV F, and anti-CedV sF polyclonal antibodies detected GhV F, but not HeV F (**Figure 5-4a**).

(a)



(b)

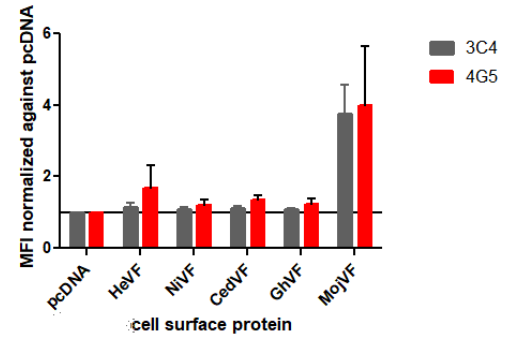


Figure 5-4: Assessment of anti-MojV F antibodies cross-reactivity to other henipavirus F glycoproteins.

BSR-T7/5 cells transiently expressing one henipavirus (HNV) F glycoprotein, or an empty pcDNA vector, were incubated with **(a)** rabbit naïve or polyclonal serum raised against each of the henipavirus glycoproteins or **(b)** one of the anti-MojV F monoclonal antibodies (4G5, 3C4). Antibodies bound to cell surface glycoproteins were detected with secondary goat anti-rabbit IgG(H+L)::AlexaFluor647® or goat anti-mouse IgG (H+L)::AlexaFluor488®. Data is presented as median fluorescent intensity (MFI) normalized against the signal obtained from cells transfected with the empty vector. The background level is set at 1 and represented by a grey line. Experiments were performed in triplicates and data was analyzed by FlowJo3 software (FlowJo LLC, Ashland, OR).

Next, immunoprecipitation experiments were performed to further characterize anti-MojV F monoclonal antibodies 4G5 and 3C4 based on a design identical to that described above in testing anti-MojV G antibodies. Both 3C4 and 4G5 antibodies were able to bind full-length S-tagged MojV F and did not recognize other henipavirus F glycoproteins, while protein expression of all henipavirus F constructs was confirmed by probing for their S-tag (**Figure 5-5**). The same amount of 3C4 and 4G5 antibody (2 μ g) was used in these experiments, yet one can note that weaker F₀/F₁ bands were detected for 3C4 immunoprecipitation of MojV F compared to 4G5 despite identical blot exposure time (**Figure 5-5**), further highlighting that these two antibodies may recognize different epitopes of MojV F with different binding strengths.

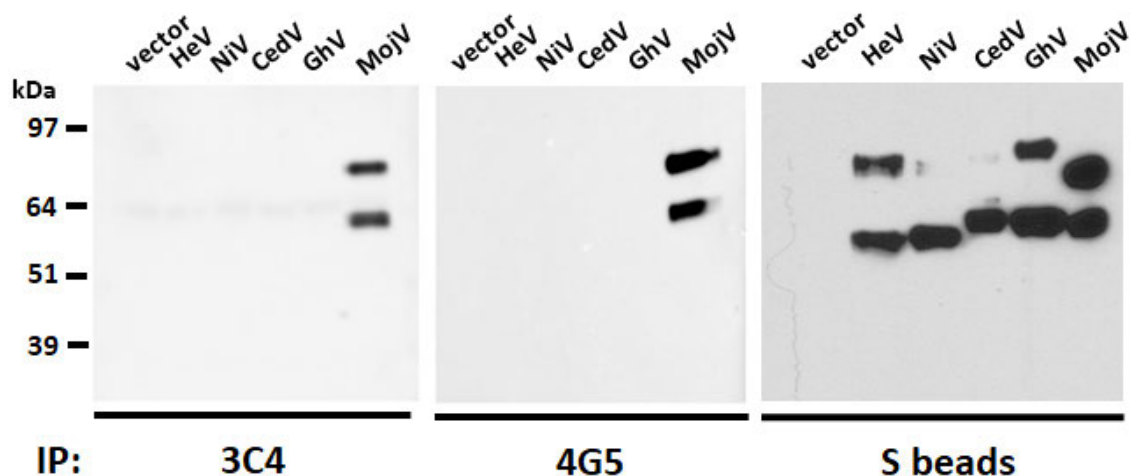


Figure 5-5: Immunoprecipitation of full-length MojV F glycoprotein by monoclonal anti-MojV sF antibodies 4G5 and 3C4.

Equal aliquots of cell lysates from BSR-T7/5 cells transfected with one S-tagged henipavirus F glycoprotein at the time or an empty vector were incubated with each monoclonal antibody. Antibody/F glycoprotein complexes were precipitated with G agarose beads or with S beads as a protein expression control. Precipitates were resolved by SDS PAGE and transferred onto nitrocellulose membranes which were probed with HRP-conjugated rabbit anti-S peptide polyclonal antibody.

Application of a multiplex microsphere immunoassay to rodent fecal samples as a non-invasive collection alternative to blood serum samples

Carvalho et al. (47) demonstrated that immunization of BALB/c mice can be monitored by assessing fecal IgG levels by ELISA as an alternative to measuring serum IgG collected via tail bleeds. These authors argue that fecal sampling allows for daily collection, decreased distress for the animals in comparison to blood collection and requires less technical skills (47). It was thus hypothesized that rodent fecal antibodies could be used to evaluate exposure to henipaviral F and G glycoproteins as an alternative to blood serum antibodies, and that this could be tested in a MMIA. In addition, secretory IgA is the most abundant immunoglobulin isotype produced by the colonic mucosa, followed by IgM and IgG (41; 42), and Soay sheep (*Ovis aries*) IgG and IgA fecal antibodies against the gastrointestinal parasitic nematode *Teladorsagia circumcincta* can be detected by ELISA (254). Furthermore, the transmission route of the Bangladeshi strain of NiV can involve the consumption of contaminated raw date palm sap allowing for exposure to viral antigens through the digestive tract mucosa (113; 211). Therefore, the reactivity of murine fecal IgG and IgA was assessed in this study. Moreover, due to the relative instability of henipavirus F glycoproteins which tend to fold into their post-fusion conformation, most of the serology studies conducted to evaluate exposure to henipaviruses or henipa-like viruses have focused on antibody reactivity to henipavirus G glycoproteins, save for recent work by Brook et al. (45) who integrated HeV, NiV and CedV sF antigens in their screening panel. Nevertheless, a proof of principle study was pursued here to assess if exposure to either F or G henipavirus glycoproteins was

detectable from fecal sample reactivity in a MMIA. The current study was focused on GhV sF and MojV sG glycoproteins, as examples of F and G glycoproteins.

Quantification of anti-GhV sF and anti-MojV sG antibodies

Two BALB/c mice per antigen were injected with GhV sF or MojV sG purified proteins (see above). Serum was obtained from tail bleeds as per schedule (**Figure 2-1**) to monitor rising titers by ELISA. Fecal pellets were collected before each tail bleed was performed and pooled per type of antigen injected each time. Polyclonal fecal antibodies were extracted by re-suspending fecal matter in 1XPBS supplemented with protease inhibitors. Supernatants were clarified by centrifugation and applied sequentially to 0.45 μm then 0.22 μm filters to further remove debris. IgG and IgA quantification of polyclonal blood serum and fecal samples was conducted with commercial ELISA kits from Mabtech, Inc. (**Figure 5-6**). As anticipated, IgA concentrations were higher than IgG concentrations in fecal samples, whereas the converse was true for serum samples, and overall antibodies levels were higher in serum samples than in fecal samples (**Figure 5-6**). Also, mice immunized with MojV sG glycoprotein seemed to have a lesser serum IgG and fecal IgG and IgA response compared to those immunized with GhV sF glycoprotein (**Figure 5-6**).

