獸醫學博士學位論文

# Vesicular stomatitis virus serotype New Jersey glycoprotein as a tool for diagnostic antigen and carrier for foot-and-mouth disease vaccine candidate

# 수포성구내염 뉴저지형 당단백질을 이용한 진단법 개발 및 구제역 백신 적용에 관한 연구

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이 향 심

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**By**

**Hyang Sim Lee**

**Supervisor: Professor Hee Jeong Youn, D.V.M., Ph.D.**

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Department of Veterinary Medicine

The Graduate School

Seoul National University

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지도교수 윤 희 정

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# Abstract

## Vesicular stomatitis virus serotype New Jersey glycoprotein as a tool for diagnostic antigen and carrier for foot-and-mouth disease vaccine candidate

Hyang Sim Lee

(Supervisor: Hee Jeong Youn, D.V.M., Ph.D.)

Department of Veterinary Medicine

The Graduate School

Seoul National University

Vesicular stomatitis virus (VSV), a member of the family *Rhabdoviridae*, genus *Vesiculovirus*, is a causative agent of vesicular disease in cattle, horses and swine. VSV is enveloped and contains a single strand of negative-sense RNA that encoded five structural proteins: glycoprotein (GP), nucleocapsid

(NC), phosphoprotein (P), matrix (M), and large polymerase (L). Two major serotypes of VSV, Indiana (VSV-IN) and New Jersey (VSV-NJ) are distinct serologically. Both strains are known to cause indistinguishable vesicular signs similar to FMD, thus necessitating differential diagnosis. Since VSV infects a broad spectrum of hosts, a serological method that can be performed irrespective of the susceptible species is preferable. In this regard, a blocking or competitive ELISA (C-ELISA) is more appropriate than an indirect ELISA. Previously, a C-ELISA using recombinant NC instead of GP have been developed. However, it showed low sensitivity relative to the VNT. Although an ELISA using VSV-IN GP has been developed, no ELISA for detecting antibodies to VSV-NJ has been available yet. Therefore, VSV-NJ blocking ELISA was established using GP and monoclonal antibody to improve sensitivity and specificity.

In the first study, an enzyme-linked immunosorbent assay (ELISA) using glycoprotein and a monoclonal antibody (MAb) was developed for detection of antibodies against VSV serotype NJ. The GP as a diagnostic antigen was extracted from partially purified VSV and the neutralizing MAb to VSV-NJ was incorporated to compete with antibodies in a blocking ELISA using glycoprotein (GP ELISA). The cut-off of GP ELISA was set up at percentage inhibition (PI) 40 which corresponded to virus neutralization test (VNT) titer

32. With this threshold, the GP ELISA exhibited 99.6% specificity for naive sera (n=3,005) comprising cattle (n=1,040), pigs (n=1,120), and horses (n=845) from domestic farms. The GP ELISA did not cross react with positive sera from foot-and-mouth disease and swine vesicular disease as well as VSV-IN.

In second study, to avoid the exposure to VSV in the manufacturing antigens, genetically engineered glycoprotein was expressed in insect cells by recombinant baculovirus system. The recombinant glycoprotein (R-GP) of VSV-NJ was expressed in insect cells by a baculovirus system. Its utility as a diagnostic antigen in a blocking ELISA was investigated as an alternative to the current native GP extracted from VSV-NJ. With the cut-off value of 73% inhibition, the R-GP ELISA exhibited 99.1% specificity for naive sera from cattle (n=1,036) and horses (n=1,016). It did not cross-react with VSV-IN positive sera and differentiated from foot-and-mouth disease and swine vesicular disease.

In final study, a chimeric construct containing the 517 aa of VSV-NJ GP as a carrier to include the FMDV type O VP1 sequence was evaluated as vaccine in field pigs. Since 2000, South Korea has experienced eleven FMD epidemics. Due to the extensive economic damage (approximately 3 billion dollars) in the 2010-2011 FMD outbreak, the South Korean government

implemented a vaccination policy throughout the country for all FMD susceptible livestock. Current FMDV vaccines are serotype-specific and consist of inactivated virus formulated in oil and aluminum hydroxide adjuvants. There are major drawbacks associated with their use, namely, the requirement of propagating virulent virus in containment facilities and the associated risk of escape from manufacturing sites. Thus, much effort has been made to develop alternative and safe vaccines utilizing the GH loop of capsid protein VP1. In final study shows that the twice immunizations one month apart in field pigs using this recombinant vaccine resulted in a significant antibody increase compared to the the commercial vaccine ($P<0.05$).

Taken together, VSV GP has a potential to be used as a diagnostic antigen for the detection of antibodies to VSV-NJ and useful tool for the development of a novel recombinant protein vaccine that could be easily produced in a general laboratory as an alternative to the current FMD inactivated vaccine.

Keywords: Vesicular stomatitis virus, Foot-and-mouth disease, FMD virus, Vaccine, glycoprotein, recombinant glycoprotein, Enzyme-linked Immunosorbent Assay

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| | |
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| **BSL** | **BioSafety Level** |
| **C-ELISA** | **Competitive ELISA** |
| **ELISA** | **Enzyme-Linked Immunosorbent Assay** |
| **FMD** | **Foot-and-mouth disease** |
| **FMDV** | **Foot-and-mouth disease virus** |
| **GP** | **Glycoprotein** |
| **GST** | **Glutathione S-transferase** |
| **MAb** | **Monoclonal antibody** |
| **NC ELISA** | **Nucleocapsid ELISA** |
| **NVSL** | **National Veterinary Services Laboratory** |
| **OIE** | **World Organisation for Animal Health** |
| **PI** | **Percentage Inhibition** |
| **R-GP** | **Recombinant Glycoprotein** |
| **VNT** | **Virus Neutralization Test** |
| **VSV** | **Vesicular stomatitis virus** |
| **VSV-IN** | **Vesicular stomatitis virus Indiana** |
| **VSV-NJ** | **Vesicular stomatitis virus New Jersey** |

# General introduction

Vesicular stomatitis (VS) is a vesicular disease of horses, cattle and pigs caused by *Vesiculovirus* genus of the *Rhabdoviridae* family. VS cannot reliably be clinically differentiated from the other vesicular diseases in the relevant susceptible species, such as foot and mouth disease (FMD), vesicular exanthema of swine (VES) and swine vesicular disease (SVD). An early laboratory diagnosis of any suspected VS case is therefore a matter of urgency.

The virus neutralization test (VNT) is recognized as a standard method for the detection of anti-VSV antibodies by the World Organisation for Animal Health (OIE). However, it is labor-intensive, requires cell culture facilities, and takes 2 to 3 days to complete. These aspects make it unsuitable for the purposes of mass serological surveillance. To address these drawbacks, several enzyme-linked immunosorbent assay (ELISA) systems have been developed and used to measure antibody titers.

Previously, a competitive ELISA using recombinant NC (NC ELISA) instead of glycoprotein was reported. However, it showed low sensitivity relative to the VNT (Alvarado *et al*., 2002). The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody (Kelly *et al*., 1972). Although an ELISA employing a glycoprotein has been developed for VSV-IN, no ELISA for the detection of anti-VSV-NJ antibodies has been available yet.

Foot-and-mouth disease (FMD) is one of the most economically and socially devastating diseases affecting animal agriculture throughout the world (Rodriguez and Gay, 2011). Vaccination against FMDV is a major strategy to control the disease during an outbreak and in endemic areas.

Current FMDV vaccines are serotype-specific and consist of inactivated virus formulated in oil or aluminum hydroxide adjuvants (Doel, 2003). Although these vaccines can induce strong humoral protective immunity, there are major drawbacks associated with their use, namely, the requirement of propagating virulent virus in containment facilities (Barteling and Vreeswijk, 1991) and the associated risk of escape from manufacturing sites (Strohmaier *et al*., 1982). Thus, many researchers have attempted to replace the current foot-and-mouth inactivated vaccine with peptides corresponding to FMDV VP1 neutralizing epitope. The Argentina research group has linked FMDV epitope to the VSV glycoprotein, ending up in limited efficacy compared to the conventional inactivated vaccine. It was postulated that the effect was lowered because only the partial outer fraction of glycoprotein was used instead of the complete VSV glycoprotein as immune carrier.

The purpose of this study was to development of a blocking ELISA for the detection of antibodies to VSV-NJ and the novel recombinant protein carrying a FMDV VP1 using a VSV-NJ glycoprotein as a vaccine carrier.

This is comprised of three chapters. As the first chapter of my study, I established an ELISA using glycoprotein extracted from VSV-NJ and a MAb specific to VSV-

NJ, and explored its feasibility in relation to the VNT for the detection of VSV-NJ antibodies. In chapter 2, I described the development of the blocking ELISA based on recombinant glycoprotein (R-GP) that could be manufactured at any laboratory without facility limitation. To this end, the R-GP gene from VSV-NJ was expressed in insect cells and investigated as a diagnostic antigen. In chapter 3, I inserted the FMDV type O VP1 129-169 codon into the VSV whole glycoprotein and evaluated for its immunogenicity as an innovative FMDV vaccine candidate.

# Literature review

## 1. Vesicular stomatitis virus (VSV)

### 1.1. Definition

Vesicular stomatitis (VS) is a vesicular disease of horses, cattle and pigs. This disease is clinically indistinguishable in relevant susceptible species from foot and mouth disease (FMD), vesicular exanthema of swine (VES), or swine vesicular disease (SVD). Sheep, goats and many other wild species can be infected. Humans are also susceptible. VS is not a newly emerged disease. As extensively reviewed by Hanson (1952), outbreaks of 'sore tongue' in horses, cattle, and swine in the eastern USA in 1801, 1802, and 1817 mirror the modern description of VS.

VS disabled 4000 horses needed to fight the Civil War in 1862. Major epidemics in US cattle and horses occurred in 1889, 1906, 1916, 1926, 1937, 1949, 1963, 1982, and 1995, with minor outbreaks during many other years. Epidemics usually involve the Southwestern states, the upper Mississippi region, and the Rocky and Appalachian mountain areas. Except for the Southeastern USA, VS disappears from the USA following each epidemic. VS is endemic in southern Mexico, Central America, northern South America, and eastern Brazil. Probable epidemics outside the American Continent occurred in horses sent from the USA to Europe during the

First World War and in South Africa in 1884, 1897, 1934, 1938, and 1943.

VS in horses, cattle, and swine is characterized by extensive vesicular and erosive lesions on the dorsal surface of the tongue, frequently accompanied by vesicles on the gums, lips, coronary band, teats, and prepuce. This is of great practical importance, because attack rates in dairies can be as high as 96% and economic consequences are in the range of $100–250 per cow. Also, VS cannot be discriminated from foot-and-mouth disease on clinical signs alone (Letchworth *et al.,* 1999).

## 1.2. Etiology and Epidemiology

Vesicular stomatitis virus (VSV) is a negative-strand RNA virus and is the prototype of the *Rhabdovirirus* family (Martinez *et al*., 2004). The virions are bullet-shaped and are generally 180 nm long and 65 nm wide (McCombs *et al*., 1966). The genome of VSV has an 11-kb in length, encoding five structural proteins: glycoprotein (GP), nucleocapsid (NC), phosphoprotein (P), matrix (M), and large polymerase (L) (Banerjee *et al*., 1977; Abraham *et al*., 1976; Banerjee *et al*., 1984). The GP forms spikes on the envelope and mediates cellular recognition and fusion, allowing the viral entry and exit from the cell (Martinez *et al*., 2003; Pal *et al.,* 1987). The M protein is located between the envelope and the nucleocapsid core, and participates in viral assembly and particle budding (Thomas *et al*., 1994). The

nucleocapsid core is composed of the viral genome tightly wound around the viral N protein, forming an RNase-resistant core environment (Brouillette *et al*., 1982). The P and L proteins combine to catalyze RNA-dependent RNA polymerization (RdRp) of genomic RNA and transcription of the mRNAs (Banerjee *et al*., 1977; Brouillette *et al*., 1982; Banerjee *et al*., 1990) in the sequential order of N-P-M-G-L (Banerjee *et al*., 1977). For genomic replication, the RdRp initiates at a different 3' end site and synthesizes a full-length positive-sense copy of the genome as a replication template (Banerjee *et al*., 1990). Due to the low fidelity rate of RdRp, VSV has a high error rate for RNA transcription, which leads to great genetic diversity and quasispecies populations (Rodriguez, 2002). Transcription and replication mechanisms of VSV are complex and not fully understood, with laboratory and natural populations yielding variable information on genetic adaptability and maintenance. Under laboratory conditions, VSV shows great capacity for genetic change and rapid adaptation (Rodriguez., 2002). Conversely, in field conditions, VSV remains relatively stable, with evolutionary patterns defined by similar ecological conditions rather than geographical origin or immunological selection (Rodriguez., 2002). Genetic fitness studies designed to investigate evolutionary pressure on the genome of VSV alternating between insect and mammalian cellular environments suggest that the stability of field populations is not due to the need of the virus to constrain adaptation between host cell types (Novella *et al*., 1999). VS viruses are classified by serotypes, which are similar in

size and morphology, but generate distinct neutralizing antibodies in infected animals (Cartwright *et al*., 1972; Kelley *et al*., 1972). There are two distinct serotypes of VSV: Indiana (VSV-IN) and New Jersey (VSV-NJ), with the latter causing the majority of outbreaks in the U.S. (Bridges *et al*., 1997; Stallknecht *et al*., 2001). Serotypes involved with the disease in livestock include VSV-NJ and VSV-IN types 1, 2, and 3 (Hanson *et al*., 1968). Serotypes, VSV-NJ and VSV-IN1, occur in North, Central, and parts of South America (Rodriguez., 2002). Serotype VSV-IN3 (or Alagoas) occurs in Brazil, and VSV-IN2 (or Cocal) occurs in Brazil and Argentina (Rodriguez *et al*., 1990; Andrade *et al*., 1980).

## 1.3. Transmission and Vectors

The transmission of vesicular stomatitis is incompletely understood. Insect vectors are thought to introduce VSV into populations of domesticated animals. Sand flies (*Lutzomyia* sp.), black flies (family Simuliidae) and *Culicoides* midges can act as biological vectors. Sand flies seem to be important vectors in endemic areas, but have a limited flight range and are not thought to spread these viruses over long distances. Black flies are believed to be particularly important vectors in parts of the western United States. Where the virus originates, before entering livestock populations, is still uncertain. Once animals develop lesions, however, insects may become infected by feeding on viruses in these lesions or contaminated secretions.

