Diagnostic approaches to transboundary and zoonotic diseases

by

Dashzeveg Bold

D.V.M., Mongolian University of Life Sciences, 2003
M.S., Mongolian University of Life Sciences, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2025

Abstract

Transboundary animal diseases (TADs), including those caused by African swine fever virus (ASFV), Classical swine fever virus (CSFV), and Foot-and-mouth disease virus (FMDV), as well as highly contagious zoonotic agents such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), pose significant threats to agriculture and public health globally. Early detection and follow-up measures are critical for the management of these infectious diseases. In the laboratory, polymerase chain reaction (PCR) is the most commonly used method for diagnosing these pathogens; however, in order to rapidly identify and prevent the spread of these highly infectious viruses, point-of-care (POC) diagnostic assays for the detection of the pathogen's genetic materials, antigens (Ag), and antibodies (Ab) in the field are necessary.

In this dissertation, lateral flow assays (LFAs) for ASFV Ag and Ab detection, multi-antigen print immunoassays (MAPIAs) for ASFV and FMDV Ab detection, and indirect ELISAs for SARS-CoV-2 Ab detection were established. It was also demonstrated that POC approaches used in a low-income country were able to identify ASFV, CSFV, and FMDV genetic sequences in suspect materials, proving the feasibility of POC diagnostics for these pathogens in low resource environments.

As a result of this thesis, valuable tools were generated for improving the detection and diagnosis of ASFV, CSFV, FMDV, and SARS-CoV-2.

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Approved by:

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Dr. Jürgen A. Richt

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Abstract

Transboundary animal diseases (TADs), including those caused by African swine fever virus (ASFV), Classical swine fever virus (CSFV), and Foot-and-mouth disease virus (FMDV), as well as highly contagious zoonotic agents such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), pose significant threats to agriculture and public health globally. Early detection and follow-up measures are critical for the management of these infectious diseases. In the laboratory, polymerase chain reaction (PCR) is the most commonly used method for diagnosing these pathogens; however, in order to rapidly identify and prevent the spread of these highly infectious viruses, point-of-care (POC) diagnostic assays for the detection of the pathogen's genetic materials, antigens (Ag), and antibodies (Ab) in the field are necessary.

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As a result of this thesis, valuable tools were generated for improving the detection and diagnosis of ASFV, CSFV, FMDV, and SARS-CoV-2.

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Acknowledgements

Dr. Jürgen Richt's mentoring of me during my PhD program has been greatly appreciated, and I would like to express my sincere gratitude to him. It is my pleasure to thank the members of my committee, Dr. Alfonso Clavijo, Dr. Natasha Gaudreault, and Dr. Roman Pogranichniy, for their valuable advice, wisdom and for reviewing my work.

Undrakhbayar Tserendorj, my beloved wife, whose love, support, and patience have guided me through every step of this journey, deserves my deepest gratitude. My greatest source of motivation and strength has been her belief in me, even during challenging times.

Furthermore, I would like to acknowledge the supportive team members, Drs. Jessie Trujillo, Gleyder Roman-Sosa, Velmurugan Balaraman, David Meekins, Igor Morozov, William Wilson, Yonghai Li, Konner Cool, Daniel Madden, Chester McDowell, Taeyong Kwon, Jayme Souza-Neto, Bianca Artiaga, and Tracy Miesner.

Thank you to all my friends who have been so supportive throughout this journey: Mrs. Joyce Nelson, Mr. Dick Nelson, Mr. Farrukh Raza, Mrs. Ghashia Kiyani, Mrs. Sharon Gardener, Mr. George Gardener, and Mr. Beni Wilson. Additionally, I would like to acknowledge the support I received from my Mongolian friends, especially Dr. Batsukh Zayat, who helped me start on the path to success.

Dedication

This work is dedicated to my parents, my dad Bold and my mom Tsetsegmaa, who have supported me unconditionally throughout my entire life, including this PhD journey. Words cannot fully express my gratitude to you.

Chapter 1 - Literature Review

History and Epidemiology

African swine fever (ASF) was first described in the early 1900s as a disease affecting domestic pigs (*Sus scrofa domesticus*). According to early observations, warthogs (*Phacochoerus africanus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hyclochoreus meinerizahageni*) are resistant to the disease; however, they are thought to play a role in the transmission of the disease to domestic pigs (Eustace Montgomery 1921). There are three distinct epidemiologic cycles: the sylvatic cycle (wild pigs-soft tick cycle), the tick-pig cycle (domestic pig-soft tick cycle), and the domestic cycle (pig-pig cycle). The sylvatic cycle is when the virus circulates between natural reservoirs, such as warthogs and soft ticks, without causing disease in warthogs. This cycle has been primarily observed in countries in southern and eastern Africa, but was also observed during the epizootic (1957-1995) on the Iberian Peninsula (Chenais, Stahl et al. 2018, Gaudreault, Madden et al. 2020). The sylvatic cycle has contributed to domestic pig populations being at risk in most sub-Saharan African countries. The tick-pig cycle, first described in East and Southern Africa in the early 20th century, the virus is maintained between soft ticks of the *Ornithodoros* species and domestic pigs. Ticks obtain the virus by feeding on infected pigs and can retain and transmit it throughout their lifespan, even passing it vertically and horizontally to their progeny and new hosts, thus acting as long-term reservoirs. This cycle historically played a central role in endemic ASF in Africa, especially in rural pig production systems where ticks lived in pig shelters and pigsties. While the domestic pig cycle, which became increasingly important after the mid-20th century when ASF spread outside Africa, involves direct pig-to-pig transmission or indirect spread through contaminated pork products, fomites, and carcasses, without the involvement of ticks. In Europe, Asia, and the

Caribbean, and more recently in Eurasia, where the absence of *Ornithodoros* ticks means the virus persists through domestic and wild boar populations (Chenais, Stahl et al. 2018, Lv, Xie et al. 2022, Jori, Bastos et al. 2023).

A genotype I ASF virus (ASFV) first spread beyond Africa in 1957, when ASFV-contaminated pork products transported by air travel led to infections in pigs in Portugal. The outbreak of ASFV led to the further spread to Spain and other countries in Western Europe, to Russia, as well as the Caribbean and Brazil; it was finally eradicated from Europe in the mid-1990s, after more than three decades (Brown, Penrith et al. 2018), except for Sardinia, Italy (Sanchez-Cordon, Montoya et al. 2018). In 2007, Georgia experienced the transcontinental spread of a genotype II ASFV through feeding of pigs with ASFV-contaminated waste originating from Africa, possibly brought in by ship to the Port of Poti in Georgia; the virus subsequently spread into neighboring countries and Eastern Europe (Vepkhvadze, Menteshashvili et al. 2017). ASFV is known to survive for extended periods in frozen or processed pork, making swill a recognized high-risk vector for long-distance transmission (Frant, Wozniakowski et al. 2017, Mazur-Panasiuk, Zmudzki et al. 2019). The spread of ASFV in Europe has led to the increased recognition of wild boar (*Sus scrofa*) as a critical virus reservoir. The disease was reported for the first time in 2018 in China and spread into neighboring countries such as Mongolia, Vietnam, Cambodia, South Korea and Indonesia as well as into Western Europe (Gaudreault, Madden et al. 2020). ASFV is currently found in Croatia, Bosnia and Herzegovina, Germany, Hungary, Italy, Poland, Romania, Moldavia, Latvia, North Macedonia, Russia, as well as in the Philippines, Malaysia, Singapore, Indonesia, Timor-Leste, Papua New Guinea, Laos, Bangladesh, Bhutan, Myanmar, Nepal, India, Thailand, Sri Lanka, and Taiwan (Figure 1.1) (FAO 2025).

It is well known that ASF poses a significant threat to the pig industry worldwide, as pig mortality rates for infected animals can reach 100%, and the costs of disease control and eradication for ASF can be extremely high. ASFV control has historically relied on mass culling of infected and exposed pigs due to the absence of effective vaccines. Vietnam began to alter this paradigm in 2022–2023 with the licensing and deployment of two live attenuated vaccines, NAVET-ASFVAC and AVAC ASF LIVE. These vaccines provide 80-95% protection for several months and are now being exported to neighboring countries (Pork 2023, Linh 2025, Times 2025). The ASFV vaccines, however, face several challenges, including doubts about long-term safety, adverse reactions, variable uptake, concerns regarding protection against diverse genotypes and emerging variants (Efeedlink 2022). Therefore, culling is the only option at this time, and implementing effective ASF management still requires an integrated approach with strict biosecurity, surveillance, and outbreak response measures, reflecting both the continuing complexity of ASFV control worldwide and its progress until an effective ASF vaccine is introduced (AVMA 2024). ASF outbreak can cause significant economic loss to pig industry. It is mainly due to mass culling and trade disruptions. These trade bans intensify the losses, greatly impacting pig-producing nations. As a result, pork prices increase, creating shortages in affected regions. Farmers also incur high costs for biosecurity measures to prevent future outbreaks. According to the financial assessment, a single outbreak of ASF in the US could cause a loss around $20 billion USD at least (Calisher 2021). In addition, farmers may face significant requirements to implement better biosecurity measures to prevent future outbreaks. (Calisher 2021).