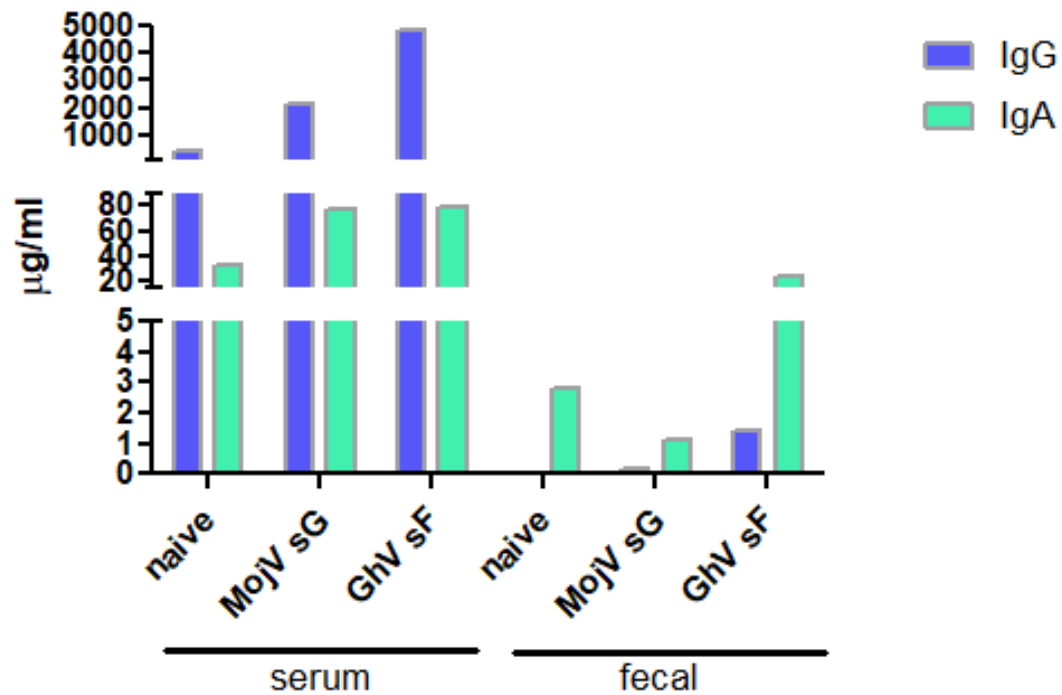


Figure 5-6: Quantification of blood serum and fecal IgG and IgA antibodies from BALB/c mice immunized with MojV sG or GhV sF glycoprotein. IgG and IgA levels in polyclonal blood serum and fecal samples obtained at the time of sacrifice from naïve mice or mice injected with MojV sG or GhV sF glycoproteins were measured with commercial ELISA kits from Mabtech, Inc.

Serum IgG obtained from final exsanguination at the time of sacrifice was purified by application to Poly-Prep® Chromatography Column (Bio-Rad) containing protein G slurry. Purified serum IgG was then concentrated with a 30kD Amicon® Ultra-15 Centrifugal Filter Unit (Millipore Sigma) and concentrations were determined with a NanoDrop™ spectrophotometer. To facilitate large scale protein purification and original detection, the soluble henipavirus F and G constructs are designed to express a S-tag, which is followed by a GCN4 trimerization motif for sF or a GCN4 tetramerization motif for sG (**Figure 4-2a and 3-4a**). Addition of a factor Xa cleavage site in the constructs allows for tag removal from purified protein preparations prior mice immunization. Work done in our lab across several S-tagged glycoproteins projects revealed that the S-tag is a highly potent immunogen and that injection of a S-tagged protein can generate a strong cross-reactive antibody response. Although the S-tag was successfully removed from GhV sF glycoproteins, Lianying Yan found in western blot experiments that the S-tag could not be removed from MojV sG purified glycoprotein. This inability to remove the S-tag from MojV sG may be due to a spatial conformation precluding access of the protease to the factor Xa cleavage site. The issue of anti-S-tag reactivity is problematic because the magnetic beads used in the MMIA are coupled to the same purified glycoproteins used to immunize mice, and therefore will still bare a S-tag as well. A positive signal for reactivity to MojV sG could then be masked by antibodies recognizing the S-tag instead of MojV sG specifically. To remove anti-S antibodies from purified serum IgG from mice immunized with MojV sG, SulfoLink agarose resin was conjugated to synthetic S peptide as per manufacturer guidelines and incubated with serum IgG. Two rounds of adsorption were performed, and loss of anti-S-tag reactivity was confirmed by

ELISA (data not shown). Intriguingly, such S-tag reactivity was not observed in fecal polyclonal antibodies from MojV sG immunized mice. The reasons of this discrepancy remain unknown. Purified serum IgG and fecal antibodies from mice immunized with tag-less GhV sF glycoprotein preparations were found to contain anti-GCN4 trimerization motif reactive antibodies (data not shown). A possible hypothesis explaining this phenomenon is that the removal of the S-tag from sF glycoprotein preparations may have allowed access to the GCN4 trimerization motif, which then became an epitope that could engage the immune response. To remedy this issue, a similar strategy was applied using SulfoLink agarose resin conjugated to synthetic GCN4 trimerization peptide to adsorb anti-GCN4 trimerization motif antibodies.

Application of multiplex microsphere immunoassay to rodent blood serum samples as a screening tool for exposure to henipavirus F and G glycoproteins

Bio-Plex Pro™ Magnetic COOH Beads were coupled to purified soluble HeV, NiV, CedV, GhV and MojV F and G glycoproteins. Quality control tests were performed to confirm successful coupling of each glycoprotein type to its corresponding bead by evaluating the ability of purified rabbit IgG antibodies to detect their respective immunogens on the coupled beads at standard concentrations of 50 and 500 ng IgG/well. Control beads provided by Lianying Yan included negative control beads coupled with mock protein preparation obtained from culture supernatant of cells that do not express recombinant soluble viral glycoproteins. In addition, beads coupled to S-tagged menangle virus glycoprotein (MenV sHN) were included as a negative control for S-tag reactivity as well as GCN4 tetramerization motif reactivity, and, MenV being a member of the genus *rubulavirus*, to serve as an outside group paramyxovirus control. Finally, beads

coupled to Marburg virus soluble glycoprotein (MARV sGp) were used as a control for GCN4 trimerization motif reactivity. Each bead type is identified by its unique fluorescence emission signature and assigned a specific number; the bead number assignments to each glycoprotein used in this study are reported in **table 2-1**. Purified murine serum IgG raised against MojV sG and GhV sF glycoproteins were tested for reactivity to HeV, NiV, CedV, GhV and MojV sG and sF panels, respectively. Purified murine naïve serum IgG was included in this study to ensure specificity of the assay and act as a negative control (**Figure 5-7c, d**).

Assessment of purified murine anti-MojV sG serum IgG reactivity to a comprehensive henipavirus sG panel by multiplex microsphere immunoassay

A titration curve for anti-MojV sG serum was established and the lower asymptote end point of this curve approached to 0.1 ng IgG/ml with an average MFI of 423 based on the reactivity to MojV sG glycoprotein (**Figure 5-7a**). The lower limit of detection (LLD) of the assay was calculated by adding two standard deviations to the mean MFI of the negative control (naïve serum) (14), and identified as an average MFI value of 580. Similarly to the analysis by McNabb et al. (155) on the evaluation of microsphere immunoassays applied to serum reactivity to HeV and NiV sG glycoproteins, a third order polynomial regression curve ($R^2=0.9944$) was fitted to the titration curve data to interpolate the anti-MojV sG serum IgG concentration corresponding to the LLD MFI (0.64 ng IgG/ml). MFI signal saturation was reached at 30464 for an anti-MojV sG serum IgG concentration of 200 ng IgG/ml (**Figure 5-7a**). The coefficient of variation (CV) was below 10% (7.65%). Anti-MojV sG serum IgG was able to detect GhV sG and reached an average MFI of 10,979 at the end

concentration of 200 ng IgG/ml, whereas it detected CedV sG and HeV sG with a maximum average MFI of 7281 and 6042 at 200 ng IgG/ml, respectively (**Figure 5-7a**). Additionally, anti-MojV sG serum IgG also bound MenV sHN, reaching an average of 5515 MFI at 200 ng IgG/ml (**Figure 5-7a**).

Assessment of purified murine anti-GhV sF serum IgG reactivity to a comprehensive henipavirus sF panel by multiplex microsphere immunoassay

The anti-GhV sF serum IgG titration curve of reactivity to GhV sF glycoprotein approached its lower asymptote at the end point concentration of 0.1 ng IgG/ml with an average MFI of 163.75 (**Figure 5-7b**). The analytical sensitivity of this assay was again evaluated by calculating the LLD and fitting a third order polynomial regression curve ($R^2=0.9911$) to the results. The LLD threshold value of 127 MFI was found to correspond to an interpolated concentration of 0.20 ng IgG/ml. Saturation of the MFI signal was recorded at 30415 at a concentration of 200 ng IgG/ml (**Figure 5-7b**). The CV approached 10% (10.78%). Cross-reactivity of anti-GhV sF serum IgG was observed toward HeV, NiV and CedV sF glycoproteins, with average MFI values of 12542, 8000 and 7269 reached at the 200 ng IgG/ml higher end point concentration of this assay (**Figure 5-7b**).