In addition, infected black flies can transmit VSV to other black flies feeding at the same time on a host, even if the host is not infected. Transovarial transmission has been demonstrated in sandflies and black flies in the laboratory, and may be possible in *Culicoides*. It might contribute to virus overwintering in cold climates (Mead *et al*., 2004). Vesicular stomatitis viruses have also been found in other insects including *Aedes* mosquitoes, Chloropidae (eye gnats), and flies in the genus Musca or family Anthomyiidae. These insects may act as mechanical vectors. Migratory grasshoppers (*Melanoplus sanguinipes*) have been proposed to play a role in spreading VSV. In laboratory experiments, these grasshoppers could be infected from plants or other sources, and cattle that ate grasshoppers developed clinical signs.

In the field, cattle may ingest large numbers of molting grasshoppers while grazing, as these insects are immobile during that stage. Once it has been introduced into a herd, vesicular stomatitis can spread from animal to animal by direct contact. Broken skin or mucous membranes may facilitate entry of the virus. Infected animals shed VSV in vesicle material. Viruses from lesions in the mouth and on the muzzle can contaminate saliva, and to a lesser extent, nasal secretions. However, VSV has also been detected in the saliva of some experimentally infected horses that did not have oral lesions. Vesicular stomatitis viruses are not considered to be shed in feces, urine or milk, although they have been detected occasionally in the feces of symptomatic, experimentally infected swine. Livestock can be infected experimentally by aerosols

in the laboratory, but this route did not result in skin lesions in most species. VSV does not appear to cross the placenta or cause fetal seroconversion.

Contaminated fomites such as food, water and milking machines are also thought to play a role in transmission. VSV in saliva was reported to survive for 3-4 days on milking pails, mangers and hay. Viruses dried onto glass, plastic or stainless steel in the laboratory lost a great deal of infectivity within the first 1-6 days at 22°C, although some infectious virus was still recovered after 2-8 days. However, survival in liquid medium that contained organic material (i.e., cell culture medium with 5% fetal bovine serum) was prolonged, especially at cold temperatures.

These suspensions did not lose significant infectivity for at least 4 weeks at 4°C. Approximately 90% of infectious virus disappeared during the first 8 days in such suspensions incubated at 28°C, but some viable viruses were still present after 4 weeks. At 37°C, 90% of infectivity had been lost by 3 days, and no live viruses could be detected after 21 days. Only 10% of the viruses suspended in cell culture medium without serum were still viable by 4-12 days at 4°C. People can be infected by contact with lesions or secretions from infected animals, particularly vesicular fluid and saliva, or when manipulating VSV in the laboratory. Aerosol transmission has been reported in laboratories, and some cases occurred after accidental inoculation (needlestick injuries). Some people are probably infected through insect bites, as antibodies to these viruses are common in endemic regions (Anna, 2016).

## 1.4. Diagnostic tests

Vesicular stomatitis viruses can be found in vesicle fluid, swabs of ruptured vesicles, the epithelium over unruptured vesicles, and epithelial flaps from freshly ruptured vesicles (e.g., epithelial tags from the mouth). Sedation is recommended before sample collection, as the lesions are very painful. If these samples are not available, esophageal/pharyngeal fluid can be collected with a probang cup from cattle, or throat swabs may be taken from pigs.

VSV can be detected in oral and nasal secretions for up to 7 days after infection. Electron microscopy of tissue samples may be helpful in distinguishing VSV from some other viruses that cause vesicular lesions, such as foot and mouth disease virus or swine vesicular disease virus. Many cell lines can be used to isolate VSV from clinical samples. Virus recovery is also possible in embryonated eggs, and animal inoculation (mice) was sometimes employed in the past. The identity of cultured virus can be confirmed with immunofluorescence, complement fixation or ELISAs to detect viral antigens, or with other tests such as reverse transcription polymerase chain reaction (RT-PCR) assays. Antigen capture (indirect sandwich) ELISAs are often used to identify the viral serotype.

Some RT-PCR tests can also distinguish New Jersey and Indiana serotypes, and one group in Brazil reported using RT-PCR to confirm the identity of isolated VSV-AV and distinguish these viruses from Cocal virus. VSV antigens can be detected in

tissue samples or vesicle fluid with an antigen capture ELISA, complement fixation or virus neutralization. Descriptions of other antigen detection assays, including lateral flow devices, have been published. Some laboratories may use RT–PCR tests to detect viral nucleic acids directly in tissues. However, this does not seem to be common at present.

Genetic identification is complicated by the variability in vesicular stomatitis viruses, including changes in epidemic viruses as they continue to circulate. Genetic assays may need to be standardized for each region where these viruses circulate. Some published multiplex RT-PCR assays can identify a wide range of vesicular stomatitis viruses, belonging to both serotypes, from North and Central America. Unexpected strains may, nevertheless, be missed.

Vesicular stomatitis can also be diagnosed by serology, using paired serum samples. A fourfold increase in titers is diagnostic. Animals usually develop serotype-specific antibodies to VSV 5-8 days after they are infected. ELISAs and virus neutralization (VN) are the preferred serological tests, according to the World Organisation for Animal Health (OIE), but early antibodies can also be quantified by complement fixation. Complement fixation cannot detect antibodies for as long as ELISA or VN. Some ELISAs for VSV are quantitative (e.g., the liquid-phase blocking ELISA), but others only report the presence or absence of antibodies to this virus. Additional serological tests have been described and/or used in the past, including agar gel immunodiffusion and counterimmunoelectrophoresis (OIE, 2018).

## 1.5. Prevention and Control

Vesicular stomatitis can spread between animals by direct contact, as well as via insect-mediated transmission. During outbreaks, uninfected livestock should kept away from any animals that could be infected. Quarantines and animal movement restrictions can help reduce virus spread. There should be no movement of animals from a quarantined property for at least 21 days after all lesions are healed, unless the animals are going directly to slaughter (OIE, 2013).

Isolating symptomatic animals may also be helpful within a herd. Horses appear to be most contagious for the first 6 days after infection. Good sanitation and disinfection can reduce the spread of the virus on fomites. Lower attack rates have been reported on dairies where feed and water troughs were cleaned regularly. Milking equipment should also be disinfected between uses, and cows with lesions should be milked last. The avoidance of hard or abrasive feeds may prevent oral abrasions that could facilitate infections. Pastured livestock are more likely to become infected than animals with access to a shelter or barn. Stabling animals during outbreaks seems to decrease the risk of disease. During one outbreak in the U.S., animals were also more likely to develop vesicular stomatitis if there were sources of running water (e.g., streams, irrigation canals) within a quarter mile, probably because water sources encourage higher vector populations. If practical (and permitted), moving animals farther from such locations during outbreaks might

reduce the risk of infection. Various insect control measures are also thought be helpful, though their efficacy is not absolute. Insecticide applications should include the inner surface of the pinna, where black flies tend to feed. Commercial vaccines are available in some endemic regions of Central and South America. Vaccines are not available in the U.S. (Anna, 2016).

## 1.6. VSV glycoprotein

There are two major serotypes of VSV, New Jersey (NJ) and Indiana (IN), which are classified based on neutralizing antibodies to the viral glycoprotein (GP). There is only 50% identity at the amino acid level between the GPs of VSV-NJ and VSV-IN. The GP is the sole surface-expressed protein on the bullet-shaped virions which anchored in the envelope and plays a critical role in the early stage of virus infection (Gallione *et al*., 1983; Marinez *et al*., 2004; Roche *et al*., 2008; Schlegel *et al*., 1985). The surface GP anchored in the membrane of vesicular stomatitis virus (VSV) forms the spike-like organ for virus adsorption to host cell receptors. The GP also appears to function in VSV pepetration of host cells based on evidence that at low pH the amino-terminal hydrophobic segment lyses erythrocytess, a property which may differ somewhat from cell fusion activity (White *et al*., 1981; Bailey *et al*., 1989; Carneiro *et al*., 2001; Roche *et al*., 2008).

The GP can be removed selectively from the surface of the virion by proteolytic enzymes, resulting in loss of infectivity, apparently due to degradation of the virion GP. The VSV GP appears to be the major antigenic determinant and is probably responsible for neutralization of the infectivity by antibody and for type specificity of the virus (Kelly *et al*., 1972).

## VSV glycoprotein as a vaccine vector

Vesicular stomatitis virus (VSV) is a highly cytopathic virus being developed as a vaccine vector due to its ability to induce strong protective T cell and antibody responses after a single dose (Melissa *et al*., 2012). Recombinant VSVs expressing foreign proteins have been studied as vaccine vectors for a number of pathogens, including HIV, influenza virus, hepatitis C virus, hepatitis B virus (HBV), measles virus, respiratory syncytial virus, severe acute respiratory syndrome virus, *Yersinia pestis*, papillomavirus, Ebola virus, and Marburg virus (Brandsma *et al*., 2007; Chattopadhyay *et al*., 2008; Cobleigh *et al*., 2010; Ezelle *et al*., 2002*;* Geisbert *et al*., 2008; Kahn *et al*., 2001; Kapadia *et al*., 2008; Roberts *et al*., 2004*;* Rose *et al*., 2001*;* Schwartz *et al*., 2001). Several candidate vaccines based on VSV were shown to elicit immune protection with strong humoral and cellular immune responses in different animal models. The GP binds to the surface of most cell types. Thus, molecular biologists often replace the envelope proteins in other viral vectors with GP to expand the host range of the vector. Multiple strategies have been developed to achieve attenuation of the VSV vaccine vector, including truncation of the

cytoplasmic tail of the VSV GP to reduce virulence, as well as modification of other structural proteins. VSV provides a variety of “danger signals” that engage the host's Toll-like receptors (TLRs) and other pattern recognition receptors, activating the innate immune response and, thus, enhancing the adaptive immune response (Kawai and Akira, 2007; Kawai and Akira, 2010). Viral vaccine vectors serve a dual function as both a recombinant protein expression vector and a vaccine adjuvant. The adjuvant properties of VSV are not sufficient, however, to induce the robust immune responses following VSV immunization. As viral vaccine vectors all express viral pathogen-associated molecular patterns (PAMPs) with adjuvant properties, the superior immune responses seen following VSV immunization may not be explained by its adjuvant properties alone and, instead, suggest that immunogenic differences could be attributed to differences in vector properties, including cytopathogenicity, protein expression levels, and temporal patterns, among others (Melissa *et al*., 2012).

# 2. Foot-and-mouth disease (FMD)

## 2.1. Definition

Foot-and-mouth disease (FMD) is one of the most highly contagious animal diseases, and the FMD virus (FMDV) rapidly replicates and spreads from infected animals via contact with susceptible animals and as an aerosol (Grubman *et al.,* 2004). FMDV is the prototype member of the *Aphthovirus* genus of the *picornaviridae* family. The virus exists in seven different serotypes: O, A, C, Asia 1, and South African Territories (SAT) 1-3 and multiple subtypes in each serotype (Mason *et al*., 2003). Vaccination or recovery from infection, with one serotype does not protect against infection from other serotypes and sometimes against another subtype within the same serotype.

## 2.2. History of FMD

The first written description of FMD probably occurred in 1514, when Hieronymus Fracastorius described a similar disease of cattle in Italy. In Germany, existence of FMD was first reported in 1754, while in Great Britain it was first recorded in August 1839, in United States of America in 1870 and, a year later in South America (Radostits *et al*., 2000). Australia has been free of FMD since 1872 and it never

occurred in New Zealand (Bachrach, 1968). Early history of FMD in Africa and Asia is not known except for South Africa where the disease was first officially recorded in 1892 (Coetzer *et al.,* 1994). In India, the earliest record of FMD dates back to 1864 when it occurred in many parts of the country. In 1898, Loeffler and Frosch discovered that the causative agent of FMD was filterable and in 1922, Vallée and Carré first showed the existence of two immunological serotypes of FMDV by cross-immunity tests in cattle. They were designated by their areas of origin, O (Oise, France) and A (Allemagne, Germany). Soon after, Waldmann and Trautwein in 1926 reported the existence of three immunologically distinct serotypes, A, B and C. Comparison of these virus types revealed that types A and B were the same as Vallée and Carré's types O and A, respectively, but type C was distinct (Brown, 2003). Later in 1940s three distinct serotypes, designated as SAT-1, 2 and 3 were identified in Africa (Brooksby, 1958). The seventh serotype, designated as Asia 1, was first recognized in the early 1950s amongst the viruses isolated from India in 1951 and 1952 (Dhanda, 1957).

## 2.3. FMDV genome

FMDV is the prototype member of the *Aphtovirus* genus on the *Picornaviridae* family. The virion compromises a single strand positive sense RNA genome

encapsidated in a non-enveloped icosahedral particle of about 25–30 nm (Grubman and Baxt, 2004). The viral genome of approximately 8500 nucleotides displays a long open reading frame (ORF) flanked by highly structured 50 and 30 untranslated regions (UTR). The 50 UTR can be divided into defined functional elements required for viral replication and translation. Translation of the viral mRNA begins at two alternative AUG codons downstream of the 50 UTR IRES element, and renders a viral polyprotein precursor of approximately 2300 amino-acids that is processed by viral proteases/enzymatic activities. The first mature product of viral translation is Lpro, a papain-like protease of fundamental role in virulence (Kleina and Grubman, 1992). Mature processed products give rise to an icosahedral acid sensitive capsid composed of 60 copies of each of the four structural proteins VP4, VP2, VP3 and VP1 (also known as 1A,1B, 1C and 1D) encoded within the P1 region (Rueckert and Wimmer, 1984). The most antigenic site in all serotypes of FMDV is located within a highly variable G-H loop in VP1 (1D). Consequently, variation at this region has prevented the development of vaccines that can provide cross protection among serotypes (Brehm *et al*., 2008). The P2 and P3 regions encode most of the non-structural (NS) viral proteins that are required for replication including 2A, 2B, 2C, 3A, 3 copies of 3B (also named VPg), 3Cpro, and 3Dpol. 3Cpro is the virus-encoded cysteine protease responsible for the majority of proteolytic cleavages in P1, P2 and P3 that result in mature cleavage products (Vakharia *et al*., 1987). 3Dpol is the viral RNA-dependent RNA polymerase (RdRp) that provides low fidelity copying during

replication (Domingo *et al*., 2002) and plays a fundamental role in the diversity of FMDV. At the end of the ORF there is a relatively short 30 UTR composed of two stem–loops and a poly (A) tract, both required for viral infectivity and known to stimulate IRES activity (Serrano *et al*., 2006).