The disease known as Hog Cholera or Classical Swine Fever (CSF) is a highly contagious hemorrhagic fever disease that affects both domestic and wild pigs. It is one of the oldest recognized viral diseases in pigs. The earliest reports of CSF date back to Tennessee, United States (US), in 1810 (Ganges, Crooke et al. 2020). Later, CSF was first reported in France in 1822 and subsequently spread to other European countries, to England, Sweden, and Denmark (Edwards, Fukusho et al. 2000). In the US, the disease was officially recognized in the Ohio Valley in 1833. In later years, outbreaks were reported in other states across the Midwest and the South. The disease has spread worldwide by the 1960s due to increased international trade in live pigs and pork products, and movement of humans and contaminated materials (Cole, Henley et al. 1960). At the end of the 20th century, CSF was present in over 60 countries, though eradicated in many high-income countries. The US achieved free status in 1978, and Canada in 1963 (USDA 2013). However, despite these successes, the disease has persisted in, or re-emerged in multiple countries. For instance, CSF reemerged in South Africa after an absence of 87 years in 2005, demonstrating the risk of reintroduction even in countries with prior control (Penrith, Vosloo et al. 2011).

CSF spread has been reduced globally due to extensive vaccination campaigns and massive surveillance efforts. Therefore, WOAH recognizes Oceania, North America, Costa Rica, Malta, Argentina, Chile, Paraguay, Uruguay, and most countries in Europe, as well as some areas of Brazil and Kazakhstan, as CSF-free. However, CSF persists in some parts of Central and South America, Europe (mainly in wild pigs), Asia (various countries), and parts of Africa. Since 2020, CSF outbreaks were reported in Japan, Indonesia, Russia, and Brazil, but the outbreaks are in resolved status (Figure 1.2).

During the early 20th century, CSF caused enormous economic losses for the United States, accounting for 90% of all swine deaths (Birch 1922). More than 12 million pigs were culled in the Netherlands from 1997 to 1998 as a result of an epidemic that affected over 400 herds (Stegeman, Elbers et al. 2000). In Colombia, between 2013 and 2018, 134 CSF outbreaks were reported; 95% of these occurred in backyard piggeries, with main transmission routes being introduction of infected pigs (38%) and human movement (37%) (Pineda, Deluque et al. 2020).

Foot-and-mouth disease (FMD) is a highly contagious animal disease that has been a major threat to the livestock industry since the 16th century. The first probable description of FMD was made in 1514 by the Italian monk Hieronymus Fracastorius, who described a disease in cattle characterized by vesicular lesions in the mouth and feet. In 1898, Loeffler and Frosch proved that FMD was caused by the FMD virus (FMDV). It was marking a pivotal moment in virology as it was the first virus ever discovered that affects animals (Brown 2003, Mahy 2005). By then, FMD had spread to almost every region of the world, except for New Zealand, Greenland, and Iceland, and some smaller islands in the Oceania region. It is profoundly influencing animal trade and agricultural policies worldwide. Early control measures heavily rely on animal movement restrictions and stamping out policies, but the introduction of inactivated vaccines in the mid 20th century revolutionized prevention from FMDV infection in animals (Paton, Sumption et al. 2009).

By the late 20th century, successful eradication programs led to the elimination of FMD from North America and Australia. FMD was last reported in the USA, Canada, Mexico, and Australia in 1929, 1952, 1954, and 1970, respectively (Woldemariyam, Kariuki et al. 2023). The European continent is free of FMD since the last major outbreak in Bulgaria in 2011 (Marschik, Kopacka et al. 2020); however, a new case of FMD was reported on January 10, 2025, in water buffaloes near Berlin, Germany (Friedrich-Loeffler-Institut 2025). Shortly thereafter, a separate incursion was confirmed in north-western Hungary in early March 2025 (Győr-Moson-Sopron County), where large dairy herds were affected and the virus was genotyped as an O/ME-SA lineage closely related to viruses previously circulating in Asia and the Middle East (WRLFMD 2025). Within weeks, Slovakia reported multiple linked outbreaks in border regions (late March–April 2025), establishment of protection and surveillance zones, depopulation of positive herds, and deployment of EU veterinary support (EC-Europa 2025). Many sub-Saharan African and Asian countries are still endemic to FMD (Figure 1.3) (Woldemariyam, Kariuki et al. 2023).

A wide range of FMD epidemiological patterns exist across the globe, with regions classified as endemic, sporadic, or FMD-free. The WOAH maintains official lists of countries and zones free of FMD. FMD is endemic in regions that include large parts of Africa, the Middle East, South Asia, and Southeast Asia. FMDV serotypes O and A have been persistently circulating in East and Southeast Asia since 2010. The virus currently circulating in serotypes SAT-1-3, O, and A is found in Africa and the Middle East (WOAH 2025). At the same time, South America has made significant progress, with most countries now FMD-free with or without vaccination. FMDV serotype Asia-1 periodically circulated in 2005-2006 and 2017-2020, since no case has been reported with the serotype (Wada, Han et al. 2025). FMDV serotype C was one of the original serotypes described early in the 20th century, but later it became less frequently involved in major outbreaks compared to serotypes O and A. The last known C serotype outbreaks were reported in Brazil and Kenya in 2004. Since then, no confirmed cases of this serotype have been reported. Hence, serotype C is widely considered to be globally extinct or effectively absent in terms of field circulation (Paton, Di Nardo et al. 2021).

FMD outbreaks result in significant economic losses due to production loss, mass culling, biosecurity concerns, and global trade restrictions. For example, in endemic regions, annual losses from visible production losses plus vaccination are estimated between ~US$ 6.5 billion and US$ 21 billion (Knight-Jones and Rushton 2013). In countries that trade livestock and livestock products, an outbreak can trigger major export bans and severe losses. For example, when a country loses its “FMD-free” status, trade losses can significantly impact the cost structure (Knight-Jones and Rushton 2013). The cost distribution is uneven: while adult animal mortality might be low, external factors such as spread, trade bans, and market closures make FMD costly beyond just the infected herd itself (Knight-Jones 2013). During severe FMD outbreaks, millions of animals were culled, incurring huge costs. For example, in a major outbreak in the UK in 2001, over 6 million animals were culled. The direct costs to the public sector and private sector, and agriculture (food chain), were estimated at £21 billion. Additionally, indirect costs from export bans and biosecurity concerns can be even higher than the direct costs (Thompson, Muriel et al. 2002).

Coronavirus disease 2019 (COVID-19) is a highly contagious viral respiratory zoonotic disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). As a result, it rapidly became the most severe global public health threat since the 1918 influenza pandemic, affecting virtually every country and disrupting societies, health systems, and economies (Lin, Lin et al. 2020). As a result, the COVID-19 led to unprecedented levels of morbidity, mortality, and socioeconomic disruptions. A global spread and impact of COVID-19 highlight the strengths and vulnerabilities of public health systems, as well as how highly transmissible infectious agents can overwhelm even the most advanced healthcare infrastructure. At the same time, the emergence of COVID-19 demonstrated the strength of animal health preparedness for diagnostics, surveillance, and research for animals, as this is a zoonotic disease globally.

The earliest known cases of COVID-19 occurred in Wuhan, Hubei Province, China in December 2019, where clusters of patients had pneumonia of unknown cause. After local health authorities noticed symptoms such as fever, cough, and dyspnea among the patients, they initiated investigations (Yang, Chen et al. 2020). Chinese scientists isolated the unknown coronavirus and sequenced its genome in early January 2020. According to genetic comparisons with SARS-CoV, the agent responsible for the 2003 SARS outbreak, SARS-CoV-2 was proven to be a novel Coronavirus (Chan, Yuan et al. 2020). A number of early cases appeared to be linked to the Huanan Seafood Wholesale Market at first, but subsequent analyses revealed no direct link (Zhu, Zhang et al. 2020). Earlier transmission of SARS-CoV-2 may have occurred among community members or from an unknown source. However, the exact zoonotic origin of the virus remains unclear (Li, Lai et al. 2021). However, the discovery of the virus sparked a global health crisis.