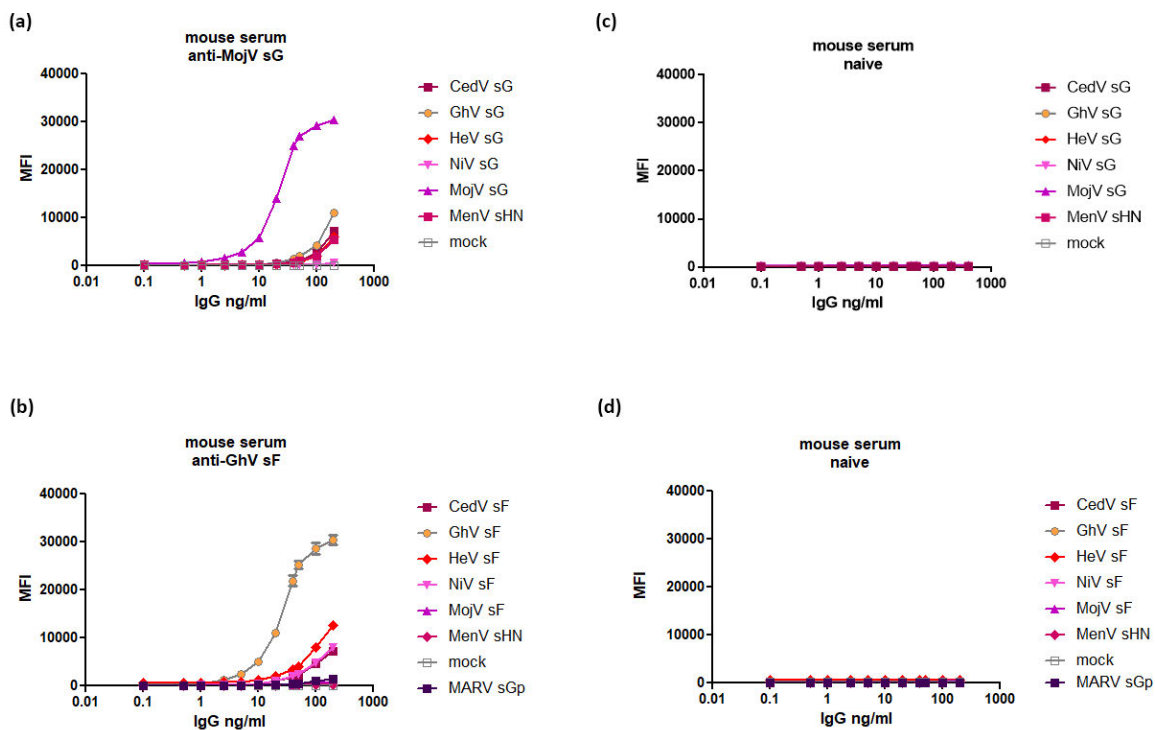


Figure 5-7: Application of multiplex microsphere immunoassay to rodent blood serum samples as a screening tool for exposure to henipavirus F and G glycoproteins.

Panels of multiplexed Bio-Plex Pro™ Magnetic COOH Beads coupled to recombinant henipavirus F or G glycoproteins and including control beads coupled to MenV sHN, MARV sGp and mock protein were incubated with different dilutions of purified murine (a) anti-MojV sG and (b) anti-GhV sF or (c) (d) naïve serum IgG. Biotin conjugated polyclonal anti-mouse IgG (1:500) was applied followed by 100 ng/well of streptavidin-R-phycoerythrin (SA-PE) to detect IgG bound to the glycoproteins coupled to the beads. Mixtures were analyzed by a Bio-Plex® 200 array reader with microplate platform for 96 well plate automated reading (Bio-Rad, Hercules, CA). Data was acquired with Bio-Plex Manager™ software (Bio-Rad, Hercules, CA).

Application of multiplex microsphere immunoassay to rodent fecal antibodies as a screening tool for exposure to henipavirus F and G glycoproteins

The same multiplex panels of henipavirus sF, sG and control beads described above were used to test the reactivity of polyclonal fecal murine anti-MojV sG and anti-GhV sF IgG and IgA antibodies.

Assessment of murine anti-MojV sG fecal IgA and IgG reactivity to a comprehensive henipavirus sG panel by multiplex microsphere immunoassay

The lowest concentration of anti-MojV sG fecal IgA evaluated was 2.82 ng IgA/mg feces at an average MFI of 486 (**Figure 5-8a**). Results from the titration curve of reactivity to MojV sG coupled beads were fitted to a fourth order polynomial regression curve ($R^2=0.9962$). The LLD of this assay was found to be 756 MFI, which corresponded to an interpolated concentration of 0.03 μ g IgA/mg feces. Saturation of the signal was attained at an average MFI of 31144 at 5.64 μ g IgA/mg feces (**Figure 5-8a**). The CV of this assay was below 10% at 7.38%. As was observed for anti-MojV sG serum IgG, anti-MojV sG fecal IgA antibodies cross-reacted with GhV sG, CedV sG, HeV sG and MenV sHN glycoproteins with maximum average MFI of 10732, 4597, 4491 and 5281, respectively (**Figure 5-8a**).

The lower asymptote end point of the titration curve for the anti-MojV sG fecal IgG curve was 0.16 μ g IgG/mg feces with an average MFI of 538 based on the reactivity to MojV sG glycoprotein (**Figure 5-8b**). A fourth order polynomial regression curve ($R^2=0.9912$) was applied to the data. The LLD was calculated to be 1395 MFI corresponding to an interpolated concentration of 3.06 μ g IgG/mg feces. Saturation of the

signal was reported at 31528 for an anti-MojV sG fecal IgG concentration of 657.77 μg IgG/mg feces (**Figure 5-8b**). The CV was 4.24%. Anti-MojV sG fecal IgG cross-reacted with GhV sG, CedV sG and HeV sG, as well as MenV sHN glycoproteins, and reached an average MFI of 13311, 6286, 2048 and 3699, respectively at the higher end point concentration of this assay (**Figure 5-8b**). These results followed a similar pattern to what was observed in the anti-MojV sG serum IgG and anti-MojV sG fecal IgA studies described above.

Assessment of murine anti-GhV sF fecal IgA and IgG reactivity to a comprehensive henipavirus sF panel by multiplex microsphere immunoassay

The highest concentration of murine anti-GhV sF fecal IgA tested was 24.20 μg IgA/mg feces and reached average MFI of 28564, whereas the lowest concentration tested was 6.20 ng IgA/mg feces with average MFI of 106 (**Figure 5-8e**). The LLD of the assay was determined to be 616 MFI and a fifth order polynomial regression curve ($R^2=0.9971$) was fitted to the titration curve to interpolate the corresponding concentration of 1.97 μg IgA/mg feces. The assay had a CV of 3.55%. Cross-reactivity of anti-GhV sF fecal IgA antibodies was recorded with CedV sF, HeV sF and MenV sHN with average MFI values reaching 1936, 6172 and 3197, respectively, at the highest concentration evaluated (24.20 μg IgA/mg feces) (**Figure 5-8e**).