## 2.4. FMDV pathogenesis

The severity of clinical FMD varies greatly depending on intrinsic virus properties as well as on the host species, genetic background, and previous history of virus exposure or vaccination. In cattle, the initial site of infection has been localized to areas of specific epithelium overlying mucosa-associated lymphoid tissue (MALT) of the nasopharyngeal mucosa (Arzt *et al*., 2010; Burrows *et al*., 1981; Pacheco *et al*., 2010; Stenfeldt *et al*., 2015) in both naive and vaccinated animals.

In non-vaccinated animals, the infection rapidly progresses to a systemic phase that is marked by the presence of large quantities of virus in the blood. This systemic dissemination of virus and occurrence of viremia further coincides with a substantial surge in shedding of infectious virus in oral and nasal secretions (Arzt *et al*., 2010; Stenfeldt *et al*., 2015). Vesicular lesions develop in the oral cavity, on coronary bands and interdigital clefts of the feet, and in other areas of non-haired skin such as the muzzle and teats (Arzt *et al*., 2011b). Infected animals shed large quantities of virus during the acute phase of infection, and due to the relatively low infectious

dose, the infection spreads rapidly among susceptible animals. Transmission may occur via direct or indirect contact between animals or through generation of airborne aerosols (Donaldson *et al*., 2001). The clinical phase of FMD usually subsides within approximately two weeks. Mortality rates amongst adult animals are generally low, although younger individuals may succumb to FMDV associated myocarditis (Arzt *et al*., 2011a).

## 2.5. Diagnosis and Surveillance

Due to the fast spread of FMD and the serious economic consequences that can arise from an outbreak, prompt, sensitive and specific diagnosis and identification of the virus serotype is essential. Initially, presumptive diagnosis is based upon clinical signs. However, FMD cannot be differentiated clinically from other vesicular diseases. Therefore, confirmed laboratory diagnosis of any suspected FMD case is a matter of urgency. Furthermore, determination of the serotype involved in field outbreaks has to be established within laboratories to enable proper control of the disease. Diagnosis of FMD is by virus isolation or by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral nonstructural proteins (NSPs) can be used as indicators of infection, irrespective of vaccination status (OIE, 2018). As part of FMD control, surveillance represents a very important fraction

directly linked to diagnostics. It requires a concerted effort by the individual livestock owner, private veterinarians, and the government's veterinary services. Unfortunately, even in countries with presumably knowledgeable livestock owners and a trained veterinary service, FMD outbreaks may still go undetected until the disease has spread beyond the initial site of infection. This can partially be due to subtle or unidentifiable FMD clinical signs of disease, to a delay in reporting the index case, or to a lack of reporting because of illegal movement of animals or contaminated animal products (Mahy, 2005). Several studies have assessed surveillance data (Chen *et al*., 2011; Kasanga *et al*., 2012) highlighting the importance of understanding local cultural and social factors that influence the agricultural community's motivations to report FMD outbreaks.

## 2.6. FMD vaccines

FMD vaccines were among the first animal vaccines developed, with efforts to immunize animals by exposure to infectious virus beginning at the end of the 19th century (reviewed in (Lombard and Fussel, 2007)). But a practical vaccine was never grasped due to the unpredictability of viral virulence and the existence of multiple viral serotypes and subtypes (Rodriguez and Grubman, 2009).

The first inactivated FMD vaccine was developed using vesicular fluid obtained from tongues of deliberately infected cattle, and subsequently inactivated with

formaldehyde, however industrial production did not begin until the 1950s after Frenkel described the successful primary culture of tongue epithelium from healthy slaughtered animals (Lombard and Fussel, 2007). Further breakthroughs in inactivated FMD vaccine production included the growth of FMDV in BHK cell suspension cultures in the 1960s (Capstick *et al*., 1962), the introduction of binary ethyleneimine (BEI) treatment for FMDV antigen inactivation (Bahnemann, 1976), and the use of oil-adjuvants in the 1970s.

# Chapter 1

# Enzyme-linked immunosorbent assay using glycoprotein and monoclonal antibody for detecting antibodies to vesicular stomatitis virus serotype New Jersey

# Abstract

In this study, an enzyme-linked immunosorbent assay (ELISA) using glycoprotein and a monoclonal antibody (MAb) was developed for the detection of antibodies to vesicular stomatitis virus (VSV) serotype New Jersey (NJ). The glycoprotein to be used as a diagnostic antigen was extracted from partially purified VSV-NJ, and a neutralizing MAb specific to VSV-NJ was incorporated to compete with antibodies in a blocking ELISA using glycoprotein (GP ELISA). The cut-off of the GP ELISA was set at 40% inhibition, which corresponded to a virus neutralization test (VNT) titer of 32. With this threshold, the GP ELISA exhibited 99.6% specificity for naive sera (n=3,005) from cattle (n=1,040), pigs (n=1,120), and horses (n=845) from domestic farms.

The GP ELISA did not cross-react with sera positive for foot-and-mouth disease virus, swine vesicular disease virus, or VSV serotype Indiana. The GP ELISA was more compatible with the VNT than was the nucleocapsid based ELISA for VSV-NJ-positive sera (n=19). Taken together, this GP ELISA could be a useful tool as an alternative to the VNT for detecting antibodies specific to VSV-NJ.



Keywords: Vesicular stomatitis virus serotype New Jersey, glycoprotein, ELISA

# Introduction

Vesicular stomatitis (VS) is an infectious disease of cattle, swine, and horses occurring throughout the Americas (Letchworth *et al*., 1999; Rainwater-Lovett *et al*., 2007; Rodríguez, 2002). It causes significant economic and production losses of livestock due not only to veterinary costs but also to trade and animal movement restrictions (Rainwater-Lovett *et al*., 2007). The causative agent of VS is vesicular stomatitis virus (VSV), a member of the genus *Vesiculovirus* in the family *Rhabdoviridae*. VSV has an 11-kb negative-sense single-stranded RNA that encodes five structural proteins: glycoprotein (GP), nucleocapsid (NC), phosphoprotein (P), matrix(M), and large polymerase (L) (Rainwater-Lovett *et al*., 2007). Of the two serotypes of VSV, the New Jersey (NJ) serotype is considered more important, since it is more pathogenic than the Indiana (IN) serotype and so accounts for the majority of clinical cases (Martinez *et al*., 2004; Rodríguez, 2002).

Since the clinical signs of VS in cattle and pigs are indistinguishable from those of foot-and-mouth disease (FMD), with vesicular lesions on the mouth, tongue, and teats (Ferris and Donaldson, 1988; Rainwater-Lovett *et al*., 2007), it is essential that VS be identified by rapid laboratory diagnostic methods. Whereas suspicious animals with clinical signs can simply be diagnosed through clinical surveillance or the detection of the virus, subclinically infected animals can be diagnosed only by serological surveillance. The virus neutralization test (VNT) is recognized as a

standard method for the detection of anti-VSV antibodies by the Office Internationale des Epizooties. However, it is labor-intensive, requires cell culture facilities, and takes 2 to 3 days to complete. These aspects make it unsuitable for the purposes of mass serological surveillance. To address these drawbacks, several enzyme-linked immunosorbent assay (ELISA) systems have been developed and used to measure antibody titers (Afshar *et al*., 1993b; Katz *et al*., 1995; Kweon *et al*., 2005; Vernon and Webb, 1985; Zhou *et al* 2001). Since VSV infects a broad spectrum of hosts (Coil and Meller, 2004), a serological method that can be performed irrespective of the susceptible species is preferable.

In this regard, a blocking or competitive ELISA is more appropriate than an indirect ELISA. Previously, a competitive ELISA using recombinant NC (NC ELISA) instead of GP was reported (Katz *et al*., 1995). However, it showed low sensitivity relative to the VNT (Alvarado *et al*., 2002). Competitive ELISAs with inactivated VSV or GP have been developed as well (Afshar *et al*., 1993b; Allende *et al*., 1992), but they employed polyclonal guinea pig serum as a competitor, which has inherent drawbacks. In contrast, the use of a monoclonal antibody (MAb) increases specificity and ensures unlimited quantity and consistent quality (Yang *et al*., 2007).

Although an ELISA employing a MAb has been developed for VSV-IN (Kweon *et al*., 2005), no ELISA for the detection of anti-VSV-NJ antibodies has been available yet.

The aim of this study is to demonstrate that an ELISA using GP and a neutralizing MAb could be a robust assay to replace the VNT for the detection of antibodies specific to VSV-NJ.

# Materials and Methods

## Virus and cells

VSV-NJ was obtained from the National Veterinary Services Laboratory (NVSL), Ames, IA. BHK-21 cells were cultured with alpha-minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, CA) and an antibiotic-antimycotic solution (Invitrogen) in a 37°C incubator under 5% CO2.

## Extraction of GP

The GP was extracted from partially purified VSV-NJ by a minor modification of methods previously described (Ferris and Donaldson, 1988). BHK-21 cells were infected with VSV-NJ at a multiplicity of infection of 0.001. When maximal cytopathic effect was observed, the supernatant was harvested by centrifugation at 10,000 × *g* for 30min. The virus in the supernatant was inactivated by the addition of 1 mM binary ethyleneimine (Sigma-Aldrich) at 37°C for 24 h, and the reaction was stopped by 10 mM sodium thiosulfate (Sigma-Aldrich) at 37°C for 1 h. The virus solution was concentrated with 7.5% polyethylene glycol 8000 (Sigma-Aldrich) at 4°C for 16 h, and the GP precipitate was collected by centrifugation at 10,000 × *g* for 30min. The resulting precipitates were resuspended in 5% of the original volume

of TEN buffer (50 mM Tris containing 1 mM EDTA and 0.1 M NaCl [pH 7.8]). The insoluble materials were removed by centrifugation at 3,500 × *g* for 20min. The supernatant was mixed with 0.03 M octyl-β-D-glucopyranoside (Sigma-Aldrich) at room temperature for 1h in order to strip the GP from the virus particles, and the mixture was then centrifuged at 85,000 × *g* for 2 h to sediment GP-free virus particles.

The supernatant containing GP was dialyzed against TEN buffer and then stored at 20°C until use. The concentration of this GP was determined by a bicinchoninic acid protein assay (Thermo Fisher Scientific).

**MAbs**

The hybridoma used to produce the MAb was generated by a minor modification of methods previously described (Coyle *et al*., 1992). Mice (BALB/c) were immunized twice via the footpad, at an interval of 2 weeks, with 100 × g of the GP extracted as described above in a mixture of incomplete Freund's adjuvant. The lymphocytes derived from the immunized mice were fused with SP2/O myeloma cells. Hybridoma cells were screened by indirect ELISA, immunofluorescence assay, and VNT. The MAb, designated 1G11, was finally selected from several MAbs by its capacity to compete with antibodies in antisera in the GP ELISA, and its isotype was determined as immunoglobulin G2b by MonoAb ID/SP kits (Zymed). The MAb was purified using the ImmunoPure IgG purification kit (Thermo Fisher Scientific)

according to the manufacturer's instructions.

**Sera**

To examine the limit of detection of the GP ELISA, one bovine and two swine serum samples were employed. One bovine serum sample positive for VSV-NJ was obtained from the NVSL, Ames, IA. Two 60-day-old pigs were immunized twice intramuscularly with binary ethyleneimine-inactivated VSV-NJ plus IMS1313 adjuvant (Seppic, France) in a final volume of 3 ml at an interval of 2 weeks. They were bled 20 days after the second immunization. Naive sera (n=3,005) from cattle (n=1,040), pigs (n=1,120), and horses (n=845) were collected from domestic farms with no history of exposure to VS. Control sera, included in the liquid-phase blocking ELISA kits, that were strongly positive for FMD virus (FMDV) serotypes O, A, and Asia 1 (Pirbright Laboratory, Surrey, United Kingdom) were employed.

A swine vesicular disease virus (SVDV) positive serum (RS2), which is an international positive control serum collected 21 days postinfection, was obtained from Pirbright Laboratory. The sera that were positive for VSV-NJ by the VNT (n=19) were derived from horses and were obtained from the NVSL, Ames, IA. The sera in the VSV neutralization test proficiency panel (n=20), comprising bovine, equine, and swine sera, were also obtained from the NVSL, Ames, IA. These sera had been tested by the VNT and the NC ELISA, and the records were provided by

the NVSL, Ames, IA.

**GP ELISA**

MaxiSorp ELISA plates (Nunc, Denmark) were coated with 1 $\mu$g/ml of VSV-NJ GP in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and were then incubated with 50 $\mu\ell$ of test sera diluted 1:5 in diluent (PBST containing 5% skim milk) at 37°C for 1 h. After a wash with PBST, 50 $\mu\ell$ of 30-ng/ml anti-VSV-NJ GP MAb 1G11 in the diluent was added and incubated at 37°C for 1 h.

After a wash with PBST, 50 $\mu\ell$ of 160 ng/ml goat anti-mouse antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific) in the diluent was added, and the plates were incubated at 37°C for 1 h. After the plates were washed five times, the colorimetric reaction was developed for 15min by the addition of 0.6 mg/ml of *o*-phenylenediamine in 0.05 M citrate phosphate buffer (pH 5.0) plus 0.015% hydrogen peroxide. The reaction was stopped by the addition of 50 $\mu\ell$ of 1.25 M sulfuric acid. The optical density (OD) was measured at 492 nm, and the OD value was converted to the percentage of inhibition (PI) by the following formula: PI = 100 x [1 - (OD of test serum well/OD of control well)], where the control well is the well containing the MAb alone.

**VNT**

The VNT was carried out, using 96-well tissue culture microplates with flat-bottom wells seeded with $5\times10^4$ BHK-21 cells per well, according to the manual of standards for diagnostic tests and vaccines of the Office International des Epizooties. In brief, a VSV-NJ suspension containing 1,000 tissue culture infective doses/well was distributed into each well. The VNT titer was expressed as the reciprocal of the final dilution of serum at which 50% of the cells in the wells were protected. Sera with VNT titers equal to or greater than 32 were considered positive.