The virus transmitted from person to person and spread rapidly due to active travel season, long incubation periods, and the ability of infected individuals to shed infectious virus before showing clinical signs (Chan, Yuan et al. 2020). As a result of its potential global spread, the World Health Organization (WHO) declared COVID-19 as a Public Health Emergency of International Concern (PHEIC) on January 30, 2020 (WHO 2020). In the following weeks, Asia, Europe, and North America reported increasing numbers of cases. The WHO declared COVID-19 a global pandemic on March 11, 2020. This was the first time a Coronavirus has been declared a pandemic, emphasizing how serious the situation is. Following the global response came lockdowns, travel restrictions, and emergency health measures. (Lamers and Haagmans 2022). The WHO investigated the COVID-19 outbreak and declared it a pandemic in early 2020.

Over seven million human deaths were reported due to virus infections worldwide as of September 2025 (Figure 1.4) (KFF 2025).

SARS-CoV-2, though primarily a human pathogen, has infected a wide range of animal species, illustrating important One Health implications. Evidence suggests a likely bat origin with a still-unknown intermediate host, and soon after the human outbreak began, natural infections were detected in domestic animals such as cats and dogs, as well as in captive wildlife like tigers, lions, gorillas, leopards, puma, mink, binturong, coatis, fishing cats, hippopotamuses, hyenas, lynxes, otters, deer, hamsters, ferrets, and gorillas at zoos (Figure 1.5) (Gaudreault, Trujillo et al. 2020, Cool, Gaudreault et al. 2021, Cui, Liu et al. 2022, Vandegrift, Yon et al. 2022, Sparrer, Hodges et al. 2023). The virus affecting animals raised concerns for public health and animals due to its implications for viral evolution, wildlife reservoirs, and potential reverse zoonosis. It was found that cats, ferrets, and mink had a high susceptibility, while dogs exhibited less susceptibility and typically showed mild disease. It has been confirmed that large-scale outbreaks of disease in farmed mink in Europe and North America were transmitted rapidly from animal to animal and even spilled over to humans, making mink the first non-human reservoir that can influence human health (Devaux, Pinault et al. 2021). White-tailed deer developed widespread natural infections, allowing the virus to circulate independently of humans and evolve. The spread of reverse zoonotic disease was also observed in zoo animals such as cats, gorillas, and hippos following their exposure to ill caretakers. However, most infected animals show mild clinical signs; particular species, especially mink and some zoo felids, experienced severe illness and mortality. As a result of these events, we can understand the complexity of SARS-CoV-2's ecology across species and the importance of integrating surveillance, biosecurity, and One Health approaches to monitor potential animal reservoirs and mitigate future zoonotic or spillback risks.

Pathogens

African swine fever virus (ASFV) is the causative agent of ASF. ASFV is the only member of the *Asfarviridae* family, and it is one of the most complex and unique DNA viruses known to date. It is a large, enveloped, double-stranded DNA virus that contains a genome of approximately 170-190 kilobase pairs (kbp) in length, encodes more than 150 open reading frames (ORFs), and many of the structural and non-structural viral proteins have unknown functions. Multiple layers surround the genome, including an outer envelope derived from the host, a nucleoid, an inner envelope, a core shell, and a capsid (Figure 1.6), and the virion diameter is ~200 nm (Alejo, Matamoros et al. 2018).

ASFV can be classified into 24 genotypes based on the B646L (p72) gene sequence, and eight serogroups based on the antibody-mediated hemadsorption inhibition assay and phylogenetic analysis of the CD2v and C-type lectin proteins encoded by the EP402R and EP153R genes (Malogolovkin, 2015). All 24 genotypes were identified in Africa, primarily in Eastern and Southern Africa. The two genotypes discovered outside Africa, genotypes I and II, were originally found in Central Africa (genotype II) and West African countries (genotype I) (Mulumba-Mfumu, Saegerman et al. 2019). As of January 2025, no ASFV has been reported in North Africa (FAO 2025). As mentioned above, the first cross-continental transmission of the virus was caused by genotype I into Europe during the mid-20th century, and the second cross-continental transmission occurred in 2007 in the Transcaucasia region with genotype II. The genotype II ASFV then spread to Russia, Europe, and in 2018 and beyond to China, Mongolia, Sri Lanka, India, Nepal, Bhutan, Bangladesh, Myanmar, Thailand, Laos, Vietnam, Papua New Guinea, Timor-Leste, Indonesia, Singapore, Malaysia, Philippines, Republic of Korea, Dominican Republic and Haiti (Wang, Sun et al. 2018, Ankhanbaatar, Sainnokhoi et al. 2021, Gonzales, Moreno et al. 2021, Stepien 2021, Ito, Kawaguchi et al. 2023, FAO 2025). Recent evidence confirms that recombinant genotype I/II ASFV is now circulating in parts of Asia, particularly in Vietnam, China, and Russia. These recombinant strains have been associated with acute, subacute, and chronic ASF, making field detection more difficult and increasing the risk of silent spread. Reports from Vietnam and neighboring countries also suggest the presence of genotype-mixed or recombinant viruses, likely linked to the movement of pigs, pork products, and unapproved live-attenuated vaccines. Overall, the emergence and spread of genotype I/II recombinant ASFV in Asia represents a significant epidemiological challenge because these strains can hinder control and eradication efforts (Zhao, Sun et al. 2023, Igolkin, Mazloum et al. 2024, Lee, Vu et al. 2024, SHIC 2024, Nguyen, Bui et al. 2025).

The ASFV is highly stable in various matrices, including feed, carcasses, water, and soil. It can survive in pH levels of 4-10 and wide ranges of temperatures (Prodelalova, Kavanova et al. 2022, Zheng, Xi et al. 2023). For instance, ASFV can remain infectious for at least 30 days in feed, water, and pork products at 4°C. However, the virus stability is significantly shortened at higher temperatures (Davies, Goatley et al. 2017, Mazur-Panasiuk, Zmudzki et al. 2019).

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF) in domestic and wild pigs (Depner, Muller et al. 1995, Blacksell, Khounsy et al. 2006, Fukai, Nishi et al. 2020). CSFV is a positive-sense, single-stranded RNA virus in the *Flaviviridae* family, genus *Pestivirus*. Three other members of the *Pestivirus* genus cause important animal diseases: bovine viral diarrhea virus-1 (BVDV-1), bovine viral diarrhea virus-2 (BVDV-2), and border disease virus (BDV). CSFV is an enveloped virus with an icosahedral symmetry, measuring 40-60 nm in diameter (Figure 1.7). The viral genome is approximately 12.3 kb in length that includes a single large open reading frame (ORF, ~3,898 amino acids) that is flanked by 5’ and 3’ untranslated regions (UTRs). The 5′ UTR folds into an internal ribosomal entry site (IRES) structure, which facilitates the translation of a single ORF into a large polyprotein consisting of 3898 amino acids. This polyprotein is then processed both co-translationally and post-translationally by the viral proteases Npro, NS2, and NS3, as well as by cellular signal peptidases, resulting in the formation of twelve viral proteins: four structural (envelope glycoproteins Erns, E1 and E2, and core protein C) and eight nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B, Npro and p7) (Meyers, Rumenapf et al. 1989, Rumenapf, Unger et al. 1993, Rijnbrand, van der Straaten et al. 1997, Zhao, Chen et al. 2024).

CSFV is a genetically diverse virus with three main genotypes and several subgenotypes. Genotype 1 has seven subgenotypes (1.1 to 1.7) and is endemic in America and Asia. Genotype 2 is the most prevalent one globally. It has three subgenotypes (2.1, 2.2, and 2.3). It has become dominant in Europe, parts of Asia, and South America since the 1990s. Genotype 3 is reported in some Asian countries (Korea, Thailand, Japan, and Taiwan) (Blome, Staubach et al. 2017, Blome, Franzke et al. 2020, Liu, Bahoussi et al. 2022, Chen, Liu et al. 2023). CSFV is stable under various conditions, depending on the environment and temperature. It can survive for days to weeks in urine, feces, and on fomites at room temperature. CSFV can persist for months in cold environments, particularly in protein-rich environments such as meat and slurry. Freezing can preserve the virus for years. CSFV can survive for days at room temperature and weeks at 4 °C, but its survival rate drops dramatically when the temperature reaches 35 °C. The virus will be inactivated within an hour at 50 °C or above (Haas, Ahl et al. 1995, Weesendorp, Stegeman et al. 2008, Botner and Belsham 2012). CSFV remains infectious between pH 5 and 10. While smoking and curing alone have little effect on virus viability, exposure to temperatures above 60 °C inactivates the virus within 5 minutes. The virus can survive for over 75 days in salami and more than 120 days in Iberian ham (Panina, Civardi et al. 1992, Mebus, House et al. 1993, Edwards 2000).