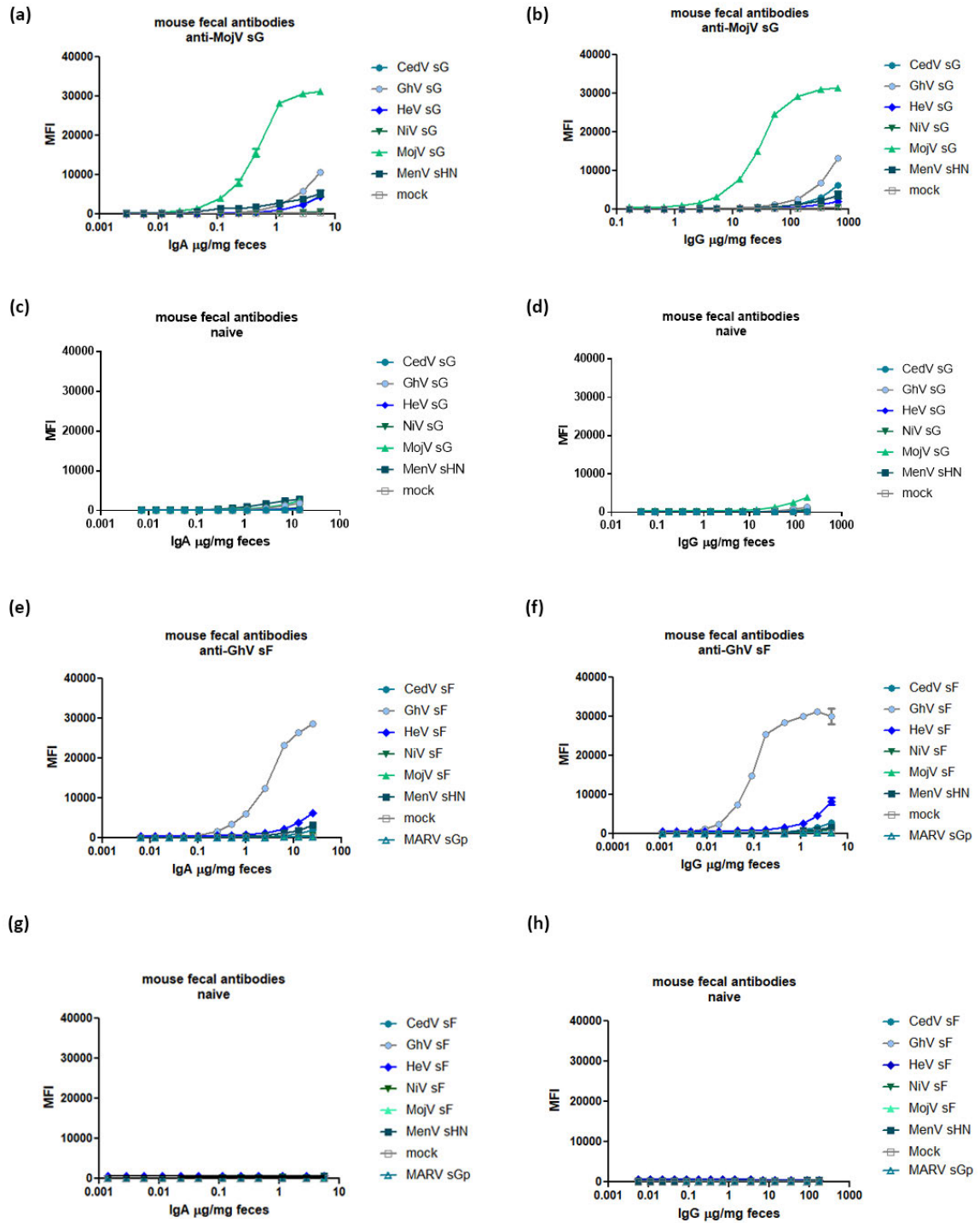
A third order polynomial regression curve ($R^2=0.9992$) was fitted to the anti-GhV sF fecal IgG titration curve data. The LLD for this assay was identified at an average MFI of 625 corresponding to an interpolated concentration of 11.79 ng IgG/mg feces, and the CV for this assay was found to be 5.5%. The lowest concentration tested was 1.11 ng IgG/mg feces and resulted in an average MFI of 226, while saturation of the signal from

GhV sF coupled beads was reached at 31169 MFI with a 2.21 μg IgG/mg feces (**Figure 5-8f**). Cross-reactivity of anti-GhV sF fecal IgG antibodies to HeV sF, CedV sF and NiV sF glycoproteins was recorded at average MFI values of 8325, 2794 and 1167, respectively, at the highest concentration tested in this assay (4.42 μg IgG/mg feces) (**Figure 5-8f**).

Overall, these results demonstrate that rodent serum can be tested in MMIA to successfully detect exposure to henipavirus F and G glycoproteins. Moreover, this study shows that fecal samples can be used as a non-invasive alternative to blood collection to screen for exposure to henipavirus F and G glycoproteins in rodents.

Figure 5-8: Application of multiplex microsphere immunoassay to rodent fecal filtrates as a screening tool for exposure to henipavirus F and G glycoproteins.

Panels of multiplexed Bio-Plex Pro™ Magnetic COOH Beads coupled to recombinant henipavirus F or G glycoproteins and including control beads coupled to MenV sHN, MARV sGp and mock protein were incubated with varying dilutions of purified murine **(a)** anti-MojV sG and **(b)** anti-GhV sF or **(c) (d)** naïve fecal filtrates. Biotin conjugated polyclonal anti-mouse IgG (1:500) or anti-mouse IgA (1:250) was applied to bead mixtures after incubation with the fecal samples, followed by 100 ng/well of streptavidin-R-phycoerythrin (SA-PE) to detect IgG or IgA bound to the glycoproteins coupled to the beads. Contents of the wells were analyzed by a Bio-Plex® 200 array reader with microplate platform for 96 well plate automated reading (Bio-Rad, Hercules, CA). Data was acquired with Bio-Plex Manager™ software (Bio-Rad, Hercules, CA).



DISCUSSION

Means of detection for the emerging henipavirus MojV are currently lacking. In the present serology study murine and leporine polyclonal antibodies as well as murine monoclonal antibodies against MojV sF and sG glycoproteins were generated as novel tools to help in the study of the molecular biology of this virus and in the elaboration of serologic assays.

Monoclonal anti-MojV sF antibody 3C4, but not 4G5, proved to be conformation dependent in western blot, flow cytometry and immunoprecipitation experiments (**Figures 5-3, 5-4 and 5-5**), making these antibodies differential tools that could be used to investigate the structure of MojV F glycoprotein. In addition, polyclonal anti-MojV sF serum cross-reacted with CedV F and HeV F in a very limited capacity when tested in flow cytometry assays (**Figure 5-4a**), suggesting that these glycoproteins share some common epitope(s) recognized by anti-MojV sF polyclonal antibodies. However, this cross-reactivity has not been evaluated in MMIA and was not apparent in the western blot analysis (**Figure 5-3**), thus requiring further investigation to be adequately confirmed.

In addition, anti-CedV sF polyclonal antibodies bound to GhV F, but not to HeV F (**Figure 5-4a**), in agreement with phylogenic analysis showing that CedV F is most closely related to GhV F than to other henipavirus F glycoproteins (**Figure 5-9b**) (282). As expected, control cross-reactivity was observed between anti-HeV sF polyclonal antibodies and NiV F, and the converse (**Figure 5-4a**). These results are supported by the close relatedness of these glycoproteins displayed in phylogenic analysis (**Figure 5-9b**) (282) and in protein sequence alignments showing 87% shared identity (**Table 3-1**). Anti-GhV sF polyclonal antibodies detected HeV and CedV F glycoproteins, but not NiV F

glycoprotein, in flow cytometry experiments (**Figure 5-4a**). However, the MMIA demonstrated that anti-GhV sF serum can bind to NiV sF, indicating that this assay can provide more sensitive detection of antibody reactivity (**Figure 5-7b**). The cross-reactivity of anti-GhV sF serum and fecal IgG, albeit to a lesser extent, to HeV, NiV and CedV sF glycoproteins can be explained in view of the close relatedness evidenced by phylogenetic analysis of henipavirus F glycoproteins (282) (**Figure 5-9b**). Moreover, GhV F percent identity of amino acid sequence alignment with HeV, NiV and CedV F is 44%, 44% and 32%, respectively (**Table 3-1**). Epitopes shared by these glycoproteins were likely recognized by polyclonal anti-GhV sF serum IgG. The lack of cross-reactivity to MenV sHN also confirmed that S-tag removal from the purified GhV sF protein preparation used to immunize mice was complete. Likewise, absence of cross-reactivity to MARV sGp indicated that anti-GCN4 tetramerization motif antibodies were thoroughly removed from the purified anti-GhV sF serum IgG used in these assays.