# Results

**Establishment of the GP ELISA.**

The GP that was used in the GP ELISA as a diagnostic antigen was extracted from partially purified VSV-NJ as usual. The selected MAb, 1G11, was specific to VSV-NJ, but not VSV-IN, by immunofluorescence assay and VNT. Since the MAb did not bind to the GP by Western blotting, it was supposed to recognize the conformational epitope of VSV-NJ GP. The GP ELISA was designed in a blocking format in which MAb 1G11 competes with antibodies in test sera. The optimal concentrations of GP antigen (1 $\mu$g/ml) and MAb 1G11 (30 ng/ml) were determined by checkerboard titration.

The optimal serum dilution turned out to be fivefold in the experiment with serially diluted rabbit sera. To determine the cut-off level for the GP ELISA, naïve sera (n=3,005) derived from domestic farms with no history of exposure to VSV were employed (Fig. 1.1). They included the main susceptible species: cattle (n=1,040), pigs (n=1,120), and horses (n=845). Since the mean PI was 8.0% and the standard deviation was 9.8%, the cu-toff was set at a PI of 40% by calculating the mean plus three times the standard deviation [8.0 + (3 x 9.8) = 37.4] to secure a high specificity irrespective of the species. With this cut-off level, there were four false positive reactions (0.4%) for bovine sera, seven for swine sera (0.6%), and one for equine

sera (0.1%). In total, only 12 sera (0.4%) were interpreted as nonspecific positive, resulting in a specificity of 99.6%.

**Limit of detection of the GP ELISA**

To examine the limit of detection of the GP ELISA in relation to the VNT, one bovine serum sample obtained from the NVSL that tested positive by the NC ELISA and two sera from swine immunized with binary ethyleneimine-inactivated VSV-NJ were employed (Fig. 1.2). The positive sera serially diluted in negative sera were assessed by the GP ELISA and the VNT. Based on the cut-off of 40% inhibition for the GP ELISA and the cut-off of a titer of 32 for the VNT, the last dilutions of sera that were scored positive by both assays were a 64-fold-diluted serum sample for cattle (Fig. 1.2A) and 400-fold-diluted sera for pigs (Fig. 1.2B). This indicates that the detection limit of the GP ELISA corresponds to the VNT titer of 32.

**Evaluation of the GP ELISA**

The GP ELISA was evaluated with equine sera (n=19) that were all positive by the VNT for VSV-NJ (Table 1.1). The panel consisted of sera with high titers by the VNT; 14 out of 19 sera showed VNT titers above 512. While the GP ELISA correctly scored all the sera as positive, the NC ELISA from the NVSL gave negative results

for five sera, which had relatively low VNT titers except for one serum sample (serum sample 2). Taken together, the GP ELISA could detect antibodies with VNT titers above 64, a finding consistent with the results shown in Fig. 1.2.

To assess the sensitivity of the GP ELISA more definitely in relation to the VNT, a panel of sera from the NVSL with titers bordering on the VNT cut-off level was employed (Table 1.2). The panel was composed of bovine, equine, and swine sera, including negative sera; the VNT titers ranged from 16 to 128. The panel included six VSV-NJ positive sera and six VSV-IN positive sera.

The GP ELISA scored five out of six VSV-NJ positive sera in the panel correctly; it scored one sample (serum sample 12) as negative, just below the cut-off value. Although serum sample 12, with a VNT titer of 64, was negative by the GP ELISA, the other sera with the same VNT titers (Table 1.1, serum sample 11; Table 1.2, serum samples 4 and 13) were defined as positive by the GP ELISA, and even the serum sample with a VNT titer of 32 (Table 1.2, serum sample 16) was also positive by the GP ELISA.

It was notable that serum sample 6 (Table 1.2), which had a VNT titer of 16 for VSV-NJ, was negative by the GP ELISA, demonstrating that the cut-off level of 40% inhibition established for the GP ELISA corresponded exactly to the cut-off level for VNT titers. In contrast, the GP ELISA scored all six VSV-IN positive sera as negative, with PIs far below the cut-off value, regardless of the VNT titers.

To investigate whether the GP ELISA could also distinguish VSV-NJ infection

from other, similar diseases, sera strongly positive for FMDV (serotypes O, A, and Asia 1) that were included in the LPB ELISA kits and a reference serum (RS2) strongly positive for SVDV were employed. The PIs of sera positive for FMDV serotypes O, A, and Asia 1 and of the SVDV-positive serum RS2 were 6% ± 1.7%, 4% ± 3.0%, 8% ± 2.1%, and 8% ± 2.1%, respectively, equivalent to the mean PIs of naive sera as shown in Fig. 1.1.

# Discussion

In this study, I established an ELISA using the GP extracted from VSV-NJ and a MAb specific to VSV-NJ, and we explored its feasibility in relation to the VNT for the detection of VSV-NJ antibodies. VSV GP is the sole protein anchored in the envelope and plays a critical role in the early stage of virus infection (Gallione *et al*., 1983; Martinez *et al*., 2004; Roche *et al*., 2008; Schlege *et al*., 1985). Therefore, the epitopes involved in virus neutralization are clustered on the GP (Kelley *et al*., 1972). An ELISA with a similar format using recombinant GP and a MAb for VSV-IN has been reported previously (Kweon *et al*., 2005). Since VSV-NJ causes more severe pathogenicity than VSV-IN (Martinez *et al*., 2004; Rodríguez, 2002), it was essential to develop a rapid assay for the detection of antibodies specific to VSV-NJ.

The GP was successfully extracted from the partially purified VSV-NJ. The yield was sufficient to test 30 plates per ml of culture volume. Even though several MAbs had higher VNT titers than 1G11, none were comparable to 1G11 in competing with anti-VSV-NJ antibodies in susceptible animal sera by the GP ELISA. This suggests that the ability of a MAb to neutralize VSV-NJ does not always parallel its usefulness as a diagnostic competitor in a blocking format of a GP ELISA for VSV-NJ.

In addition, whereas some other MAbs showed variable competitive reactivities depending on the species, the competition of 1G11 with serum antibodies was consistent irrespective of the species; this was the key determinant in selecting 1G11

as a detector in the blocking format of the GP ELISA. Since 1G11 did not show reactivity to the GP by Western blotting, it is considered to recognize a conformational epitope. The exact epitope of 1G11 needs to be defined in order to elucidate the importance of this locus in relation to other, previously reported epitopes (Keil *et al*., 1989; Lefrancois *et al*., 1983; Nagata *et al*., 1992).

The advantage of the NC ELISA is that it distinguishes between animals vaccinated with VSV GP and VSV-infected animals (Ahmad *et al*., 1993). However, considering that no VSV vaccine is currently available, it was more important to develop a rapid assay to detect anti-VSV-NJ antibodies in animals expressing early-stage infection than to distinguish between infected animals and those that might be vaccinated. In this regard, the ELISA using the GP and an anti-GP MAb was established in a blocking format to be applied regardless of species.

The detection limit of the GP ELISA was determined as the point corresponding to a VNT titer of 32, the cut-off level for bovine and swine sera. Even though the detection limit remains to be determined with more sera, this result suggests that the GP ELISA is feasible as a surrogate assay in place of the VNT for bovine and swine sera. This was expected, because the GP ELISA and the VNT target the same GP, even though the numbers of epitopes on the GP recognized by the GP ELISA and the VNT are different. It has been reported that more than one epitope on the antigen surface can be recognized directly by a competitor MAb or blocked by steric hindrance or conformational changes induced by antibodies binding to other epitopes

(Sugiyama *et al*., 1997). These phenomena could compensate for the small number of epitopes recognized by 1G11 in the GP ELISA compared to the VNT. There is also another possibility, that the 1G11 epitope may be the predominant antigenic site on the GP of VSV-NJ. Even though we did not use the sequential sera post-VSV infection to investigate how early the GP ELISA could detect VSV-NJ antibodies in comparison to the NC ELISA or the VNT, evidence that the sensitivity of the GP ELISA is comparable to that of the VNT was provided by the data showing that the GP ELISA was more sensitive than the NC ELISA in detecting antibodies to VSV-NJ in sera (n=19) that were positive by the VNT (Table 1.1). This result was in accordance with previous reports that discrepant results between the NC ELISA and the VNT may be due to different antibodies in the NC ELISA and the VNT (Alvarado *et al*., 2002; Katz *et al*., 1995).

Actually, the GP ELISA and the VNT should be equivalent, because they recognize the same neutralizing antibodies. Among the proficiency panel sera employed to examine the correlation between the GP ELISA and the VNT, the negative result for one serum sample (serum sample 12) for which the PI was 32% ± 9.4% could be explained by certain individual differences between sera. The PI of the serum sample (Table 1.2, serum sample 16) with a VNT titer of 32 was 48% ± 3.1%, demonstrating that the cut-off of the GP ELISA is at least comparable to that of the VNT. This suggestion was corroborated by the result that serum sample 6 in Table 1.2, with a VNT titer of 16, was negative by the GP ELISA.

The GP ELISA could be a useful tool to distinguish VSV-NJ from VSV-IN, in contrast to the NC ELISA, as shown in Tables 1.1 and 1.2. This is reflected in the previous report that the NC ELISA could not replace the VNT for serotyping purposes, because the NC ELISA titers were less serotype specific than the titers measured by the VNT (Katz *et al*., 1995). This may be explained by the fact that the amino acid similarity of the NC of VSV-NJ to the NC of VSV-IN is 69%, as opposed to 50% for the GP (Ahmad *et al*., 1993; Katz *et al*., 1995, Martinez *et al*., 2004; Martinez *et al*., 2005).

Since the amino acid similarity between the GPs of VSV-NJ and VSV-IN amounts to 50%, it was speculated that there would be some degree of cross-reactivity between the serotypes by the GP ELISA. Contrary to our expectation, there was no cross-reactivity toward VSV-IN, even for the serum sample with a VNT titer of 128.

This may be due to the use of a MAb instead of polyclonal sera as a competitor in the GP ELISA. In particular, the PIs of sera positive for VSV-IN were equivalent to the mean PI of naive sera, suggesting low chances that VSV-IN-positive sera could cause interference with the interpretation of results for VSV-NJ by the GP ELISA. Of course, this GP ELISA should be further examined with sera with VSV-IN neutralization titers above 128 in the future.

Since VS is clinically indistinguishable from other vesicular diseases, it is essential to differentiate VS from other vesicular diseases, such as FMD and SVD, by a rapid serological assay. Particularly in South America, where VS and FMD occur

frequently, most cattle are vaccinated annually with FMDV vaccines (Allende *et al*., 1992).

The GP ELISA was found to be specific to VSV-NJ and did not react with sera that were strongly positive for FMD or SVD. In particular, this serotype specificity of the GP ELISA should be useful for epidemiological analysis in VS outbreak regions.

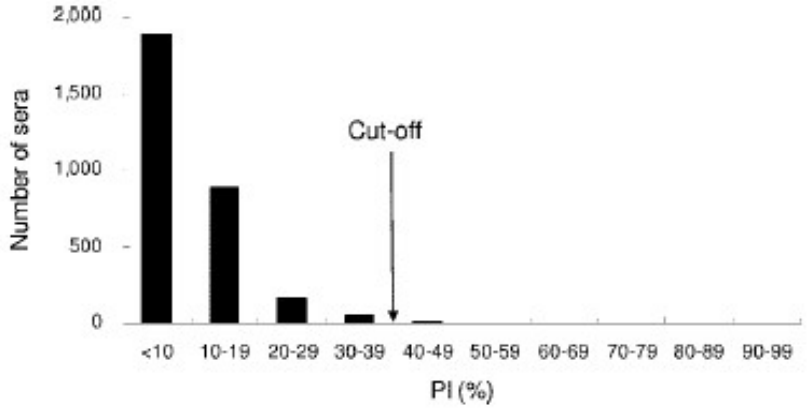


Figure 1.1. Determination of the specificity of the GP ELISA. The frequency distribution of the PI by the GP ELISA was recorded for naive sera (n=3,005) from cattle (n=1,040), pigs (n=1,120), and horses (n=845). The cut-off value (PI above background) was set at 40% inhibition.

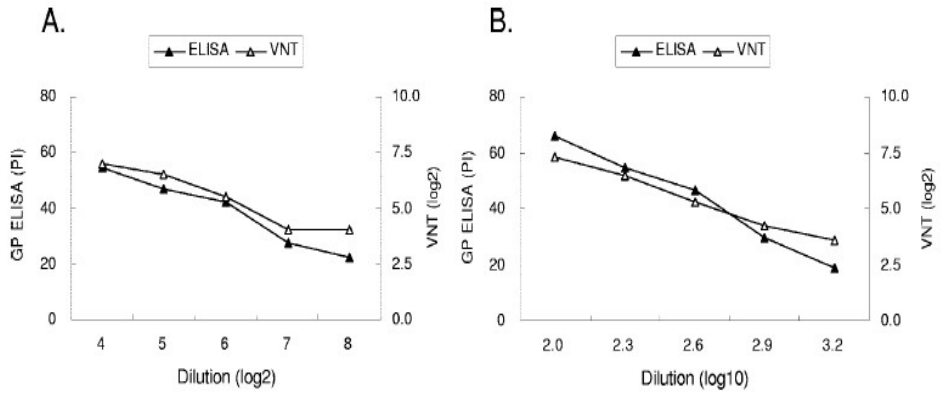


Figure 1.2. Defining the limit of detection of the GP ELISA in relation to that of the VNT. One positive bovine serum sample infected with VSV-NJ (A) and two swine sera immunized with binary ethylenimine-inactivated VSV-NJ (B) were serially diluted with the respective VSV negative sera and tested by the GP ELISA and the VNT. The results were analyzed comparatively by identifying the last serum dilution defined as positive by each assay, based on the cut-off of 40% inhibition for the GP ELISA and a titer of 32 for the VNT. The results in panel B are means for two swine sera.

Table 1.1. Relative sensitivities of the GP ELISA and the NC ELISA for VSV-NJ positive sera

| Serum sample | VNT titer [a] | PI [b] (%) by: | |
|---|---|---|---|
| | | NC ELISA | GP ELISA |
| 1 | **128** | 36 | **57 ± 0.9** |
| 2 | **>512** | 49 | **94 ± 1.6** |
| 3 | **>512** | **64** | **83 ± 1.5** |
| 4 | **>512** | **56** | **90 ± 0.4** |
| 5 | **>512** | **57** | **94 ± 1.7** |
| 6 | **>512** | **64** | **94 ± 1.3** |
| 7 | **>512** | **63** | **82 ± 1.2** |
| 8 | **256** | **67** | **79 ± 3.2** |
| 9 | **>512** | **52** | **74 ± 8.0** |
| 10 | **>512** | **63** | **87 ± 2.8** |
| 11 | **64** | 44 | **69 ± 2.7** |
| 12 | **>512** | **76** | **78 ± 0.6** |
| 13 | **128** | 44 | **80 ± 0.2** |
| 14 | **>512** | **59** | **93 ± 2.5** |
| 15 | **>512** | **70** | **89 ± 1.5** |
| 16 | **>512** | **80** | **88 ± 0.1** |
| 17 | **>512** | **76** | **88 ± 0.3** |
| 18 | **256** | 34 | **63 ± 3.0** |
| 19 | **>512** | **76** | **68 ± 18.2** |

[a] from the records of the NVSL, Ames, IA.