FMDV is a member of the *Aphthovirus* genus, the *Picornaviridae* family and is the causative agent of foot and mouth disease (FMD). The FMDV genome is approximately 8.5 kilobases of single-stranded, plus-sense RNA. It is surrounded by an icosahedral capsid measuring 25-30 nm in diameter that is composed of 60 protomers and four structural proteins (Figure 1.8). Each of the protomers is assembled by the structural proteins VP1, VP2, and VP3, while VP4 is an internal protein linked to the RNA genome. FMDV protein VP1 binds to integrin receptors on host epithelial cells, initiating virus entry via endocytosis. While VP2, VP3, and VP4 support viral structure and are target for the host immune response. The non-structural proteins (NSPs) of FMDV, including Lpro, 2A, 3C, 2B, 2C, 3A, 3B, and 3D, play a crucial role in viral replication, transcription, and immune evasion. As NSPs are expressed during natural infection, but not in inactivated vaccines, they are often targeted for diagnostic assays that differentiate between infected and vaccinated animals (DIVA). Protective immunity is mainly induced by FMDV structural proteins (SPs). The VP1 protein is essential in identifying FMDV and serotyping the virus. Additionally, it is involved in receptor binding and has the main antigenic sites that stimulate neutralizing antibodies (Grubman and Baxt 2004, Gao, Sun et al. 2016, Peng, Yi et al. 2020). FMDV has seven distinct serotypes, including A, Asia 1, C, O, SAT 1, SAT 2, and SAT 3, each with multiple topotypes. The most common serotype is serotype O, while serotype C has not been reported in recent years. SAT (Southern African Territories) serotypes have been mainly identified in sub-Saharan Africa, but SAT 2 has lately been found in Western Asia and the Middle East. Asia 1 was primarily found in Asia but later also reported in the Middle East and Europe (Jamal and Belsham 2013). FMDV is relatively stable across various environments, particularly in cool, moist conditions. The virus can survive for months at 4°C and even longer when frozen in tissues, such as bone marrow and lymph nodes. FMDV can survive for several weeks in feces and up to six months under cold conditions, as well as on fomites. Higher temperatures successfully inactivate the virus, such as temperatures 70°C or above inactivate it immediately. Although FMDV is highly sensitive to acidic (pH below 6.0) and alkaline (pH above 9.0) environments, it remains quite stable in neutral pH, especially in protein-rich materials. A drying process, salting process, or smoking process might not completely inactivate the virus. FMDV is susceptible to a variety of disinfectants, such as sodium hydroxide, citric acid, and formaldehyde-based disinfectants. These qualities suggest the importance of strict biosecurity, proper meat processing, and effective disinfection procedures to maintain biosafety and preventing the spread of FMDV (Bachrach, Breese et al. 1957, Mebus, House et al. 1993, Yang, Zhao et al. 2017, Mielke and Garabed 2020).

The SARS-CoV-2 virus (Severe Acute Respiratory Syndrome Coronavirus 2) causes COVID-19. It is an enveloped, positive-sense RNA virus belonging to the *Coronaviridae* family, *Betacoronavirus* genus. It is a large RNA virus with a diameter of ~80 nm. The virus genome consists of approximately 30 kb of RNA surrounded by a protein coat. The virus has four structural proteins: Spike, Membrane, Envelope, and Nucleocapsid. The spike protein is a trimeric glycoprotein that extends from the viral envelope and forms a crown-like ("Corona") appearance on the virus (Figure 1.9). It is a crucial glycoprotein that plays a main role in virus entry and is the target for neutralizing immune antibodies. The spike (S) protein is divided into two functional subunits. A receptor binding domain (RBD) of the S1 subunit binds to the Angiotensin-Converting Enzyme 2 (ACE2) host cell receptor, while the S2 subunit facilitates membrane fusion it enables virus enter the host cells. Many SARS-CoV-2 strains have mutations in the spike protein that affect transmissibility, immune evasion, and vaccine effectiveness. The membrane (M) protein is the most abundant structural protein within the virus and is essential in assembling virion and maintain the virus structure. The envelope (E) protein, on the other hand, is a small protein that helps viruses assemble and interact with their hosts. The nucleocapsid (N) protein plays a crucial role in packaging viral RNA and in virus replication. The N-terminal domain of N protein binds viral RNA to form the ribonucleoprotein (RNP) complex. While the C-terminal domain facilitates interactions with viral and host proteins, which are crucial for viral replication and assembly. Furthermore, SARS-CoV-2 encodes several nonstructural proteins, which are encoded by two large open reading frames, ORF1a and ORF1b, that support RNA replication and immune modulation. The S gene sequence is utilized for genotyping SARS-CoV-2, since it contains variable regions that can differentiate virus variants. The World Health Organization (WHO) genotyped SARS-CoV-2 mainly based on genetic differences in the spike protein. The strains in early were categorized as lineage A (originating from Wuhan, China, in 2020) and lineage B (which emerged as the dominant strain globally in 2020). In the subsequent years (2021-2025), the virus was classified into several major variants of concern (VOCs), including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (BA.1, BA.2, BA.2.86, BA.5, XBB, JN.1). The Alpha VOC was first identified in September 2020 in the UK, and it spread around the world rapidly in early 2021. The Beta VOC was first detected in South Africa in May 2020; however, despite being less transmissible than the Alpha VOC, it spread in Africa and Europe. The Gamma VOC was first identified in Brazil in November 2020. Although it was less transmissible than the Alpha variant, it still spread to Africa. The Delta VOC was first detected in India in October 2020 and became the dominant strain worldwide by mid-2021. The Omicron VOC was first recognized in November 2021 in South Africa and quickly replaced the Delta variant on a global scale. By mid-2022, the BA.5 strain had become a dominant, immune-evasive strain, superseding the initial subvariants BA.1 and BA.2. The strain XBB had emerged as a dominant variant by early 2023. The BA.2.86 and JN.1 variants identified in 2023-2024 are highly mutated subvariants of Omicron. By summer 2025, variants like XFG ("Stratus") and NB.1.8.1 ("Nimbus") were dominating global circulation, and the WHO designated other JN.1 descendants as Variants Under Monitoring (GISAID 2025, WHO 2025).

Pathogenesis and Clinical Signs

ASFV causes hyperacute, acute, subclinical, or chronic infections in pigs; the mortality rate in hyperacute/acute cases may reach 100% (Kleiboeker, 2002). Incubation periods range from two to thirteen days, depending on virulence of the strain and whether the disease is acute or subacute (Pershin, Shevchenko et al. 2019). Depending on the individual virus isolate, exposure, or dose, the severity and duration of the clinical signs can vary. Bush pigs (*Potamochoerus larvatus*), giant hogs (*Hylochoerus meinertzhageni*), and warthogs (*Phacochoerus africanus*) can be persistently infected and serve as reservoirs for the virus. Also, *Ornithodoros* ticks transmit the virus to warthogs within the sylvatic cycle, which is maintained in Africa (Sanchez-Cordon, Montoya et al. 2018). The ASF sylvatic cycle refers to the natural transmission cycle of the African Swine Fever virus involving wild animals, particularly wild pigs and soft ticks of the genus *Ornithodoros*. This cycle plays a main role in maintaining and spreading ASFV among wild populations. It can also cause spill-over infections to domestic pigs in at-risk areas. ASFV transmits among domestic pigs through multiple routes, many of which are independent of the sylvatic cycle. One of the primary routes of transmission is direct contact between infected and susceptible pigs through bodily fluids, including blood, saliva, urine, and feces. Indirect transmission is also important, since the virus can survive on fomites such as equipment, vehicles, clothing, and feed. It is very risky to feed pigs uncooked or undercooked pork products from infected animals, since the ASFV can remain infectious for a very long time in processed meats. Another major factor that contributes to human-mediated transmission is the use of contaminated clothing, shoes, and tools, especially in low-biosecurity settings. Additionally, ASFV can survive in the environment, including in pens, water, and slurry, for weeks to months, creating a long-term source of infection. These domestic transmission pathways highlight the need for strict hygiene practices, controlled animal movements, and bans on feeding swill to prevent ASF outbreaks (Guinat, Gogin et al. 2016, Gaudreault, Madden et al. 2020, Lohse, Nielsen et al. 2022).

Evidence suggests that ASFV initially enters the body through the tonsils or dorsal pharyngeal mucosa. It then amplifies in the tributary lymph nodes located in the retropharyngeal or mandibular areas, before spreading systematically from these lymph nodes into the bloodstream and subsequently throughout nearly all tissues (Greig 1972). In domestic pigs, ASFV strains with high virulence can cause hyperacute or acute hemorrhagic disease with 100% mortality rates. One example of this is the genotype II ASFV Georgia 2007 (Georgia/07) strain, which caused significant mortality in pigs in the Republic of Georgia (Chapman, Darby et al. 2011). Symptoms of the disease include high fever, anorexia, cutaneous erythema, nasal hemorrhages, melena, skin cyanosis, and bloody diarrhea in some cases (Kleiboeker 2002).