Monoclonal anti-MojV sG antibodies 2B2, 6E5 and 10G2 were shown to be conformation dependent and can be used for the detection of full-length cell surface expressed MojV G in flow cytometry and immunoprecipitation experiments (**Figures 5-1 and 5-2**). Polyclonal and monoclonal anti-MojV sG antibodies were found to be specific and did not cross-react with other henipavirus G glycoproteins in western blot and flow cytometry experiments. However, cross-reactivity of anti-MojV sG serum IgG with GhV, CedV and HeV sG glycoproteins was observed in the more sensitive MMIA (**Figure 5-7a**). Similar patterns of cross-reactivity to GhV, CedV and HeV sG glycoproteins were observed with anti-MojV sG fecal IgG (**Figure 5-8b**) and IgA (**Figure 5-8a**). This could be explained by the fact that MojV G is most closely related to GhV G, then only to

CedV G and HeV G (**Figure 5-9a**) (282). Interestingly, no cross-reactivity of serum and fecal antibodies from MojV sG immunized mice was detected with NiV sG in the MMIA even though HeV and NiV G share 78% identity in their amino acid sequences (**Table 3-1**). This suggests that the epitopes recognized by polyclonal anti-MojV sG serum IgG are present in GhV, CedV and HeV G, but not in NiV G. Anti-MojV sG serum IgG also detected MenV sHN glycoprotein in the same concentration and MFI range as CedV and HeV sG in the MMIA. Cross-reactivity to MenV sHN may be due to residual S-tag reactivity of anti-MojV sG serum IgG incompletely adsorbed of its anti-S antibodies rather than to the MenV sHN glycoprotein itself. Successful removal of cross-reactive anti-S antibodies was confirmed by ELISA. Nevertheless, Luminex technology-based assays provide higher sensitivity than traditional ELISA experimental designs. Therefore, more than likely, although residual anti-S antibodies were still present in very low quantities in the purified and adsorbed anti-MojV sG serum IgG preparations tested in this study, they were not detectable in the context of an ELISA, again demonstrating the superior sensitivity of the Luminex technology-based microsphere immunoassay. Cross-reactivity to the S peptide may be masking a possible true cross-reactivity to CedV and HeV G glycoproteins as well. To confirm or refute this hypothesis, control beads coupled to synthetic S peptide could be included in multiplex panels tested in future assays.

The results of these studies confirm the applicability of the MMIA to the detection of rodent antibodies specific to henipavirus glycoproteins present in both serum and fecal materials. This highly sensitive assay can be regarded as a valuable option in techniques applied to surveillance work, to assess henipavirus exposure and detect

possible spillover events. Knowledge regarding the presence of henipviruses and henipa-like viruses in rodent populations is extremely limited. To date, the study by Sasaki et al.(224), which detected henipavirus-related paramyxoviruses in Zambian *Crocidura hirta* (lesser red musk shrew) and *Rattus rattus* (black rat) by RT-PCR analysis, is the sole publication reporting on henipaviruses in rodents since the identification of MojV. The discovery of MojV (266), highlights the need for a better understanding of the role that rodents may play in the circulation of henipaviruses. The present study shows that MMIA can be applied to rodent samples and support future serologic surveillance in these populations of potential henipavirus carriers. Furthermore, the results demonstrate that rodent fecal material may be used as a non-invasive alternative to blood samples in this assay, increasing opportunities and sources for sample collection in the field. Indeed, the wide availability of rodent fecal material and ease of collection makes it an attractive option to conduct such studies.

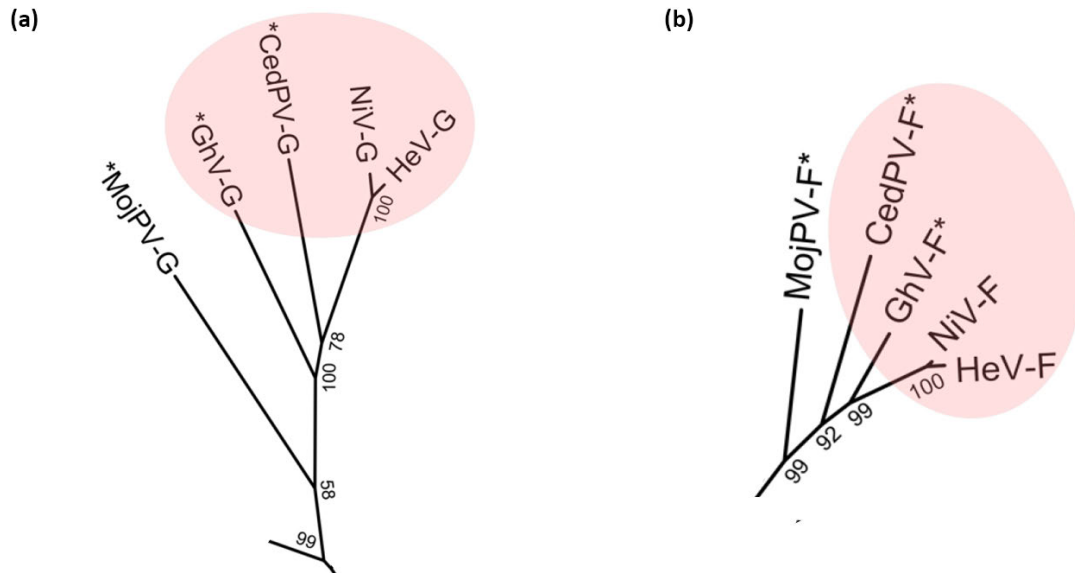


Figure 5-9: Maximum likelihood phylogeny of henipavirus attachment and fusion glycoproteins sequences.

Relatedness between (a) G and (b) F glycoproteins of different henipavirus species are represented in phylogenetic trees generated with MEGA6 (Molecular Evolutionary Genetics Analysis Version 6.0). Numbers at the nodes represent bootstrap values (1,000 replicates). Uncategorized viruses awaiting taxonomic evaluation by the International Committee on Taxonomy of Viruses at the time of publication of the original manuscript are marked with an asterisk. Virus names (abbreviations) and GenBank accession numbers are as follows: Mojjiang virus (MojPV) NC_025352; Ghanaian bat henipavirus (GhV) HQ660129; Cedar virus (CedPV) NC_025351; Nipah virus (NiV) NC_002728; Hendra virus (HeV) NC_001906; Adapted from Zeltina A, Bowden TA, Lee B (2016) Emerging Paramyxoviruses: Receptor Tropism and Zoonotic Potential. PLoS Pathog 12(2): e1005390. <https://doi.org/10.1371/journal.ppat.1005390> (creative commons license).

CHAPTER 6: Discussion

Three Chinese miners from Mojiang Hani Autonomous County, Yunnan Province, died from severe pneumonia of unknown etiology in 2012, prompting a search for possible causative novel zoonotic pathogens (266). Collection of anal swabs from twenty *Rhinolophus ferrumequinum* bats, nine *Rattus flavipectus* rats, and five *Crocidura dracula* musk shrews from the abandoned cave was conducted six months after the workers' deaths, and led to the discovery of a novel henipavirus, Mojiang virus (MojV), in three of the *Rattus flavipectus* samples (266). It may seem surprising that none of the bat samples processed were positive for henipavirus nucleic acids, given that these mammals have been identified as the natural reservoir of henipaviruses (266). However, the sample size of this study was notably limited and *Rhinolophus ferrumequinum* bats, also called greater horseshoe bats, have not been reported to carry henipaviruses in previous surveillance studies. Nevertheless, their geographic distribution, which encompasses Europe, Northern Africa, Central Asia and Eastern Asia (205), may in some localities overlap with that of established henipavirus pteropid hosts with whom they may interact or co-roost. Moreover, detection of henipavirus nucleic acid in bats can be challenging as these hosts are able to control infection well and tend to display transient viremia with low viral titers that can only be detected during a narrow time frame (100). Therefore, *Rhinolophus ferrumequinum* bats may still serve as yet-to-be-confirmed carriers of henipaviruses. Expanding sample size and rodent and bat species range would provide a more accurate estimate of the extent of the circulation of henipa-like or henipaviruses in wildlife and peridomestic populations of the Yunnan area and other regions.