[b] The NC ELISA results were provided by the NVSL, Ames, IA. The GP ELISA results were obtained in this study and are means ± standard deviations (n=3). Boldface numbers indicate positive results by each assay, based on a cut-off of 50% inhibition for the NC ELISA and 40% inhibition for the GP ELISA.

Table 1.2. Serotype specificity of the GP ELISA versus the VNT using a panel of sera (n=20)

| Serum sample | VNT titer [a] for: | | PI [b] (%) by: |
|---|---|---|---|
| | VSV-IN | VSV-NJ | GP ELISA |
| 1 | <8 | <8 | 11 ± 13.8 |
| 2 | <8 | <8 | 1 ± 2.7 |
| 3 | <8 | <8 | 1 ± 0.7 |
| 4 | <8 | **64** | **56 ± 2.8** |
| 5 | **64** | <8 | 5 ± 3.0 |
| 6 | <8 | 16 | 18 ± 3.6 |
| 7 | **32** | <8 | 5 ± 3.9 |
| 8 | 16 | <8 | 7 ± 4.1 |
| 9 | **64** | <8 | 4 ± 4.4 |
| 10 | **32** | <8 | 3 ± 1.5 |
| 11 | <8 | **128** | **83 ± 4.4** |
| 12 | <8 | **64** | **32 ± 9.4** |
| 13 | <8 | **64** | **73 ± 1.9** |
| 14 | **128** | <8 | 2 ± 2.3 |
| 15 | **64** | <8 | 1 ± 1.3 |
| 16 | <8 | **32** | **48 ± 3.1** |
| 17 | <8 | <8 | 3 ± 3.4 |
| 18 | <8 | **128** | **41 ± 6.0** |
| 19 | <8 | <8 | 1 ± 1.5 |
| 20 | <8 | <8 | 2 ± 2.4 |

[a] Provided by the NVSL, Ames, IA. Boldface numbers indicate positive results by each assay, based on a cut-off titer of 32 for the VNT (for VSV-NJ and VSV-IN) and a cut-off PI of 40% for the GP ELISA.

[b] Determined in this study. Results are means ± standard deviations (n = 3).

# Chapter 2

# Development of a blocking ELISA using a recombinant glycoprotein for the detection of antibodies to vesicular stomatitis New Jersey virus

# Abstract

A recombinant glycoprotein (R-GP) of vesicular stomatitis New Jersey virus (VSV-NJ) was expressed in insect cells by a baculovirus system. Its utility as a diagnostic antigen in a blocking ELISA was investigated as an alternative to the current native GP extracted from VSV-NJ. With the cut-off value of 73% inhibition, the R-GP ELISA exhibited 99.1% specificity for naive sera from cattle and horses. It did not cross-react with VSV-Indiana (VSV-IN) positive sera and differentiated from foot-and-mouth disease and swine vesicular disease. Taken together, this is the first report that the R-GP has a potential to be used as a diagnostic antigen in place of the native GP for the detection of antibodies to VSV-NJ in cattle and horses.



Keywords: Vesicular stomatitis New Jersey virus, recombinant glycoprotein

# Introduction

Vesicular stomatitis (VS) is a contagious disease of livestock including horses and cattle throughout the Americas (Letchworth *et al*., 1999; Rodriguez, 2002). VS is characterized by vesicular lesions on the tongue, lips, coronary band, and teats (Letchworth *et al.,* 1999). The causative agent is vesicular stomatitis virus (VSV), a member of the genus *Vesiculovirus* in the family *Rhabdoviridae*. VSV has an 11 kb single-stranded RNA of negative polarity that encodes five structural proteins: glycoprotein (GP), nucleocapsid (NC), phosphoprotein (P), matrix (M), and large polymerase (L) (Rainwater-Lovett *et al*., 2007). Two main serotypes of VSV have been described: New Jersey (NJ) and Indiana (IN) (Rodriguez, 2002; Martinez *et al.,* 2004; Martinez and Wertz, 2005).

Since clinical signs of VS are similar to those of foot-and-mouth disease (FMD) and swine vesicular disease (SVD), VS should be differentiated from other vesicular diseases by a rapid laboratory diagnostic method (Ferris and Donaldson, 1988).

Although the virus neutralization test is a golden standard assay, it is laborious and time-consuming, and requires high security conditions, thus being unsuitable for large scale serosurveillance. To overcome these shortcomings, several ELISAs have been developed as alternative methods (Vernon and Webb, 1985; Allende *et al*., 1992; Afshar *et al.,* 1993a, b; Ahmad *et al*., 1993; Katz *et al.,* 1995; Zhou *et al*., 2001; Alvarado *et al*., 2002; Kweon *et al*., 2005). Since the sensitivity of a competitive

ELISA using a recombinant nucleocapsid (NC ELISA) was lower than that of the virus neutralization test (Alvarado *et al*., 2002), an ELISA employing a native GP extracted from VSV-NJ has recently been developed (Lee *et al*., 2009).

However, production of the native GP requires biosafety facility, and live virus manipulation always poses a risk of accidental release into the environment.

Therefore, the aim of this study was to replace the native GP with a recombinant GP (R-GP) expressed by a baculovirus system as a safe diagnostic antigen.

# Materials and Methods

## Cell and viruses

Vero cells were grown in alpha minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotic–antimycotic solution in a 37°C incubator. *Spodoptera frugiperda* (Sf9) cells (Invitrogen, Carlsbad, CA, USA) for the propagation of a recombinant baculovirus were grown in Grace's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, antibiotic–antimycotic solution, and lactalbumin hydrolysate (Invitrogen) in a 27°C incubator. The VSV-NJ was obtained from the National Veterinary Services Laboratories (Ames, IA, USA).

## Construction of the recombinant baculovirus plasmid

Viral RNA was extracted from Vero cells infected with VSV-NJ using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Complementary DNA of VSV-NJ GP RNA was produced using random hexamers and AccuPower RT Premix (Bioneer, Daejeon, Korea). The GP gene was amplified from cDNA using nPfu DNA polymerase (Enzynomics, Seoul, Korea). Primers containing *Bam*HI and *Xho*I were designed based on the sequence of M21425 as follows: forward, 5'-

GCGCGGATCCATGTTGTCTTATCTACTAATC-3‘; reverse, 5‘-ATCCCTCGAGTTAAC GGAAATGA GCCAT-3‘. The amplification of the GP gene was carried out in a thermal cycler, with an initial denaturation at 95°C for 2min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, with a final extension at 72°C for 10min. The amplified GP gene was cloned into pBacPAK8 transfer vector (Clontech, Mountain View, CA, USA) using *Bam*HI and *Xho*I. The amino acid sequence of the amplified GP gene was found to be the same as that of M21425.

**Expression of the R-GP**

The recombinant baculovirus was produced by co-transfecting the recombinant transfer vector and pBacPAK6 baculovirus linear DNA (Clontech) into Sf9 cells. It was then stored at 4°C until use. Sf9 cells grown in tissue culture flasks were infected with the recombinant baculovirus and harvested when the maximal cytopathic effect was observed. The cell pellet was washed three times with phosphate buffered saline (PBS) and resuspended in TEN buffer (50mM Tris, 1mM EDTA, 0.1M NaCl, pH 7.8) in 5% of the initial culture volume. The cells were disrupted using a sonicator (Sonics and Materials, Newton, CT, USA) with 10 cycles of 5 s on and off. The crude cell lysate was used as a diagnostic antigen in this study. For Western blot analysis, the protein samples were separated using NuPAGE Novex Bis–Tris gels with Xcell SureLock Mini-Cell (Invitrogen) according to the manufacturer’s

instructions. Separated proteins were transferred from the gels onto nitrocellulose membranes. After blocking 1 h with a diluent consisting of Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% skim milk, MAb A13 to VSV-NJ GP was added at 1:100 in diluent and incubated for 1 h at 37°C. Following washing with TBST, the secondary antibody, goat anti-mouse labeled with alkaline phosphatase (KPL, Frederick, MD, USA), was added at 1:3000 in diluent and incubated for 1 h at 37°C. After washing the membrane, the commercial membrane substrate BCIP/NBT (KPL) was added and incubated at room temperature to develop the colorimetric reaction.

**Immunogenicity of the R-GP**

To obtain a soluble form of the R-GP, the crude cell lysate was clarified to remove cell debris by centrifuging at 14,000 × g for 20min, and then partially purified using a Resource Q, anion exchange column, with the AKTA-purifier system (GE Healthcare, Piscataway, NJ, USA). Four cattle were immunized intramuscularly with 0.2 mg of soluble GP in a mixture with IMS 1313 adjuvant (Seppic, Paris, France) and received a booster 22 days after the primary immunization. They were bled 22 days after primary immunization and again 19, 31, and 38 days post-booster.

**Sera**

Sera (n=19) that were collected from horses in the field and positive for VSV-NJ with high virus neutralization titres, were obtained from the National Veterinary Services Laboratories, which also provided the records of the virus neutralization test and NC ELISA. Another panel of sera (n=20) included low virus neutralization titres of sera positive for VSV-NJ and VSV-IN collected from experimentally infected cattle, horses and pigs. Of 20 sera, VSV-NJ positive sera were four bovine and two equine sera, and six VSV-IN positive sera consisted of four bovine and two swine sera. These sera and the records of the virus neutralization test were provided by the National Veterinary Services Laboratories. Naive sera (n=2,052) from cattle (n=1,036) and horses (n=1,016) were collected from domestic farms with no history of exposure to VSV.

Strong positive control sera included in the liquid phase blocking (LPB) ELISA kits for FMD serotype O, A and Asia 1 (Institute for Animal Health, Pirbright, Surrey, UK) were employed as FMD positive sera.

The positive serum (RS2) of SVD is an international reference serum collected 21 days post-infection and was obtained from Pirbright Laboratory (Surrey, UK).

**Monoclonal antibodies**

Monoclonal antibodies (MAbs) were generated according to the methods described previously (Coyle *et al*., 1992). The neutralizing MAb, 1G11, was selected as a

competitor antibody in a blocking ELISA as described previously (Lee *et al.,* 2009) and the other MAb, A13, was used to identify the native and recombinant GPs by Western blotting. The MAbs were purified using the Imunopure IgG purification kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

**R-GP ELISA**

MaxiSorp ELISA plates (Nunc, Glostrup, Denmark) were coated with the crude cell lysate diluted 1:200 in 0.05M carbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed with PBS containing 0.05% Tween 20 (PBST) and then incubated with test sera diluted 1:5 in a diluent (PBST containing 5% skim milk) at 37°C for 1h. Following washing, 2 $\mu$g/ml MAb 1G11 in diluent was added and incubated at 37°C for 1h. After washing, 200 ng/ml goat anti-mouse antibody labeled with horseradish peroxidase (Thermo Fisher Scientific) in diluent was added and the plates were incubated at 37°C for 1 h. After washing, 0.6 mg/ml of O-phenylenediamine plus 0.015% hydrogen peroxide were added and incubated for 15 min. The reaction was stopped by adding 1.25Msulfuric acid. Optical density (OD) was measured at 492 nm.

The OD value was converted to percentage inhibition (PI) using the formula:

$$\text{PI}\ (\%) = \left[1 - \frac{\text{OD of test serum well}}{\text{OD of control well}}\right] \times 100$$

Where OD of control well represents the well containing MAb alone.

**Virus neutralization test**

The virus neutralization test was carried out using tissue culture 96-well microplates with flat-bottomed wells according to World Organisation for Animal (OIE) standards for diagnostic tests and vaccine (OIE, 2008). In brief, a VSV-NJ suspension containing 1000 $TCID_{50}/50\mu\ell$ and Vero cells was employed. The virus neutralization titre was expressed as a reciprocal of the final serum dilution where 50% of the wells were protected. The serum with virus neutralization titre equal to or greater than 32 was considered positive.

# Results

## Characterization of recombinant VSV-NJ GP

The morphology of Sf9 cells infected with the recombinant baculovirus differed from that of normal cells. Prominent multinucleate syncytia were observed 3–4 days post-infection, in contrast to control Sf9 cells that remained as single cells showing round morphology (Fig. 2.1A).

To examine expression of the R-GP in insect cells, Western blot was performed with the MAb, A13, which is specific to VSV-NJ (Fig. 2.1B). The R-GP was the same molecular weight as the native GP extracted from VSV-NJ. However, several smaller bands of native GPs, presumed to be degraded products, were also observed.

To investigate whether the R-GP preserved the neutralizing epitopes intact, the crude cell lysate was further processed and partially purified to obtain a soluble fraction of the R-GP. Four cattle were immunized twice with the soluble R-GP. At 22 days after the primary immunization, the virus neutralization titres of two (A and C) were 9 log 2 and those of the other two (B and D) were 10 log 2 (Fig. 2.1C). After the booster, the virus neutralization titres of cattle A to D rose sharply to 14.2 log 2, 16 log 2, 14.7 log 2, and 16.5 log 2, respectively, after which they declined somewhat, but remained high at above 13 log 2 throughout the experimental period.

**Defining the cut-off value of the blocking ELISA**

As the R-GP preserved neutralizing epitopes in cattle, it was then evaluated as a diagnostic antigen of a blocking ELISA. As an initial standardization of the R-GP ELISA, the optimal conditions were determined following checkerboard titrations (data not shown). The R-GP crude cell lysate was optimal at a dilution of 1:200 in coating buffer. The optimal concentration of the neutralizing competitor, MAb 1G11 was 2 $\mu$g/ml and the test serum was optimal at five-fold dilution. To define the cut off value of the R-GP ELISA, naive sera (n=2,052) from domestic farms with no history of exposure to VSV were employed. The cut-off value was established at 73% inhibition by calculating the mean PI value plus three times the standard deviation [25% + (3×16%) = 73%] for naive sera from horses (n=1,016) and cattle (n=1,036) (Fig. 2.2). Using this cut-off value, the R-GP ELISA showed three false positive reactions (0.3%) for equine sera and 15 false positive (1.4%) for bovine sera. In all, the R-GP ELISA exhibited 18 false positive reactions (0.9%), resulting in 99.1% specificity.