Moderate/low virulence strains of ASFV have a more extended incubation period and can cause up to 70% mortality. While the clinical signs of moderate to low virulence strains are usually less noticeable, vascular changes, specifically hemorrhage and edema, are rather severe. Low virulent strains can cause chronic forms of ASF, which have a relatively low mortality rate. However, they may also lead to symptoms like delayed growth, swollen joints, skin ulcers, and secondary bacterial infections. (Sanchez-Vizcaino, Mur et al. 2015).

CSFV mainly transmits between hosts through direct contact with infected pigs or their bodily fluids, like saliva, blood, or feces. Contaminated feed, water, and surfaces can also be sources of infection. This allows the virus spread rapidly in the situations where large numbers of pigs are kept in limited space. Additionally, the virus can be transmitted from pregnant sows to their fetuses, resulting in transplacental, congenital infections (Choe, Kim et al. 2019). Once the virus replicates in a pig, blood vessels and lymphoid tissues are damaged, resulting in bleeding and tissue damage as well as in critical drops in white blood cells and platelets.

The clinical signs of CSFV depend on the virulence of the strain, age of the pigs, and their immune status. Infected pigs often have high fever, lethargy, depression, and loss of appetite. They may also experience diarrhea, respiratory distress, and skin hemorrhages. Highly virulent strains of CSFV can cause cyanosis of the ears and snout, and neurological symptoms such as tremors and convulsions. Infection often leads to death within 1-2 weeks and causes high mortality rates. Low virulence strains may cause milder clinical signs, such as transient fever and reduced growth rates, and more prolonged survival. The subacute form is similar to the acute form, but it has milder signs, lasting up to 3 weeks, with moderate mortality. The chronic form leads to intermittent fever, poor growth, and prolonged clinical signs. Congenital infections may result in stillborn or weak piglets that can shed the virus (Lohse, Nielsen et al. 2012, Blome, Staubach et al. 2017, Ganges, Crooke et al. 2020).

FMDV causes vesicular lesions and systemic symptoms in infected animals. The virus spreads through aerosol droplets from one animal to another. FMDV typically enters the body through respiratory tract. The arginine glycine aspartic acid (RGD) motif on VP1 capsid protein of FMDV binds to integrin αvβ6 receptor of epithelial cell, and it enables the virion to penetrate the cell membrane and infect the host cell (Kotecha, Wang et al. 2017, Sarry, Vitour et al. 2022). Once the virus enters cells, it can hijack the host cells' machinery to replicate and spread. FMDV primarily replicates within the epithelial cells in upper respiratory system, and it causes local inflammation and damage. Following initial infection, the virus enters the bloodstream and targets epithelial tissues in the mouth, tongue, feet, and teats. This results in cell damage and the formation of fluid-filled blisters that contain an enormous amount of infectious virus, which is highly contagious (Li, Wang et al. 2021). It is generally believed that the host's immune system will eventually eliminate the virus; however, some animals, particularly cattle, may carry the virus persistently without exhibiting any symptoms.

Cloven-hoofed animals, such as cattle, pigs, sheep, goats, yaks, camels, and wild ungulates, are affected by FMD. The clinical signs vary according to the species, virus strain, and environmental conditions. The incubation period ranges from 2 to 14 days. Early symptoms include high fever (40–41°C), depression, lethargy, and decreased milk production. Vesicles develop on the tongue, lips, gums, teat, and feet. When these vesicles rupture, they form painful erosions. The severe lesions in the mouth cavity lead to profuse, foamy, and stringy salivation, as well as painful lameness resulting from foot lesions. Additionally, anorexia and weight loss can be observed. In pigs, severe lesions on the feet and snouts are notable, while sheep and goats typically exhibit mild symptoms, often manifesting as lameness or gum lesions. The condition can lead to complications such as bacterial infections and chronic myocarditis. There is a possibility that some cattle could become asymptomatic carriers of the virus, which can persist in the pharynx. These are the most common clinical signs of FMDV infection in domestic animals (Li, Wang et al. 2021).

SARS-CoV-2 invades respiratory epithelial cells by using its RBD of the S protein to attach to the ACE2 receptor of these cells, found in the lung, kidney, heart, intestine, and blood vessels. The transmembrane protease serine 2 (TMPRSS2) is involved in the cleavage of the S protein, which enables membrane fusion and virus entry. Following viral entry and replication, infected cells experience cytopathic effects that triggers local inflammation. This causes tissue damage and dysregulated immune responses (Jackson, Farzan et al. 2022). In humans, infection severity can range from asymptomatic to severe pneumonia. However, it depends on viral load, or patient's immune status, and underlying health conditions. In severe cases, infection may cause a “cytokine storm,” characterized by an excessive release of pro-inflammatory cytokines. It can result in severe lung injury, acute respiratory distress syndrome (ARDS), and multiorgan failure (Baj, Karakula-Juchnowicz et al. 2020). In animals, pathogenesis depends on species susceptibility and ACE2 receptor compatibility. Experimental and natural infections have shown that cats, ferrets, mink, hamsters, deer, and some non-human primates are susceptible, and the virus can replicate efficiently in the upper respiratory tract of these animal species. The infection is generally mild or subclinical in most animals. However, severe disease has been documented in mink and aged non-human primates. As a result of their low ACE2 binding affinity, livestock species such as pigs, cattle, poultry, and horses are mostly resistant to virus replication, while dogs showed limited viral replication (Mahdy, Younis et al. 2020, Hamer 2023, Usai, Mateu et al. 2023).

Clinical signs range from mild flu-like illness to severe respiratory difficulties in humans. Fever, fatigue, dry cough, sore throat, ageusia (loss of taste), and anosmia (loss of smell) are the common signs in human. In severe cases, may progress to pneumonia, hypoxia, dyspnea, and respiratory failure, and they often accompanied with cardiac complications or coagulopathy (Wilk, Rustagi et al. 2020, Lamers and Haagmans 2022). Depending on their species, animals show different clinical signs. While some cats show mild respiratory symptoms, such as sneezing, nasal discharge, coughing, and lethargy, others remain asymptomatic. During farm outbreaks, mink have experienced high levels of morbidity and mortality due to interstitial pneumonia. A mild cough, fever, or reduced activity may occur in ferrets. Dogs are usually infected subclinically, but sometimes mild respiratory or gastrointestinal symptoms may occur. Primates show pathological findings similar to those in humans, such as interstitial pneumonia, alveolar damage, and inflammation (Cui, Liu et al. 2022, Halfmann, Iida et al. 2022, Kannekens-Jager, de Rooij et al. 2022).

Wild-type laboratory mice are resistant to the ancestral SARS-CoV-2 strain because of its weak affinity to murine ACE2 receptors. However, with the emergence of SARS-CoV-2 VOCs, particularly those carrying the N501Y mutation in the spike protein, such as Alpha, Beta, and Gamma, increased susceptibility has been observed in wild-type mice. The spike mutation enhances the ability of the virus to bind to mouse ACE2, allowing for more efficient replication and mild to moderate respiratory signs in infected mice. Although the Delta VOC shows limited infectivity in wild-type mice, the Omicron variant and its sublineages (e.g., BA.1, BA.2, BA.5), which possess multiple spike mutations including N501Y, Q493R, and Q498R, have demonstrated improved murine ACE2 binding and replication capability. Despite this, Omicron generally causes milder disease in mice compared to earlier VOCs. For consistent modeling of severe and lethal SARS-CoV-2 infection, transgenic mice expressing human ACE2, such as K18-hACE2 mice, remain the most reliable approach (Knight, Montgomery et al. 2021, Jimenez de Oya, Calvo-Pinilla et al. 2024, Kondo, Suzuki et al. 2025).

Hamsters are naturally highly susceptible to SARS-CoV-2 and are widely used in vaccine and therapeutics SARS-CoV-2 research. Shortly after infection, they experience significant weight loss. Infected hamsters may show symptoms such as sneezing, nasal discharge, and labored breathing. In addition to lethargy and loss of appetite, behavioral changes may occur, such as reduced grooming. Experimentally infected hamsters showed significant viral replication in the respiratory tract, leading to inflammation and lung damage. As a result, they transmit the virus to each other (Castellan, Zamperin et al. 2023).