Importantly, the emerging MojV is the first henipavirus fully sequenced from rodent field samples discovered to date (266). However, rodents are known hosts of paramyxoviruses. Murine paramyxoviruses include Sendai virus (SeV) (84), Nariva virus (NarPV) (134; 243), J virus (JV) (114; 117) and murine pneumonia virus (MPV) (77), whereas Mossman virus (MoV) (161), Beilong virus (BeiV) (141; 265), Tailam virus (TaiV) (264), Mount Mabu Lophuromys paramyxovirus 1 (MMLPV-1) and Mount Mabu Lophuromys paramyxovirus 2 (MMLPV-2) (246) were first reported in rats. To the best of our knowledge, only two paramyxoviruses have been discovered in shrews: Tupaia virus in Asian tree shrew (*Tupaia belangeri*) (TupV) (242) and henipa-related paramyxoviruses in Zambian musk shrew (*Crocidura hirta, luna*, sp.) (224). More recently, a novel paramyxovirus, Pohorje Myodes paramyxovirus 1 (PMPV-1), was identified in a bank vole (*Myodes glareolus*) in Slovenia (246). It is therefore possible that rodents may play a more significant role than currently understood as natural hosts of henipaviruses.

In light of the limited knowledge available on MojV, in particular regarding its host and/or reservoir range, as well as its cellular and receptor tropism features, the present research project was centered on the overall hypotheses that MojV, like other henipaviruses, could gain host cell entry via mechanisms that require its attachment (G) and fusion (F) glycoproteins, potentially employing an ephrin ligand(s) as an entry receptor, and that MojV would display species-specific tropism suggesting that different henipaviruses could be found in animal reservoirs other than bats.

Summary and discussion of the results in the context of the aims

Aim 1:

The first aim of this study was to assess MojV species and entry receptor tropism. However, no MojV isolate has ever been recovered and this henipavirus is known only from nucleic acid sequence data (266). Further, because MojV has unknown pathogenic potential a recombinant virus rescue by reverse genetics approach is not possible outside of BSL-4 containment. Therefore, the development of recombinant molecular tools was required to perform the proposed research goals within BSL-2 settings. In order to examine MojV species and entry receptor tropism the generation and characterization of recombinant MojV F and G envelope glycoproteins were required to determine that these sequence-identified glycoproteins were structurally and functionally competent. In the present study, tagged and untagged recombinant MojV F and G glycoprotein constructs were designed and expressed in rodent cells. The size, migration patterns and oligomerization status of these glycoproteins, as well as the cellular processing of the precursor MojV F₀ into a mature F₁+F₂ form of the fusion protein, confirmed that the sequence of MojV F and G glycoproteins reported from the original discovery of this virus does encode henipaviral glycoproteins with apparent integrity. The functionality of the recombinant MojV F and G constructs used in this work was demonstrated qualitatively (syncytia formation assay) and quantitatively (Nano Luciferase cell-cell fusion reporter-gene assay). Taken together, these results confirm the nature of the MojV sequence identified and support the predicted relatedness and classification of MojV as a novel henipavirus. Moreover, the syncytia and cell-cell reporter-gene fusion assays performed in this study allowed for the investigation of MojV F and G species

susceptibility and demonstrated that these glycoproteins can mediate membrane fusion with human, non-human primate and rodent cell lines, in particular with rat glioma cells. This suggests that the zoonotic potential of MojV should be considered, and further investigation of MojV, perhaps through reverse genetics rescue of replication-competent virus while abiding to appropriate safety containment, is needed.

The fact that HeV, NiV, CedV and GhV G glycoproteins all mediate cell-cell fusion by interacting with one or more ephrin ligands as a functional entry receptor(s) prompted the hypothesis that MojV G glycoprotein could mediate cell-cell fusion in an ephrin ligand-dependent manner. Moreover, as CedV G can bind and utilize murine ephrin-A1 but not human ephrin-A1 as an entry receptor, assessing the possible species specificity of MojV, the first henipavirus discovered in a rodent, was warranted. Entry receptor specificities play a critical role in restricting interspecies transmission of viruses. Mutations in key residues of the binding domains of entry receptors can result in expanding species tropism of viruses. For example, Li et al. (138; 139) have shown that mutations in residues 82, 83, 84 and 353 of rat angiotensin-converting enzyme 2 (ACE2) can convert it to a competent entry receptor for severe acute respiratory syndrome-related coronavirus (SARSr-CoV), which utilizes human ACE2 more efficiently to gain host cell entry. Moreover, a single residue mutation in the G-H loop of ephrin ligand A1 from human T₁₁₅ to murine I₁₁₅ restricts CedV G to interactions with murine ephrin ligand A1 (131). Findings from this study did suggest that MojV F and G glycoproteins could mediate cell-cell fusion by interacting with target cells expressing murine ephrin-A1, rat ephrin-A4 and human ephrin-A5 in over-expressed ephrin ligand transfection experiments. Because MojV G was able to mediate cell-cell fusion, albeit at very

inefficient levels, with cells expressing rat ephrin-A4, but not human ephrin-A4, a possible species specificity was further investigated by creating mutants mimicking rat ephrin-A4 G-H loop (S119P) in a human ephrin-A4 background and the converse (P122S). However, these additional cell-cell fusion assays revealed that the contribution of rat ephrin-A4 to MojV F and G mediated membrane fusion is not determined by the rat amino acid residue P₁₂₂, suggesting that domains outside of the G-H loop of rat ephrin-A4 might play a role in some functional interaction that allowed for limited MojV F and G glycoprotein mediated cell-cell fusion. Overall, however, the suggestive functional use of some ephrin ligands (murine ephrin-A1, rat ephrin-A4 and human ephrin-A5) as entry receptors for MojV could not be confirmed due to the low levels and slow kinetics of cell-cell membrane fusion observed in both the qualitative (syncytia formation assay) and quantitative (reporter-gene cell-cell fusion assay) evaluations of MojV F and G mediated cell-cell fusion. In addition, the putative interactions between MojV G and ephrin ligands were assessed by co-precipitation assays and more sensitive Bio-Layer Interferometry (BLI) assays, which both failed to show significant binding between MojV G and any ephrin ligand.

One possible explanation for the lack or limited interactions of MojV G with ephrin ligands, may rest on the fact that the only MojV G sequence currently available originated from the report that identified MojV as a novel henipavirus based on viral nucleic acid amplification and sequencing alone (266). Therefore, the original sequencing data reported by Wu et al. (266) may contain possible inaccuracies, which could have impacted residues in the central binding cavity of the β -propeller domain of the globular head of MojV G that may be critical to sustained and productive interactions with ephrin

ligands. However, this hypothesis does not stand considering that Rissanen et al. (215) have solved the crystal structure of MojV G based on the expression of this very sequence. Their analysis concluded that a “closed” conformation limiting MojV G binding interface, at least in the case of ephrin ligands B2 and B3, was the result of steric hindrance involving two of the six blades of the β -propeller domain of MojV G, blades one and five (215). The fact that such an unfavorable spatial arrangement would be the result of sequencing errors seems improbable. Studies modeling docking of A-class ephrin ligands as well as ephrin-B1 to MojV G could help confirm that this steric hindrance would similarly limit binding of ephrin ligands other than B2 and B3 to the central cavity of MojV G. Presently, the primary cognate entry receptor of MojV G and the nature of the contribution, albeit limited, of A-class ephrins to MojV F and G mediated cell-cell fusion remain unknown. In silico screens of protein ligand structure databases may help identify potential binding partners and putative entry receptor candidates of MojV G, opening new avenues of investigations in the search of MojV primary entry receptor(s). While MojV is currently classified as a henipavirus by the ICTV, the surprising inability of MojV F and G to mediate membrane fusion with ephrin-B2, the canonical entry receptor of henipaviruses, and the unusually low levels of fusion recorded with target cells expressing murine ephrin-A1, rattine ephrin-A4 and human ephrin-A5 raise questions as to the position of MojV in the evolution of the henipavirus lineage, its distance to a common ancestor to HeV, NiV, CedV and GhV, and its possible re-categorization as henipa-related or henipa-like paramyxovirus.