**Evaluation of the blocking ELISA**

The relative sensitivity of the R-GP ELISA in comparison to the virus neutralization test was measured using VSV-NJ positive sera (n=19) with high virus

neutralization titres (Table 2.1). Whereas the NC ELISA did not detect five sera, the R-GP ELISA correctly scored all sera as positive regardless of virus neutralization titres. This result was the same as that of the native GP ELISA (Lee *et al*., 2009).

To gauge the sensitivity of the R-GP ELISA with reference to the virus neutralization test, we used sera with low virus neutralization titres derived from experimentally infected animals (Table 2.2). While the native GP ELISA detected five out of six VSV-NJ positive sera, the R-GP ELISA correctly scored all six sera as positive, being comparable to the virus neutralization test. The R-GP ELISA even scored the serum with a virus neutralization titre of 64 as positive, whereas the native GP ELISA scored it negative. Notably, the R-GP ELISA defined the serum with a virus neutralization titre of 16 as negative and the serum with a virus neutralization titre of 32 as positive. The six VSV-IN positive sera were all negative by the R-GP ELISA.

To examine whether the R-GP ELISA could also differentiate VS from other similar vesicular diseases, FMD strong-positive sera (serotypes O, A, and Asia 1) and strong-positive serum (RS2) for SVD were tested by the R-GP ELISA. The PI values of FMDV O, A, Asia 1 and SVD RS2 were 30 ± 1.7, 8 ± 0.6, 5 ± 2.1, and 31 ± 2.6, respectively.

# Discussion

A blocking ELISA using the native GP extracted from VSV-NJ and a neutralizing MAb was recently developed (Lee *et al*., 2009). However, use of the native GP derived from VSV-NJ requires handling the live virus, thus posing a risk of virus release into the livestock field. In contrast, the R-GP could be manufactured at any laboratory without facility limitation. To this end, the GP gene from VSV-NJ was expressed in insect cells and investigated as a diagnostic antigen.

The entire R-GP gene, cloned in a baculovirus vector, encodes four domains composed of amino-terminal signal region, extracellular domain, transmembrane domain, and carboxy-terminal cytoplasmic region (Bailey *et al.,* 1989; Roche *et al.,* 2006, 2008). It was reported syncytia form when VSV GP faces acidic pH as a membrane protein (White *et al*., 1981; Bailey *et al*., 1989; Carneiro *et al*., 2001; Roche *et al.,* 2008). The R-GP expressed in this study also induced a fusogenic reaction among insect cells that were maintained in acidic (pH 6.0) medium.

Baculovirus-expressed GP from VSV or rabies has been reported to exhibit a smaller size compared to the native GP likely because of the different glycan complexities made in the distinct cell types, as insect cells is the lack sialyltransferase activity (Bailey *et al.,* 1989; Prehaud *et al.,* 1989). Contrary to these reports, the R-GP of this study had the same size band as the native GP. The cause of this discrepancy remains to be addressed in the future. However, another

baculovirus-expressed VSV-IN GP (Kweon et *et al*., 2005) had the same molecular weight as the R-GP in this study.

The GP is a transmembrane protein that protrudes from the VSV surface, which explains its exclusive role in eliciting neutralizing antibodies in susceptible hosts (Kelley *et al.,* 1972; Pinschewer *et al*., 2004). As expected, the R-GP expressed in this study exhibited a potent immunogenic property. One immunization was sufficient to elicit a high titer of neutralizing antibodies, indicating that it preserved neutralizing epitopes. This result allowed to develop a blocking ELISA using the R-GP as an alternative to the native GP reported previously (Lee *et al*., 2009).

Although the soluble fraction of the cell lysate was used as an immunogen, it did not work as well as the crude cell lysate in terms of a diagnostic antigen when it was applied in the ELISA (data not shown). I presume that the epitope recognized by 1G11, may be correctly exposed only when tethered to the cell membrane. This would be consistent with a previous report that a competitive ELISA for rabies employed cell lysate suspension from transformed BHK-21 cell lines as a diagnostic antigen (Zhang et al., 2009).

The cut-off value of the R-GP ELISA was established at 73% inhibition, which was higher than the 40% cut-off value of the native GP ELISA (Lee *et al*., 2009). This high cut-off value was also observed in a MAb-based ELISA for SVD (Brocchi *et al*., 1995), a prescribed test in the OIE manual (OIE, 2008). The R-GP ELISA exhibited a higher sensitivity than the NC ELISA as reported previously for the

native GP ELISA (Lee *et al.,* 2009). Although sequential sera were not employed to assess the early antibody response, the R-GP ELISA worked as well as the native GP ELISA for VSV-NJ positive sera (n=19) with high virus neutralization titres and panel sera (n=20) with low virus neutralization titres for VSV-NJ and VSV-IN.

In addition, the R-GP ELISA differentiated VSV-NJ from other vesicular diseases such as FMD and SVD in a preliminary test, indicating that the R-GP ELISA may be useful in South America where FMD and VS outbreaks occur. For serotype specificity, the R-GP ELISA did not cross-react with VSV-IN sera that had virus neutralization titre up to 128. In summary, the R-GP ELISA may be a useful tool as an alternative to the native GP ELISA for the detection of antibodies to VSV-NJ in cattle and horses.

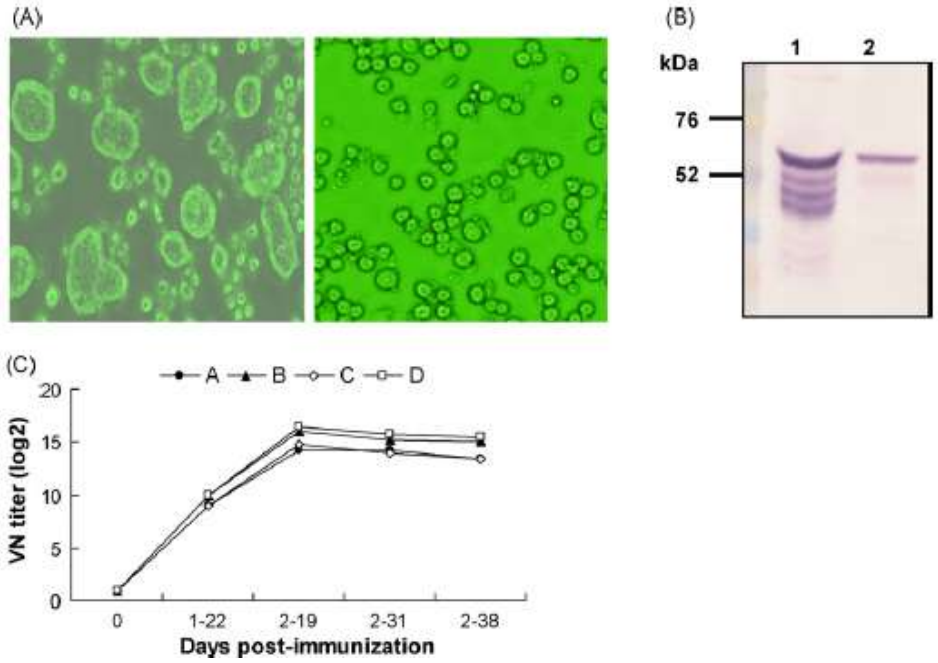


Figure 2.1. Characterization of the R-GP in insect cells. (A) Comparison of morphologies between recombinant baculolvirus-infected Sf9 cells (left panel) and mock-infected control Sf9 cells (right panel). (B) Native GP (lane 1) and R-GP (lane 2) were identified by Western blotting with MAb A13 specific to VSV-NJ. (C) Four cattle (A–D) were immunized intramuscularly with the soluble fraction of the R-GP. They were bled 22 days after the primary immunization and then 19, 31, and 38 days post-booster. A virus neutralization titre less than 16 is represented as 1 log 2 for simplicity.

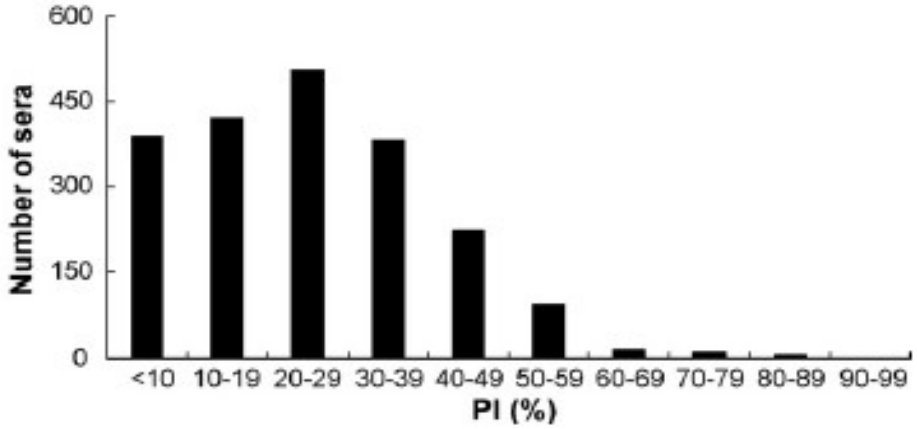


Figure 2.2. Determination of the R-GP ELISA specificity. The frequency distribution of percentage inhibition (PI) was recorded for naive sera (n=2,052) from cattle (n=1,036) and horses (n=1,016) by the R-GP ELISA.

Table 2.1. Evaluation of the R-GP ELISA for panel sera with high virus neutralization titres (n=19)

| VNT[a] | | NC ELISA[a] | | R-GP ELISA[b] | |
|---|---|---|---|---|---|
| Titer | No. sera | No. pos. | No. neg. | No. pos. | No. neg. |
| >512 | 14 | 13 | 1 | 14 | 0 |
| 256 | 2 | 1 | 1 | 2 | 0 |
| 128 | 2 | 0 | 2 | 2 | 0 |
| 64 | 1 | 0 | 1 | 1 | 0 |

[a] Virus neutralization test. The results were recorded by the National Veterinary Services Laboratories (Ames, IA, USA).

[b] Recorded in this study, based on the cut-off value of 73% inhibition.

Table 2.2. Evaluation of the R-GP ELISA for panel sera with low virus neutralization titres (n=20)

| Serotype | VNT[a] | | Native GP ELISA[b] | | R-GP ELISA[c] | |
|---|---|---|---|---|---|---|
| | No. pos | No. neg | No. pos. | No. neg. | No. pos. | No. neg. |
| VSV-NJ | 6[d] | 14[e] | 5 | 15 | 6 | 14 |
| VSV-IN | 6[f] | 14 | 0 | 20 | 0 | 20 |

[a] Virus neutralization test. The results were recorded by the National Veterinary Services Laboratories (Ames, IA, USA).

[b] Recorded previously by the native GP ELISA (Lee *et al*., 2009).

[c] Recorded in this study, based on the cut-off value of 73% inhibition.

[d] The number of sera with virus neutralization titres of 32, 64, and 128 was one, three, and two, respectively.

[e] One had a virus neutralization titre of 16, just below the cut-off value of the virus neutralization test.

[f] The number of sera with virus neutralization titres of 32, 64, and 128 was two, three, and one, respectively.

# Chapter 3

# Recombinant vesicular stomatitis virus glycoprotein carrying a foot-and-mouth disease virus epitope as a vaccine candidate

# Abstract

Foot-and-mouth disease is one of the most highly contagious animal diseases. In an effort to overcome the drawbacks of the currently used inactivated foot-and-mouth disease virus vaccine, a novel recombinant protein carrying foot-and-mouth disease virus VP1 GH loop epitope linked to vesicular stomatitis virus glycoprotein was expressed in a baculovirus system. Its antigenicity was confirmed with ELISA using monoclonal antibody against foot-and-mouth disease virus. Twice immunizations one month apart in field pigs resulted in a significant antibody increase compared to the glutathione S-transferase carrier containing the same epitope and the commercial vaccine.

To my knowledge, this is the first report that the recombinant protein vaccine was superior to the current vaccine. Although further studies are required to examine their immunogenicity in a large number of animals, this study sheds light on the development of a novel recombinant protein vaccine that could be easily produced in a general laboratory as an alternative to the current FMD vaccine, which requires a biosafety level 3 containment facility for vaccine production.



Keywords: epitope, Foot-and-mouth disease, vaccine, vesicular stomatitis virus glycoprotein

# Introduction

Foot-and-mouth disease (FMD) is one of the most highly contagious animal diseases, and the FMD virus (FMDV) rapidly replicates and spreads from infected animals via contact with susceptible animals and as an aerosol (Grubman and Baxt., 2004). FMDV is the prototype member of the *Aphthovirus* genus of the *picornaviridae* family. The virus exists in seven different serotypes: O, A, C, Asia 1, and South African Territories 1 (SAT 1), SAT 2, and SAT 3, but a large number of subtypes have evolved within each serotype (Mason *et al*., 2003).

Since 2000, South Korea has experienced eleven FMD epidemics (March 2000, May 2002, January 2010, April 2010, November 2010, July 2014, December 2014, January 2016, February 2017, March 2018, and January 2019). Due to the extensive economic damage (approximately 3 billion dollars) in the 2010-2011 FMD outbreak, the South Korean government implemented a vaccination policy throughout the country for all FMD susceptible livestock.

Disadvantages of the current chemically inactivated FMDV vaccine include the risk of viral release during vaccine production, and problems in serological distinction between infected and vaccinated animals (Doel, 2003). Thus, much effort has been made to develop alternative and safe vaccines utilizing the GH loop of capsid protein VP1 (Bittle *et al*., 1982; Cao *et al.,* 2017; Cubillos *et al*., 2008; Lee *et al*., 2017; Shao *et al*., 2011; Wang *et al.,* 2003). However, the immunogenicity of these recombinant

vaccines was substantially lower than those of the traditional inactivated FMDV vaccines (Rodriguez *et al.,* 2003).

Vesicular stomatitis virus (VSV) glycoprotein is known to strongly elicit neutralization activity similar to those induced by the live virus (Kelley *et al*., 1972). In order to address the low immunogenicity of the peptide vaccine, one research group inserted a B cell target antigenic site into a VSV glycoprotein with the first 193 amino acids (aa) out of 517 aa as a candidate vaccine, resulting in limited efficacy compared to the conventional inactivated FMDV vaccine (Capozzo *et al.,* 2011). Another study showed that recombinant glycoprotein whole cell lysate was a better diagnostic antigen than the glycoprotein soluble fraction when applied in ELISA (Heo *et al.,* 2010).