Diagnosis and control measures

The diagnosis of ASF involves the isolation of the virus or the detection of its genetic material, antigens, or antibodies. An accurate diagnosis begins with a quality sample collected in the first step. For ASF diagnostics, blood is often taken for viral, antigen, and serology tests, as well as the spleen, lymph nodes, tonsils, liver, and kidney for viral or antigen tests. Virus detection can be performed using molecular techniques, such as the Polymerase Chain Reaction (PCR). PCR is highly sensitive and detects small fragments of the DNA genome of ASFV. ASF PCR tests target the B646L gene, which encodes the major capsid protein p72 of ASFV. Virus isolation can be performed using primary porcine monocyte macrophages or one of the following cell lines: MA-104, WSL, and PIPEC. The method is not routinely used for diagnostic purposes since it is time and labor intensive and needs a BSL-3 laboratory. The Lateral Flow Assay (LFA) is a practical method for rapid on-site detection of ASFV antigen. However, it is less sensitive than the antigen ELISA (Enzyme-linked immunosorbent assay) or qPCR (quantitative polymerase chain reaction). For serological tests, ELISA, IPT (immunoperoxidase test), IBT (immunoblotting test), and IFAT (indirect fluorescent antibody test) are commonly used to identify ASFV-specific antibodies. Molecular tests can also be performed on portable devices, either PCR (polymerase chain reaction) or LAMP (loop-mediated isothermal amplification) assays. In an ASF outbreak, rapid antigen tests using LFA can be used for diagnosis (WOAH 2024). Since there are no effective vaccines available in non-endemic countries, control measures focus on early detection, movement restriction, biosecurity, surveillance, and quarantine. In order to prevent ASF from spreading, it is crucial to identify ASFV early, eliminate infected animals, and dispose of carcasses and waste in the right way. Zoning involves dividing a country into areas for infected animals, surveillance zones, and free zones with limited movement of pigs. Movement controls restrict on-farm traffic, and no potentially contaminated products are allowed for sale. Staff training, sanitation, and controlling movement of animals between pens are the most important aspects of biosecurity. Surveillance programs conducting targeted investigations and enforcing quarantine procedures during repopulation of pigs in surveillance zones (Liu, Zhang et al. 2021, WOAH 2025). Monitoring ASF in wild boar populations is crucial for controlling the disease in affected regions. In addition to regulating hunting practices, carcass removal is important to prevent disease transmission (Kim, Park et al. 2021, Licoppe, De Waele et al. 2023).

The diagnosis of CSF involves observing clinical signs, considering epidemiological factors, and performing laboratory tests. In CSFV-infected pigs, postmortem findings include petechial skin hemorrhages, enlarged spleen, and hemorrhagic lymph nodes as well as ulcerative gastrointestinal lesions. Epidemiological investigations, including herd health history, vaccination records, recent animal movements, and potential sources of infection, are important for implementing control measures. In addition, a differential diagnosis of CSF from other similar disease symptoms, such as ASF, PRRS, Salmonellosis, and Erysipelas in the field is critical. However, clinical signs, epidemiological factors and postmortem findings help to diagnose the disease; a comprehensive approach is necessary to diagnose CSF accurately. Laboratory tests for CSFV include RT-PCR, ELISA, virus isolation, VNT, immunohistochemistry, and fluorescent antibody tests. RT-PCR is routinely used in the diagnosis of CSF by amplifying specific viral genetic material, allowing for highly sensitive and rapid identification of the viral gene. Additionally, confirmatory testing is necessary to confirm the presence of the virus. Overall, to control outbreaks of CSF, early diagnosis is crucial, since the virus infection transmits rather efficiently, and the disease has significant economic implications (WOAH 2024).

A laboratory diagnosis of FMD requires the collection of bodily fluids and/or tissue samples. Various tests are used to diagnose FMD, including virus isolation, RT-PCR analysis, ELISA, CFT, VNT, serotyping, and genotyping. Several advances in FMD diagnostic techniques have enhanced the accuracy and speed of characterizing the outbreak virus. Portable RT-PCR devices enable rapid on-site testing with results available in hours rather than sending samples to a laboratory and delaying test results for days. Moreover, next-generation sequencing techniques are able to characterize viral strains in detail, enabling outbreak tracking and vaccination planning. Differentiating FMD from other diseases with similar symptoms is crucial. POC diagnostics are becoming increasingly popular for fast field detection of FMDV. Laboratory tests are essential for confirming the presence of FMDV, particularly in disease-free areas (WOAH 2024).

Laboratory diagnostic tests for COVID-19 are crucial for identifying infections with SARS-CoV-2. A variety of assays are available for diagnosing COVID-19. Nasal or throat samples are collected for RT-PCR, which identifies virus RNA within a few hours. The viral genome can be detected by RT-PCR only during active infection, and it is a highly accurate and reliable test. An antigen test detects specific proteins of the virus in a nasal or throat swab using a rapid detection lateral flow kit. Results are usually available within minutes, but they are less sensitive than RT-PCR. Antibody tests, such as VNT, ELISA, IFA, and LFA, detect viruses-specific antibodies in blood samples from individuals or animals who have infected with SARS-CoV-2 in the past. The antibody tests provide valuable information about infection or immunization, but they are not suitable for diagnosing early stages of infection (WHO 2020).

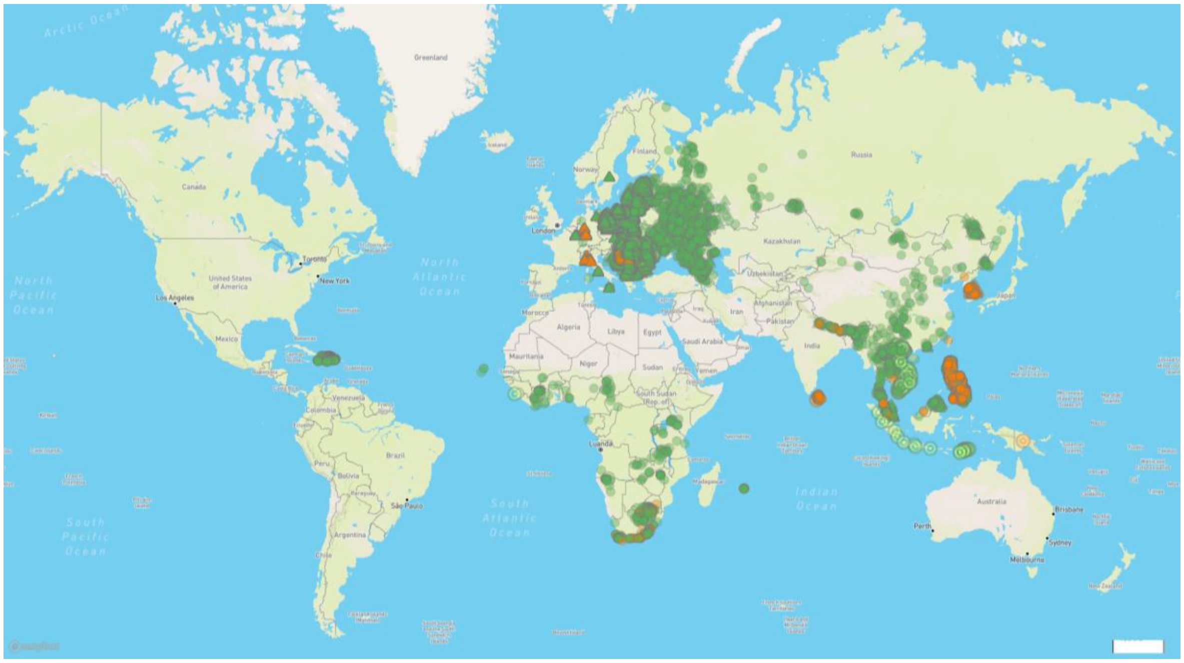

Figure 1.1. Geographic distribution of ASF outbreak status in October 2025.
The map displays approximate locations and intensity of reported ASF outbreaks involving domestic and wild species. Outbreaks are categorized by status: currently reported outbreaks (orange), and resolved outbreaks (green). The shape of the marker indicates the type of animals involved: circles for domestic animals, and triangles for wild species. Outbreak locations are approximate and based on data submitted by national veterinary authorities. The map was created by the World Animal Health Information System (WAHIS).