Aim 2:

The second aim of this study was to investigate possible structural and/or mechanistic causes for the low levels of MojV F and G mediated cell-cell fusion and the atypical (slow) kinetics observed in the present study and also reported by others (215). The oligomerization status of MojV F and G glycoproteins expressed at the cell surface was assessed in protein cross-linking experiments. These assays revealed that each glycoprotein assembles as their expected higher order oligomeric forms, and properly traffics to the surface of expressing cells. Furthermore, and critically important, the ability of MojV sF to undergo a measurable pre- to post-fusion conformational transition, that is required for facilitating membrane fusion, was confirmed using an in vitro heptad-repeat capture assay, which also suggested that the MojV F pre-fusion trimer is unstable in comparison to CedV (**Figure 4-3b**), NiV (48; 68) and HeV (48) F pre-fusion trimers. Possible sequence inaccuracies introduced during the RT-PCR step of sample processing that yielded the assembled sequences reported by Wu et al. (266) in their discovery of MojV may be causing the atypical features of MojV F and G mediated membrane fusion we and Rissanen et al. (215) observed. Until viral isolates can be recovered from MojV infected specimens, the reference sequence currently available cannot be positively confirmed, and this question remains open to debate. However, Nano luciferase reporter-gene cell-cell fusion assays were performed to evaluate the respective contributions of MojV F and G glycoproteins by testing the functionality of heterotypic F and G complementation pairings including HeV, NiV, CedV, GhV and MojV glycoproteins. These data indicated that MojV F could facilitate more robust cell-cell fusion in the context of triggering by a heterotypic G glycoprotein, GhV G, known to engage with ephrin-B2 as its receptor (**Figure 3-5c**) (136; 256). This suggests that the yet unknown

MojV entry receptor may be absent or expressed at extremely low levels on the surface of cells susceptible to MojV F and G mediated membrane fusion identified in this study.

Taken together, results from these experiments suggest that the lower levels and slower kinetics of cell-cell fusion mediated by MojV F and G, compared to what is observed for other henipaviruses F and G glycoproteins, may be the result of receptor restrictions rather than failing functionality due to structural or mechanistic defects in MojV F and G glycoproteins. However, novel characteristics specific to the MojV F glycoprotein were revealed in the heptad-repeat capture assays and did established that MojV F, contrary to HeV, NiV or CedV F glycoproteins, seems to be less stable in its pre-fusion conformation.

Additional investigations into this phenomenon are warranted. In silico modeling analysis of the structure of MojV F or determination of the molecular structure of MojV F in its pre- and post-fusion conformations could help identify similarities and differences with other henipavirus F glycoproteins, and explain the observed relative instability of MojV F. Still, it is possible that MojV F would be appropriately stabilized by cytoplasmic tail domain interactions with MojV M protein to prevent excessive flexibility or premature triggering of the pre- to post-fusion conformational cascade. Although this hypothesis lays beyond the scope of this work and the experimental designs developed in the present study, it could be examined in the context of a chimeric recombinant CedV/MojV live infection system (further discussed below).

Aim 3:

The third aim of this project was to generate tools to support serologic surveillance studies that can help evaluate exposure to MojV or MojV-like viruses and

detect possible spillover events. An additional goal of this work was to apply a Luminex-based MMIA to screen rodent fecal samples for exposure to henipavirus F and G glycoproteins to provide a non-invasive alternative to blood serum sampling in field studies. Such novel means could help determine the reservoir of MojV and assess the geographic range of circulation of this emerging henipavirus. To address this aim, immunogenic recombinant MojV sF and MojV sG glycoproteins that recapitulate the native oligomerization status of cell surface expressed full length F and G glycoproteins were produced and served to obtain polyclonal and monoclonal antibodies characterized by western blot, flow cytometry and IP experiments. All three monoclonal anti-MojV sG antibodies (2B2, 6E5 and 10G2) obtained were found to be specific and to detect MojV G glycoprotein in a conformation-dependent manner, whereas monoclonal anti-MojV sF antibody 3C4, but not 4G5, was shown to be conformation-dependent, indicating that 3C4 and 4G5 can be utilized as differential tools to further examine the structure of MojV F and its immunogenic epitopes. Cross-reactivity of polyclonal anti-MojV sG antibodies with GhV, CedV and HeV sG, but not NiV sG glycoproteins, was only detected in the context of highly sensitive MMIAs, suggesting that these glycoproteins share some conserved epitope(s) with perhaps limited immunogenic potential. Similarly, polyclonal anti-MojV sF antibodies demonstrated minimal cross-reactivity with HeV F glycoprotein in flow cytometry experiments. However, further investigation is required to confirm the cross-reactivity of anti-MojV sF antibodies, as it was not observed in the context of western blot analysis and has not been evaluated using the more sensitive MMIAs.

In addition, the postulate that fecal rodent antibodies, as a non-invasive alternative to blood serum, could be screened to gauge exposure to henipavirus F and G glycoproteins

and that reactivity of these fecal antibodies could be detected by MMIA was explored. Proof of principle experiments examining murine serum and fecal samples from animals immunized with MojV sG, as an example of henipavirus G glycoprotein, and GhV sF, as an example of henipavirus F glycoprotein, successfully demonstrated that the MMIA evaluated here constitutes a highly sensitive tool and additional option to the current arsenal of techniques used in serologic surveillance. Moreover, findings from the present study indicate that rodent fecal material should be considered as a viable alternative to blood samples and an attractive option to increase sources and opportunities for field sample collection. Secretory IgA, mostly present in its dimeric form, is the most abundantly produced colonic immunoglobulin isotype (41; 42), and can readily be detected in fecal samples by ELISA (254). Dimeric secreted IgA, furthermore, can be potent virus neutralizing antibodies (42). Because transmission routes of the Bangladeshi strain of NiV can involve the consumption of contaminated raw date palm sap, exposure to viral antigens may occur through the digestive tract mucosa (113; 211). This study, therefore, also evaluated the application of the MMIA to the detection of fecal IgA reactivity, offering additional options in testing parameters. The neutralizing power of secreted IgA antibodies in henipavirus infections and possible correlations to degrees of reactivity and specificity, however, are unknown and would require further investigation.

Contributions to the field

Key findings within the present study that included the low level fusion functionality and slow kinetics of cell-cell fusion mediated by the MojV F and G glycoproteins, and the lack of ephrin-B2 and -B3 use by MojV as entry receptors were discovered early during the course of this research project. While additional work was

ongoing, similar findings were reported by Rissanen et al. (215), showing that MojV F and G can mediate cell-cell fusion with human alveolar epithelial (A549), human embryonic kidney (HEK293T) and baby hamster kidney (BHK-21 and BSRT7/5) cells and that ephrin ligands B2 and B3 cannot act as binding partners to MojV G. Nevertheless, the findings presented here have not only confirmed the work by Rissanen et al. (215), but also significantly expanded the knowledge available on species susceptibility to MojV by establishing that additional human (HeLa-USU), non-human primate (Vero) and rattine (L2, C6 and Rat2) cells can support MojV F and G mediated cell-cell fusion. Furthermore, because our group published findings on the expanded ephrin tropism of CedV, this work also widened the search for the entry receptor of MojV by investigating the role of A-class ephrin ligands as well as that of ephrin-B1. The results here suggest that murine ephrin-A1, human ephrin-A5 and rat ephrin-A4 may play some role in MojV F and G mediated fusion, although the preponderance of data indicate that the primary cognate entry receptor of MojV G remains unknown.

This work also developed a highly sensitive reporter-gene assay based on T7-driven expression of the Nano luciferase enzyme to detect low levels of cell-cell fusion that could have been missed by other assays currently in use in the field, including reporter systems based on the expression of *Gaussia* luciferase (215) or β -galactosidase (29).

This study revealed atypical levels and kinetics of MojV F and G mediated fusion, a fusion phenotype also observed by Rissanen et al. (215). Structural and mechanistic causes were thus explored here. Through this investigation, additional reagents, heptad-repeat peptides, were designed to mimic the HR2 domain of MojV F and CedV F and

novel characteristics of MojV F were identified. Such reagents could be used in future studies to evaluate fusion and entry inhibition of MojV and CedV. In the case of MojV inhibition, two mechanisms of action may be examined due to the exposition of MojV F HR1 domain at the pre-fusion conformation stage, before receptor engagement by MojV G, and after receptor engagement as MojV F is triggered to undergo conformational cascade and adopts a PHI structure vulnerable to heptad-repeat peptide interaction.