Therefore, I constructed a recombinant protein vaccine using a whole VSV glycoprotein with 517 aa residues as a carrier to include the FMDV type O VP1 GH loop epitope corresponding to 129-169 aa and examined antigenicity and immunogenicity of this recombinant protein vaccine.

# Materials and Methods

## Cells

*Spodoptera frugiperda* (SF9) cells (Invitrogen, Carlsbad, CA, USA) for the propagation of the recombinant baculovirus were grown at 27°C in Sf-900™ II medium (Invitrogen) with Antibiotic-Antimycotic solution (100X; Invitrogen).

## Cloning recombinant proteins containing the FMDV VP1 GH loop epitope

The FMDV type O VP1 GH loop epitope 129-169 aa was synthesized by replacing the 158th amino acid in proline with cysteine from the nucleotide sequence isolated in Jincheon, South Korea in 2014 (Bioneer, Daejeon, Korea); the sequence is shown in Table 3.1. The FMDV sequence was derived from O/SKR/JC/2014 (GenBank KX162590). The VSV glycoprotein originated from recombinant BacPAK8 (Clontech, Mountain View, CA, USA) containing the VSV New Jersey GP (Heo *et al.,* 2010). The VSV glycoprotein was excised and cloned into pFastBac HT-B vector according to the bac-to-bac system (Invitrogen) using *Bam*HI and *Xho*I. The recombinant VSV glycoprotein with FMDV epitope was constructed by inserting the FMDV VP1 GH loop epitope sequence between codons 160 and 161 of the VSV whole glycoprotein (517 aa). As a control antigen, the FMDV type O VP1 GH loop epitope 129-169 aa was cloned into pGEX 4T-1 (GE Healthcare, Piscataway, NJ, USA) using *Bam*HI and *Xho*I.

**Expression and purification of recombinant proteins**

For recombinant glutathione S-transferase (GST)-VP1, *E. coli* strain BL21 was transformed with pGEX 4T-1 containing the FMDV type O VP1 epitope and grown overnight at 37°C with shaking. Cultures were diluted at a 1:10 ratio with fresh Luria Broth containing ampicillin and incubated for 3 h at 37°C with shaking. Protein expression was induced by the addition of Isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM, and the cultures were incubated for 4 h. Pellets were harvested from the culture by centrifugation at 3000 × *g* for 10min, resuspended in phosphate-buffered saline (PBS; 50 mM, pH 7.4) and disrupted by sonication at a 20% amplitude. The lysate was centrifuged at 3000 × *g* for 10min, and the soluble protein factions were collected and purified by affinity column chromatography using Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. The GST control was also expressed by the above method using only pGEX 4T-1.

For recombinant VSV glycoprotein with FMDV epitope (VSV GP-VP1), $5\times10^5$ Sf9 cells were plated out per well in a 6-well plate. Twelve hours later, the cells were washed twice with serum and antibiotic free Grace media. The recombinant bacmid DNA was transfected using Cellfectin II (Invitrogen) according to the manufacturer's instructions. The primary virus was harvested after 4 days and used for amplification; the virus titer was calculated by plaque assay.

The optimal condition for expression of VSV GP-VP1 was determined by multiplicity of infection (MOI) tests. The Sf9 cells $2\times10^4$/well were cultured in a monolayer 24-well culture dish inoculated with baculovirus at a range of 0.05-10 MOI, culture supernatants were harvested every 24 hours for 6 days, and protein expression level was analyzed by SDS-PAGE and western blotting.

The VSV GP-VP1 fused to a histidine affinity tag at its N-terminus were purified using nickel-nitrilotriacetic acid resin (Life Technologies) according to the manufacturer's instructions. Briefly, Sf9 cells at a density of $1.5\times10^6$/ml in shaker flasks were infected with VSV GP-VP1 at 1.0 MOI and incubated with shaking for 96 h at 27°C. The infected cells were harvested by centrifugation at 500 × *g* for 5 min at 4°C. The pellet was resuspended in lysis buffer (20 mM $Na_2HPO_4$, 500 mM NaCl, pH 7.4, and 1 mM PMSF). After incubation for 15min at 4°C, the samples were sonicated on ice and centrifuged at 10,000 g for 20min. The insoluble fraction was resuspended in 50 ml of binding buffer (20 mM Tris, 100 mM $NaH_2PO_4$, pH 8.0, 8 M urea, and 20 mM imidazole) at 4°C. After centrifugation at 10,000 g for 20min, the supernatants were filtered through a 0.45 μm membrane. The samples were loaded into a nickel-nitrilotriacetic acid column, washed with binding buffer (20 mM Tris, 100 mM $NaH_2PO_4$, pH 8.0, 8 M urea, and 20 mM imidazole). The final eluted samples in elution buffer (20 mM Tris, 100 mM $NaH_2PO_4$, pH 8.0, and 500 mM imidazole) were analyzed by 10% SDS-PAGE. Protein concentrations were determined according to the BCA method (Thermo Fisher Scientific).

**Western blot analysis**

The recombinant proteins were mixed with 4× LDS sample buffer (Invitrogen, Carlsbad, USA). The pretreated samples were heated at 99ºC for 10min. Samples were separated on 12% Bis-Tris gels (Invitrogen, Carlsbad, USA). The resolved samples were electro-transferred to a polyvinylidene difluoride membrane using an iBlot™ gel-transfer device (Invitrogen, Carlsbad, USA). The membranes were blocked with 2% (w/v) skim milk in Tris-buffered saline containing 0.1% of Tween 20 and incubated with GST antibody (GE Healthcare, Piscataway, NJ, USA), monoclonal antibody (76.5E) against FMDV epitope, or antibody (NVSL, Ames, IA, USA) against VSV glycoprotein. This was followed by incubation with a goat anti-mouse horseradish peroxidase conjugated secondary antibody (Invitrogen, Rockford, USA) diluted to 1: 5,000. Proteins were visualized with Pierce ECL Western Blotting Substrate (Invitrogen) using an Azure C600 device (Azure Biosystem, Dublin, USA).

**Antigenicity test of recombinant vaccine candidate**

The antigenicity of the recombinant proteins was measured by ELISA using neutralizing monoclonal antibody against FMDV. Maxisorp ELISA 96-well plates (Nunc, Denmark) were coated with serially two-fold diluted recombinant proteins starting from 2 µg/ml in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST), then

incubated with 100 μl of monoclonal antibody (76.5E) against FMDV VP1 conjugated to horseradish peroxidase (0.5 μg/ml) diluted in diluent (PBST containing 5% skimmed milk) at room temperature for 1 h. After washing the plates, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate was added. The color was developed for 15min at room temperature, and the reaction was stopped by adding 50 μl of 0.5 M sulfuric acid. The optical densities of the samples were measured at 450 nm.

**Vaccination and sample collection**

Pigs raised on a farm in Gyeongbuk province were examined in advance, and those negative by PrioCHECK FMDV Type O ELISA (Prionics, Lelystad, Netherlands) were selected for vaccination experiment. Six pigs were vaccinated with the VSV GP-VP1 and six pigs with GST-VP1. One of the GST-VP1 vaccinated pigs died suddenly during the experiment. The recombinant protein immunogens (100 μg) were mixed with the ISA 206 adjuvant (Seppic, Paris, France) in a 1:1 ratio as recommended by the manufacturer's instructions. A commercial vaccine, an oil-adjuvanted inactivated FMDV vaccine (GCVP FMD Vaccine, South Korea) that had been imported from Merial Animal Health Ltd. and filled in the bottle at Green Cross Ltd. in South Korea was also immunized into four pigs. Pigs were intramuscularly immunized twice with the recombinant proteins, four weeks apart. The blood samples were collected, allowed to clot at room temperature, and centrifuged (2000

× *g*, 10min, 4°C). Sera samples were stored at -20°C until the serological test was performed. Animal experiments in this study were approved (APQA 2016-337) and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Immunogenicity test of vaccine candidates**

PrioCHECK FMDV Type O, a blocking ELISA that detects antibodies against FMDV type O, was employed to detect type O antibodies in the sera samples according to the manufacturer's instructions. Raw data have been expressed as PI values. If the PI value of the sample was more than 50%, the result was considered positive. If the PI was below 50%, the result was considered negative.

**Data analysis**

Pairwise t-tests were conducted with *p*-values below 0.05 (95% confidence interval) being considered statistically significant.

# Results

## Expression of recombinant vaccine candidates carrying the FMDV type O VP1 epitope

SDS-PAGE showed that GST-VP1 was approximately 31 kDa compared to the GST control, which was 27 kDa and they were mainly expressed in the soluble fractions (Fig. 3.1A). In addition, FMDV type O VP1 epitope expression was confirmed by western blot using monoclonal antibody against FMDV type O VP1 neutralizing epitope, and there was no reactivity against the GST control (Fig. 3.1B).

The VSV GP-VP1 was expressed in the insoluble fraction from insect cells with a molecular weight of around 64 kDa, consisting of VSV glycoprotein 517 aa, FMDV type O VP1 41 aa, and His tag 26 aa. Any protein band corresponding to the molecular weight (64 kDa) of VSV GP-VP1 in the soluble fraction was not observed by SDS-PAGE as shown in Fig. 3.1C. Western blot analysis showed that the VSV GP-VP1 reacted with monoclonal antibodies against FMDV capsid and VSV glycoprotein (Fig. 3.1D). The antigenicity of the recombinant proteins was measured by ELISA using neutralizing monoclonal antibody against FMDV. All the recombinant proteins containing the FMDV epitope showed distinct reactivity in comparison to the GST control, which did not react with the antibody (Fig. 3.1E).

**Antibody responses elicited by recombinant vaccine candidate in pigs**

Field pigs were twice immunized, with a one-month interval, by the two chimeric recombinant proteins and commercial vaccine. The sera collected from all the pigs were tested by FMDV type O ELISA for the detection of antibodies against the FMDV type O capsid epitope. Before immunization, all pigs showed a PI level of approximately 20, regardless of experimental group. Out of them, only the VSV GP-VP1 group exhibited significant differences ($p < 0.05$) after immunizations in comparison to the pre-immunization. The GST-VP1 group and the commercial vaccine group did not show a significant difference from the pre-immunization (Fig. 3.2).

# Discussion

In this study, a novel recombinant protein carrying FMDV type O VP1 GH loop epitope linked to vesicular stomatitis virus glycoprotein was constructed and evaluated for its immunogenicity as an innovative vaccine candidate.

Although a previous report introduced VSV glycoprotein as vaccine candidate, it resulted in limited efficacy compared to the conventional inactivated FMDV vaccine (Capozzo *et al.,* 2011). It was postulated that the low effect could be attributed to the fact that the partial outer fraction of the glycoprotein was used as the immunogen carrier instead of the complete VSV glycoprotein, and that the peptide size was not long enough to induce sufficient immunogenicity in cattle. Based on the previous results that extension of the FMDV epitope size from 140-160 aa to 129-169 aa increased the neutralizing index (Wang *et al.,* 2001), and that the whole protein of the VSV glycoprotein was more antigenically effective than the soluble form (Heo *et al*., 2010), I inserted the FMDV type O VP1 129-169 codon into the VSV whole glycoprotein. The FMDV type O VP1 GH loop epitope was incorporated between the amino acids at positions 160 and 161 of the VSV glycoprotein (Capozzo *et al.,* 2011), The GST was employed as a control carrier because GST is the most frequently used fusion partner protein in recombinant protein expression systems. Since the massive FMD outbreaks in December 2010 in South Korea, the nationwide

local veterinary services have carried out a serological test for the detection of the antibody elicited by vaccination using an FMDV type O ELISA kit every year.

Therefore, in this study, the immunogenicity of the candidate vaccine was examined by FMDV type O ELISA to reflect the field situation. In addition, serological surveillance conducted every year in South Korea showed that pigs exhibited less immunogenicity in comparison to cattle with the same commercial FMD vaccine (Park, 2013). Therefore, a successful novel vaccine candidate should provide evidence of potential efficacy in local field pigs.

The VSV GP-VP1 groups showed higher immunogenicity than the GST-VP1 group in field pigs, considering that the FMDV type O VP1 epitope linked to VSV glycoproteins showed a significant increase at a 95% CI level compared to the preimmunization stage. This might reflect the strong immunogenicity of the VSV glycoprotein as previously reported (Kelley *et al.,* 1972).

Theoretically, nationwide immunization of FMD susceptible livestock could result in a large difference in antibody prevalence among pig farms. Also, since this experiment was conducted at only one pig farm, the low antibody prevalence induced by the recombinant protein and the commercial vaccine might be attributed to the characteristics of the experimental farm. In future studies, it will be necessary to experiment with a large number of pigs from a large number of farms.

The relatively low immunogenicity of the recombinant proteins might also be due to the low immunogen concentration. While mouse or specific pathogen-free pigs

were previously immunized with relatively low concentrations (20 or 100 μg) of immunogen (Lee *et al.,* 2017; Wang *et al.,* 2001), local field pigs were immunized with a high concentration (500 μg) of immunogen (Cao *et al.,* 2017). Further study using various immunogen concentrations may be required.

In conclusion, a novel vaccine platform was constructed to carry FMDV type O VP1 GH loop epitope linked to a whole VSV glycoprotein as a novel vaccine candidate. The findings of this study provide groundwork for the development of a novel recombinant protein vaccine that could be easily produced in a general laboratory as an alternative to the current FMD vaccine, which requires a biosafety level 3 containment facility for vaccine production.