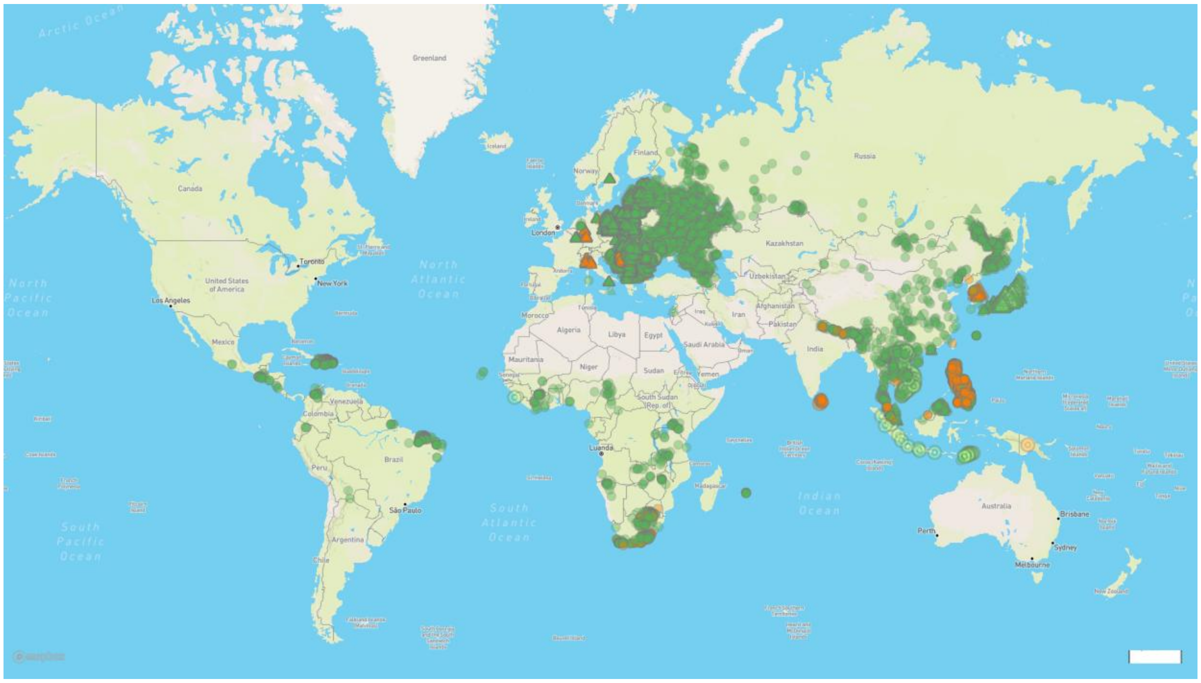

Figure 1.2. Geographic distribution of CSF outbreak status in October 2025.
The map displays approximate locations and intensity of reported CSF outbreaks involving domestic and wild species. The recent outbreaks have been resolved and are now marked as green. The shape of the marker indicates the type of animals involved: circles for domestic animals, and triangles for wild species. Outbreak locations are approximate and based on data submitted by national veterinary authorities. The map was created by the World Animal Health Information System (WAHIS).

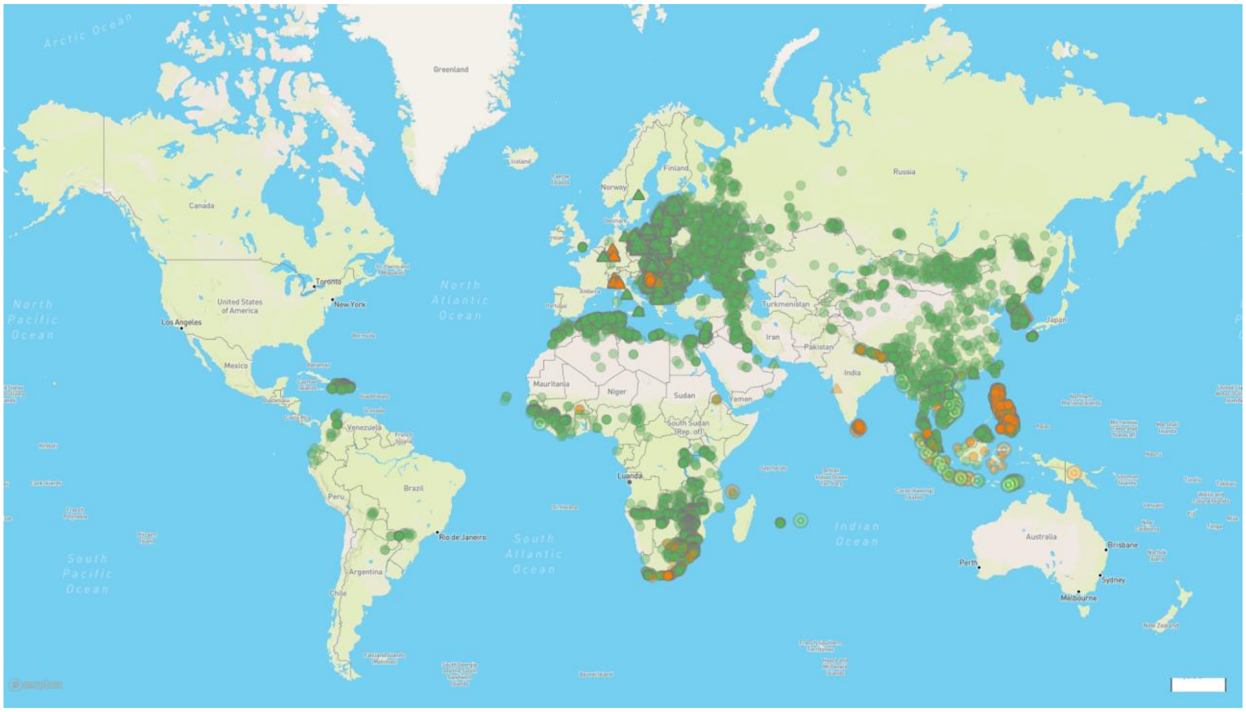

Figure 1.3. Geographic distribution of FMD outbreak status in October 2025.

The map displays approximate locations and intensity of reported FMD outbreaks involving domestic and wild species. Outbreaks are categorized by status: currently reported outbreaks (orange), and resolved outbreaks (green). The shape of the marker indicates the type of animals involved: circles for domestic animals, and triangles for wild species. Outbreak locations are approximate and based on data submitted by national veterinary authorities. The map was created by the World Animal Health Information System (WAHIS).

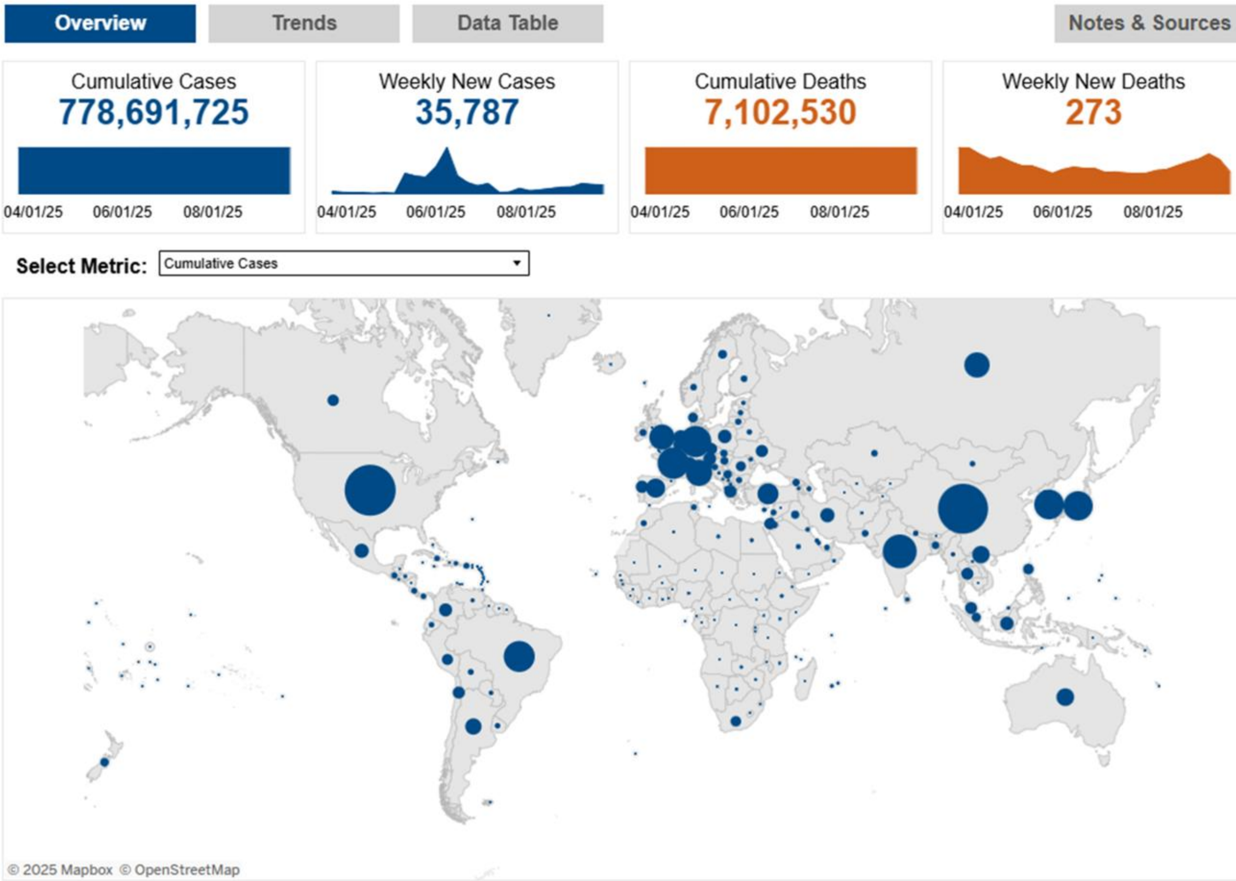

Figure 1.4. Global COVID-19 human cases and deaths as of September 2025.