In addition, this project included the production of soluble versions of MojV F and G glycoproteins, providing novel tools to investigate the structure and immunogenic characteristics of MojV F and G glycoproteins. Moreover, anti-MojV sF (3C4 and 4G5) and anti-MojV sG (2B2, 6E5 and 10G2) monoclonal antibodies produced here can aid in the examination of structure of MojV F and G glycoproteins, but also in the identification of immunogenic epitopes that could be structurally mapped to their respective glycoprotein targets. Further, the neutralizing potential of these antibodies could also be evaluated and structure/function studies may divulge such neutralizing mechanisms as demonstrated, for example, in publications analyzing interactions between the fragment antigen binding (Fab) of monoclonal anti-NiV F antibody 66 with NiV F and monoclonal anti-HeV F antibody 36 with HeV F (17), or between NiV F and HeV F and the murine anti-NiV sF monoclonal antibody 5B3 as well as its humanized version (68), and between the HeV/NiV G cross-reactive monoclonal antibody m102.4 and HeV G (271). Furthermore, the MojV sF and sG constructs produced here may be tested in subunit recombinant anti-MojV vaccine designs, while the monoclonal and polyclonal antibodies they helped generate may be assessed for passive immunotherapy regimens, should the need arise.

The production of MojV sG and MojV sF has also permitted the expansion of the MMIA panels being currently used in serologic viral surveillance studies. MojV sG coupled microspheres have been integrated in a multiplex henipavirus sG panel applied to screen sera from Malaysian *Hipposideros* bats, *Pteropus Hypomelanus* bats and *Macaca fascicularis* (cynomolgus monkey); these assays revealed exposure to MojV or MojV-like henipaviruses (130).

Since the relative importance of MojV F and G glycoproteins in viral entry has not been elucidated and the reservoir of MojV has not been confirmed, screening for exposure to both MojV sF and MojV sG glycoproteins may improve the sensitivity to multiplex microsphere immunoassays. The MMIA performed here were conducted using freshly produced henipavirus sF glycoproteins and coated beads, which allowed for the inclusion of all five henipavirus species of sF glycoproteins in addition to the five sG glycoproteins. However, due to the propensity of henipavirus sF glycoproteins to thermal instability and folding into their post-fusion conformation, MMIA performed in henipavirus serologic surveillance works have primarily employed G glycoproteins, with the exception of a panel including HeV, NiV and CedV sF in recent work by Brook et al. (45). Disulfide bond-stabilizing mutations can be introduced in henipavirus sF constructs to remedy this issue (68; 260). In the future, the introduction of mutated henipavirus sF glycoproteins stabilized in their pre-fusion conformation to MMIA panels may offer more sensitive and detailed serologic surveillance data.

Additionally, in testing rodent serum and fecal samples, this project attested that such an assay can be a valuable alternative in extending the panoply of techniques available to answer questions about emerging potentially zoonotic and pathogenic

viruses, and providing increased flexibility as to the type of sampling that can be obtained and used in such studies. The present work was limited, as a proof of principle, to comparing the use of murine serum from tail bleeds and fecal polyclonal antibodies in assessing reactivity against MojV sG and GhV sF, as examples of henipavirus G and F glycoproteins. Nevertheless, the proven application of the MMIA to the detection of virus-specific antibodies isolated from fecal material from immunized rodents constitutes a significant expansion of this assay, which now offers a new approach and non-invasive option to serological virus surveillance.

Future directions

Knowledge about the emerging henipavirus MojV remains limited and numerous questions pertaining to the host and reservoir range of MojV, its tissue and receptor tropism, or its potential for zoonotic transmission and pathogenicity, as well as the extent of its circulation, remain unanswered. Building on the foundations provided by the work presented here, the following projects constitute the next steps to be undertaken in exploring such questions.

The overall percent identity of protein sequences of ephrin ligands is highly conserved across mammalian species. For instance, rat ephrin ligand A4 shares 83% identity with its human homologue (**Table 1-1**). Moreover, the G-H loop of ephrin ligands was identified as the domain responsible for functional interactions with henipavirus G glycoproteins in mediating fusion (38; 131; 238; 268), and only one the four G-H loop residues critical to secure such interaction differs between the G-H loop of rat and human ephrin-A4 (**Figure 3-7a**). Yet, findings of the present study suggest that functional interactions between MojV G and rat ephrin-A4, if present, may occur outside

of the G-H loop domain. Solving the molecular structures of human and rat ephrin-A4 could allow for structural comparisons and in silico modeling of putative docking sites of MojV G onto rat ephrin-A4, which may be absent from human ephrin-A4. Such data, combined with domain mutation studies, would help identify regions or residues of rat ephrin-A4 and MojV G for which interaction is required to result in fusion.

Additionally, future work should assess bat cell lines for susceptibility to MojV F and G mediated membrane fusion, as frugivorous pteropid bats are known to serve as a reservoir to henipaviruses. Alignments of all eight ephrin G-H loop sequences from *Pteropus alecto* bats were found to share 100% identity with human sequences (data not shown). However, the findings of the present study strongly suggest that ephrin ligands do not act as the primary functional entry receptors of MojV. Nevertheless, MojV may utilize an entry receptor that would be expressed by bat cells. Further investigation of MojV F and G fusogenicity with *Pteropus alecto* kidney, lung and brain cells, for example, would help shed light on a possible species-specific receptor tropism of MojV and, coupled with serologic investigations, on a potential role of bats in the circulation and transmission of MojV to other species, such as rodents, which may act as spillover hosts.

Although the ORFs of interferon response antagonists V and W proteins were identified in the genomic sequence of MojV (266), the ability of MojV to express a functional and competent form of these proteins is, at present, unknown. Further investigation of the capacity of MojV V and W to interact with RIG-I, MDA-5 or STAT1/2 and to consequently inhibit the type I IFN response is therefore needed to help assess if MojV has high pathogenic potential.

Recently, a wild type recombinant CedV (rCedV-wt) and variants expressing GFP (rCedV-GFP) (129) and luciferase (unpublished data) were rescued using a reverse genetics approach. This advance, in absence of a MojV isolate and of the evaluation of its pathogenic potential, provides an exciting opportunity to create a replication competent recombinant chimeric virus expressing MojV F and G glycoproteins within the background of the non-pathogenic CedV (rCedV Δ F Δ G-MojV F/G), as well as variants expressing GFP or luciferase reporters. These chimeras would allow for live virus experiments to be performed safely in BSL-2 settings, and circumvent BSL-4 requirements that would apply to the rescue and use of a recombinant MojV (rMojV). Such tools could be employed to address multiple questions, such as the inefficient membrane fusion activity of MojV F and G glycoproteins when analyzed outside the context of a replication competent recombinant virus. Live rCedV Δ F Δ G-MojV F/G infections could shed light on the relative instability MojV F. One hypothesis explaining this characteristic postulates that the instability of MojV F may be artificial and the result of a lack of proper interactions between the cytoplasmic tail ends of MojV F and matrix (M) glycoproteins. Absent this stabilizing effect, MojV F may spontaneously and irreversibly transition from pre- to post-fusion conformation, preventing the completion of the fusion mechanism upon engagement of MojV G with its cognate receptor. Expression of MojV F in the context of an infectious viral particle would help address this hypothesis. Confirming the cell-cell fusion results obtained from heterotypic complementation of MojV F with GhV G by evaluating a recombinant chimeric virus expressing MojV F and GhV G glycoproteins within a CedV background (rCedV Δ F Δ G-

MojV F/GhV G) would also provide information on the contribution of receptor restriction and MojV F instability to cell entry.

In addition, replication competent rCedV Δ F Δ G-MojV F/G infections of susceptible host cells, followed by mass spectrometry analysis, can be applied to the search for the primary entry receptor of MojV. Alternatively, MojV sG could be used as a co-precipitating reagent to identify receptor(s) expressed on C6, BSR-T7/5 or BHK-21 cells, as the present study indicates that these cells may express a functional MojV entry receptor.

Furthermore, rodent or immune-deficient mouse infection studies with rCedV Δ F Δ G-MojV F/G could help answer questions on receptor contingent-infection routes and tissue tropism. Supplementary studies on MojV F and G mediated tissue tropism could give some indications on the possible pathologic profile of MojV infection by exposing the susceptibility of different cell types that could play a role in establishing an infection or dissemination of the virus to otherwise inaccessible sites. In addition, the present study was limited to the use of *Rattus norvegicus* derived cell lines and recombinant protein (rat ephrin-A4). However, experiments with cells from *Rattus flavipectus*, as well as bats of the genus *Pteropus* and *Hipposideros* are also warranted.

Finally, the application of soluble MojV F and G glycoproteins in MMIAAs could be extended to the screening of rodent, bat and human populations to gain further insights into the geographic distribution of MojV, and the possible roles of rodents and bats as natural hosts of MojV.

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