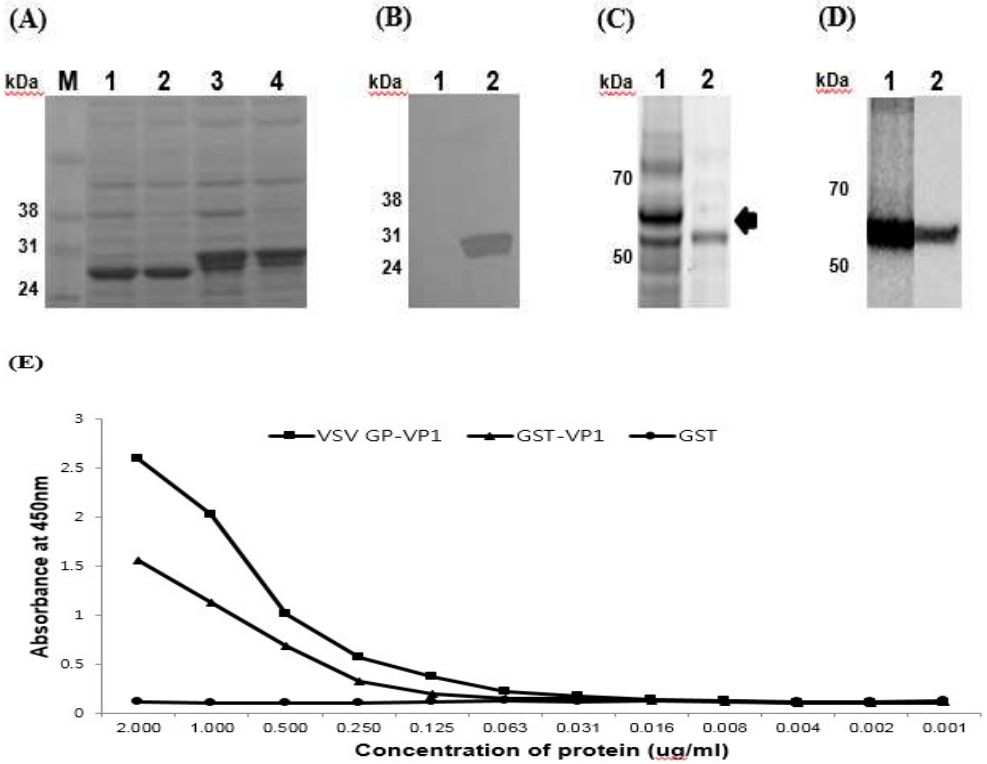


Figure. 3.1. Expression and antigenicity of recombinant vaccine candidates carrying the FMDV type O VP1 GH loop epitope. (A) SDS-PAGE analysis: Lane 1, total lysate of GST; lane 2, soluble fraction of GST; lane 3, total lysate of GST-VP1; lane 4, soluble fraction of GST-VP1. (B) Western blot analysis with monoclonal antibody against type O FMDV: Lane 1, purified GST; lane 2, purified GST-VP1. (C) SDS-PAGE analysis: Lane 1, total lysate of recombinant glycoprotein fused to FMDV epitope; lane 2, soluble fraction of recombinant fused to FMDV epitope. The arrow indicates the band corresponding to the VSV GP-VP1. (D) Western blot analysis: Lane 1, total lysate of the VSV GP-VP1 with monoclonal antibody against type O FMDV; lane 2, total lysate of the VSV GP-VP1 with monoclonal antibody against VSV glycoprotein. (E) Antigenicity of the VSV GP-VP1, GST-VP1, and GST control.

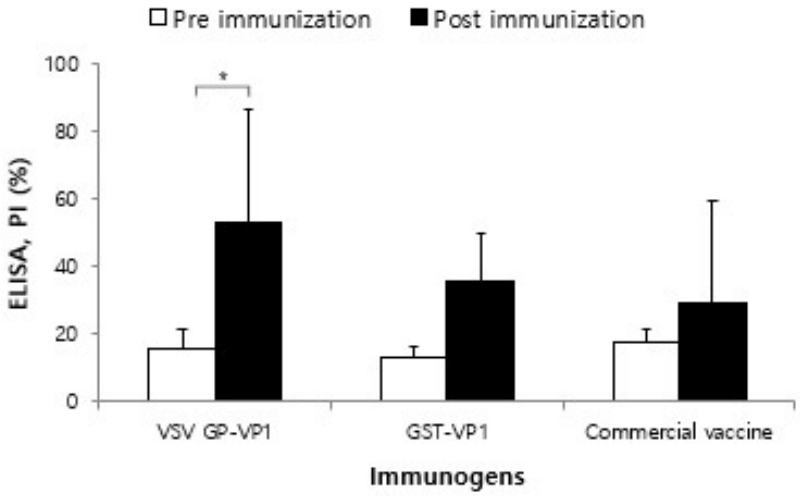


Figure. 3.2. Antibody responses elicited by various immunogens in pigs. Six pigs were vaccinated with the VSV GP-VP1, five pigs with GST-VP1, and four pigs with commercial inactivated FMDV vaccine. Pigs were immunized twice intramuscularly, four weeks apart. The antibody responses were measured by FMDV type O ELISA, and the results were presented as average PI (%). The statistical significance of differences between the booster immunization and pre-immunization stage was determined at a 95% confidence level, and the asterisks represent a statistically significant difference ($p < 0.05$).

Table 3.1. Amino acid sequences of the FMDV type O VP1 GH loop epitope insertion site within the VSV glycoprotein and GST

| Group | Amino acid sequence (N to C) |
|---|---|
| VSV GP-VP1[a] | GP-WIVYNGNCKYTGGSLPNVRGDLQVLAPKAARCLPTSFNYGAIKDP-162 |
| GST-VP1[b] | GST-VYNGNCKYTGGSLPNVRGDLQVLAPKAARCLPTSFNYGAIK |

Underlined sequences indicate VP1 129 to 169 amino acid residues from FMDV (GenBank: KX162590) isolated in 2014, South Korea, with displacement of proline to cysteine at residue 158 to enhance neutralization activity.

[a)] Recombinant VSV glycoprotein fused to FMDV VP1 epitope (129-169)

[b)] Recombinant GST fused to FMDV VP1 epitope (129-169)

# General conclusions

Vesicular stomatitis (VS) is an economically important disease in cattle, horses and swine, characterized by the appearance of vesicles and ulcers in the tongue, oral tissues and hooves. Of the two serotypes of VSV, the New Jersey (NJ) serotype is considered more important, since it is more pathogenic than the Indiana (IN) serotype and so accounts for the majority of clinical cases. Since the clinical signs of VS in cattle and pigs are indistinguishable from those of foot-and-mouth disease (FMD), it is essential that VS be identified by rapid laboratory diagnostic methods.

In this study, an ELISA using glycoprotein (GP) and monoclonal antibody (MAb) was developed for detection of antibodies against vesicular stomatitis virus (VSV) serotype New Jersey (NJ). In first study, the GP was successfully extracted from the partially purified VSV-NJ and a neutralizing MAb specific to VSV-NJ was incorporated to compete with antibodies in a blocking ELISA. The GP ELISA was found to be specific to VSV-NJ and did not react with sera that were strongly positive for FMD or SVD. However, use of the GP extracted from VSV-NJ requires handling the live virus, thus posing a risk of virus release into the livestock field. Therefore, the GP gene from VSV-NJ was expressed in insect cells and investigated as a diagnostic antigen. The ELISA using a recombinant GP worked as well as the native GP ELISA for VSV-NJ positive sera (n=19) with high virus neutralization titres and panel sera (n=20) with low virus neutralization titres for VSV-NJ and VSV-IN.

These methods differentiated VSV-NJ from other vesicular disease such as FMD and SVD in a preliminary test, indicating that this diagnosis may be useful in south America where FMD and VS outbreaks occurs. The GP of VSV is known to strongly elicit neutralization activity similar to those induced by the live virus.

Disadvantages of the current chemically inactivated FMDV vaccine include the risk of viral release during vaccine production. Thus, much effort has been made to develop alternative and safe vaccines utilizing the GH loop of capsid protein. Therefore, a recombinant protein carrying FMDV type O VP1 epitope linked to vesicular stomatitis virus GP was constructed and evaluated for its immunogenicity as an innovative vaccine candidate. The VSV GP-VP1 groups showed higher immunogenicity than the GST-VP1 group and commercial vaccine group in field pigs.

To my knowledge, this is the first report that the recombinant protein vaccine was superior to the current vaccine. In conclusion, a novel vaccine platform was constructed to carry FMDV type O VP1 GH loop epitope linked to a whole VSV GP.

Taken together, this GP has a potential to be used as a diagnostic antigen for the detection of antibodies to VSV-NJ in cattle and horses and useful tool for the development of a novel recombinant protein vaccine that could be easily produced in a general laboratory as an alternative to the current FMD vaccine.

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# 국문초록

# 수포성구내염 뉴저지형 당단백질을 이용한 진단법 개발 및 구제역백신 후보주에 관한 연구

**이 향 심**

**(지도교수: 윤 희 정, D.V.M., Ph.D.)**

**서울대학교 대학원**
**수의학과 수의병인생물학 및 예방수의학 전공**

수포성구내염은 소, 말 및 돼지에서 발생하는 수포성질병으로 원인체는 *Rhabdoviridae* 과의 수포성구내염바이러스이다. 수포성구내염바이러스는 11kb (-) 극성의 단일가닥 RNA 를 함유하고 5 종의 단백질 (당단백질, 뉴클레오캡시드, 인단백질, 매트릭스, 유전자중합효소)로 구성되어 있다. 수포성구내염은 인디아나형과 뉴저지형의 두가지 혈청형이 있으며, 입과 제관부, 유두에 수포 등이 형성되면 구제역과 임상적으로 구분이 불가능하기 때문에 감별진단이 필요하다.

수포성구내염 혈청검사를 위한 표준검사법은 중화시험법이나 생바이러스를 취급하고 차폐실험실을 사용해야하며 2-3 일의 반응시간이 소요되는 단점이 있어서 대량의 혈청을 검사하기에는 부적합하다. 이를 대체하기 위하여

재조합뉴클에오캡시드를 진단항원으로 사용한 진단법 (NC ELISA)이 OIE 매뉴얼에 권장진단법으로 사용되어져 왔으나 중화시험과의 상관성이 낮아서 최근 개정된 OIE 매뉴얼에는 중화항체가를 결정하는 당단백질을 진단항원으로 사용할것을 권고하고 있다. 따라서 본 연구에서는 뉴클에오캡시드를 진단항원으로 사용한 기존 수포성구내염 뉴저지형의 진단법 (NC ELISA)의 민감도와 특이도를 개선하기 위하여 당단백질을 진단항원으로 사용하여 항체진단법을 개발하였다.

첫 번째로 수포성구내염 뉴저지형에서 당단백질을 추출하고 중화단클론항체 1G11 을 이용하여 경쟁방식의 ELISA 를 구축하였고, 검출한계를 측정해보니 반응저해도 40% 기준으로 설정하였을때 중화시험법 판정기준인 32 배에 해당하였다.

수포성구내염 표준연구실인 미국 농무부산하 NVSL 에서 구입한 표준혈청을 적용해 본 결과에서도 중화시험 판정기준인 32 배는 양성으로, 16 배는 음성으로 판정하였기 때문에 대량의 혈청검사시에 표준진단법인 중화시험법을 대체할 수 있는 신규진단법으로서의 유효성을 확인하였다. 또한, 국내에서 사육하는 소, 돼지, 말 혈청을 검사하였을 때 축종별로 차이없이 99.6% 특이도를 나타내어 모든 축종에 대해서도 검사가 가능할 것으로 판단되었다.

NC ELISA 에서 중화시험법 양성혈청 19 점중 5 점을 음성으로 판정한 반면에 GP ELISA 는 19 점 모두 양성으로 정확하게 판정하였으며, 구제역 및 돼지수포병

양성혈청과 VSV 인디아나 양성혈청에서 모두 음성으로 판정하여 뉴저지형 특이 진단법으로 사용할 수 있음을 보여주었다.

두 번째 연구에서는, 수포성구내염 바이러스를 취급시 발생할 수 있는 위험요인을 제거하기 위하여 유전자재조합 방법으로 진단항원을 제작하여 진단법 (R-GP ELISA)을 확립하였으며, 수포성구내염 표준연구실인 미국 농무부산하 NVSL 에서 구입한 양성혈청으로 유효성을 평가한 결과, 첫번째 연구의 GP ELISA 결과와 동일하게 모두 양성판정하였다. 또한, 정확한 민감도 평가를 위해서 수포성구내염 중화시험법 proficiency 혈청패널을 적용한 결과, GP ELISA 에서 음성이었던 12 번 혈청도 양성판정하였으며 중화역가 16 배인 6 번 혈청도 양성으로 판정하였기 때문에 중화시험법보다 민감도가 우수하여 중화시험법을 대체하여 대량의 혈청검사를 위한 신규진단법으로서의 활용가능성을 확인하였다. 국내에서 사육하는 소와 말 혈청에 대해서 특이도 99.1%를 나타내었으며 구제역 및 돼지수포병 양성혈청과 VSV 인디아나 양성혈청에서 모두 음성으로 판정하였다.

마지막 연구에서는, 수포성구내염바이러스 당단백질에 구제역바이러스 VP1 유전자 부위를 연결하여 재조합 항원단백질을 제작하고 오일 보조제와 혼합하여 돼지에 접종하여 면역원성을 확인하였다. 한국에서 구제역은 2000 년 이후 11 번 발생하였으며 2010-2011 년 발생으로 약 3 조원의 경제적 손실을 입은 후 구제역 감수성이 있는 동물에 백신접종을 실시하였다. 현재 사용되는 구제역 불활화

백신을 생산하기 위해서는 Biosafety level 3 시설을 사용해야 하며, 백신생산중 구제역바이러스의 외부유출 등의 위험성 및 백신접종축과 감염축을 감별해야하는 단점이 있다. 두번째 연구에서 유전자재조합 당단백질이 중화항체를 대량유도한다는 사실을 발견하여 백신으로서의 개발가능성을 확인하였기 때문에, 수포성구내염 바이러스의 당단백질에 구제역바이러스의 VP1 유전자 부위를 결합하여 재조합 항원 단백질을 제작하여 오일보조제와 혼합하여 돼지에 접종하여, 병원성이 나타나지 않으면서 상업적으로 판매중인 구제역 불활화 백신보다 우수한 면역원성을 확인하였다 ($P<0.05$).

이러한 결과를 통해 수포성구내염바이러스 당단백질이 뉴저지형의 특이 진단항원으로서 유용하고 또한 구제역바이러스 면역원성 부위를 수포성구내염 바이러스의 당단백질에 결합하여 제작한 재조합 단백질이 현재 사용중인 불활화백신보다 유의적인 차이를 나타내어, 재조합 발현백터로서 당단백질의 활용가능성을 확인하였다.

**핵심어:** 수포성구내염 바이러스, 구제역, 구제역바이러스, 백신, 당단백질, 재조합 당단백질, 효소결합면역흡착측정법

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