The dashboard presents cumulative and weekly global statistics on COVID-19 cases and deaths. As of the reporting date, there were 778,691,725 cumulative cases and 7,102,530 cumulative deaths worldwide, with 35,787 new cases reported weekly and 273 new deaths reported weekly. The map visualizes the weekly number of new deaths by country, with circle size indicating the relative number of deaths. Data sourced from KFF (Kaiser Family Foundation).

1. USA: B, Ca, Co, De, Do, Fe, FC, G, Hy, Li, Ly, M, O, P, SL, T
2. Spain: Ca, Do, Fe, Li, M, O
3. Canada: Ca, Do, De, M
4. Netherlands: Ca, Do, G, M
5. Argentina: Ca,Do, P
6. China: Ca, Do, P
7. France: Ca, Do, M
8. Italy: Ca, Do, M
9. Sweden: Li, M, T
10. Germany: Ca, Do
11. Japan: Ca, Do
12. Brazil: Ca, Do
13. Switzerland: Ca, Do
14. Columbia: Ca, Do
15. Croatia: Ca, Do
16. Egypt: Ca, Do
17. Thailand: Ca, Do
18. Belgium: Ca, Hi
19. Greece: Ca, M
20. Latvia: Ca, M
21. Denmark: Ca, M
22. Belarus: Ca, M
23. Slovenia: Fe, M
24. India: Ha, Li
25. South Africa: Li, P
26. Czech Republic: Li, T
27. Russia: Ca
28. UK: Ca
29. Chile: Ca
30. Iran: Ca
31. Peru: Ca
32. Portugal: Ca
33. Bosnia and Herzegovina: Do
34. Mexico: Do
35. Estonia: Li
36. Sri Lanka: Li
37. Singapore: Li
38. Lithuania: M
39. Poland: M

Figure 1.5. Global distribution of SARS-CoV-2 infections in animals (2020–2022). Countries with animals that tested positive for SARS-CoV-2 are marked in orange. The animal species positive for SARS-CoV-2 in each country are listed. As of May 12, 2022, a total of 39 countries have reported animals testing positive for the virus. B: binturong; Ca: cat; Co: coati; De: deer; Do: dog; Fe: ferret; FC: fishing cat; G: gorilla; Ha: hamster; Hi: hippopotamus; Hy: hyena; Li: lion; Ly: lynx; M: mink; O: otter; P: puma; SL: snow leopard; T: tiger.

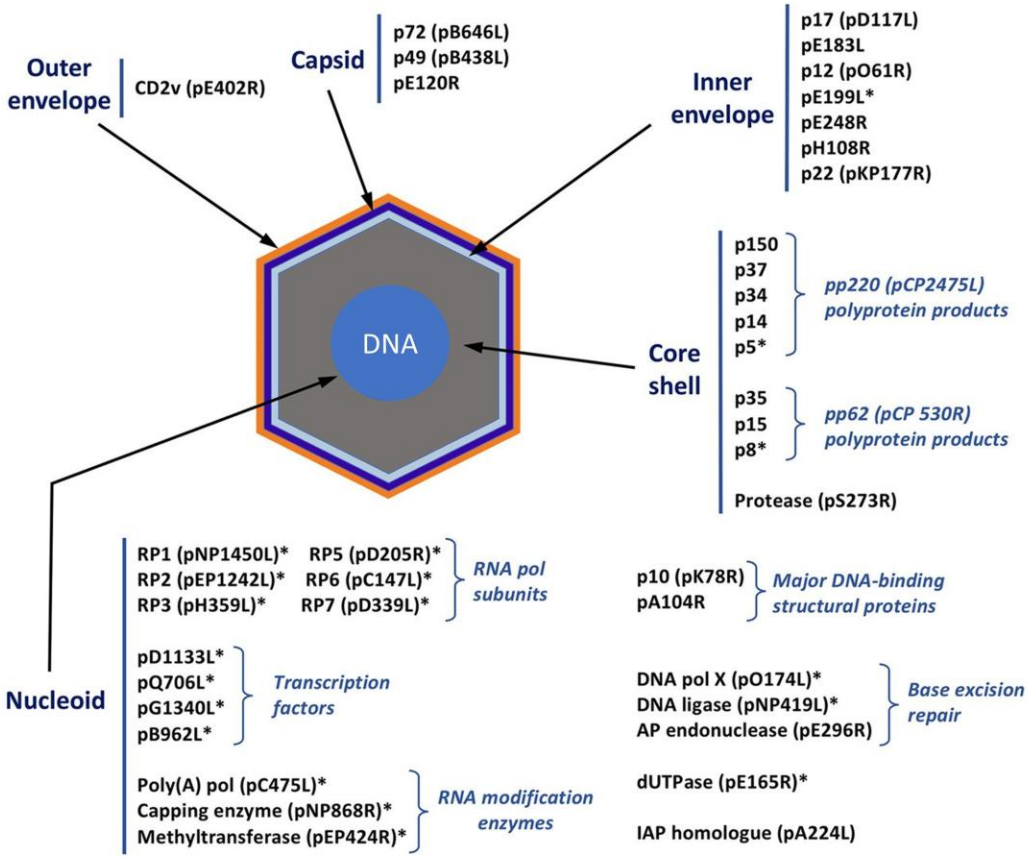

Figure 1.6. ASFV Particles.

The subviral localization of 40 viral proteins among the five structural domains of the ASFV particle is shown. The distribution of proteins marked with an asterisk was inferred from the predicted or known role, while that of the remaining proteins was determined by immunoelectron microscopy (adapted from Alejo et al., 2018).

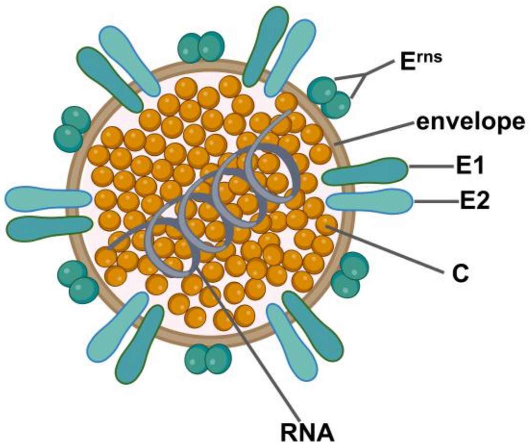

Figure 1.7. The structure of CSFV.

There are three envelope proteins on the surface of virus particles: Erns, E1, and E2. As a transmembrane protein, E2 is present as a homodimer or forms E1–E2 heterodimers on the virion. The C protein binds to the viral RNA genome with a nonspecific affinity for nucleic acids. The CSFV genome is approximately 12.3 kb long. It consists of one large open reading frame (ORF). Four structural proteins (C, Erns, E1 and E2) are encoded by the ORF, along with eight nonstructural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (modified from Guo et al., 2023). The figure was created with BioRender.com.

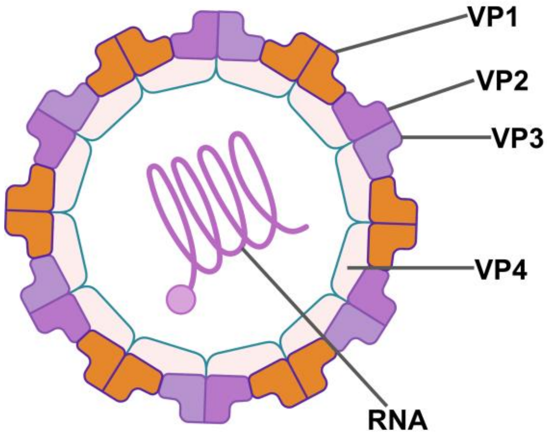

Figure 1.8. The structure of FMDV.

The FMDV genome encodes four structural proteins (VP1, VP2, VP3, and VP4) that form the virion capsid. VP1-3 are external proteins that contribute to antigenic variability, while VP4 is an internal protein that stabilizes the virion structure. Moreover, there are eight nonstructural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D). The virus genome consists of a single-stranded, positive sense RNA that is approximately 8,300 nt long. It is divided into untranslated regions (5' UTR and 3' UTR) and an open reading frame (ORF). The ORF (~ 7,000 nt) encodes both structural and nonstructural proteins. The figure was created with BioRender.com.

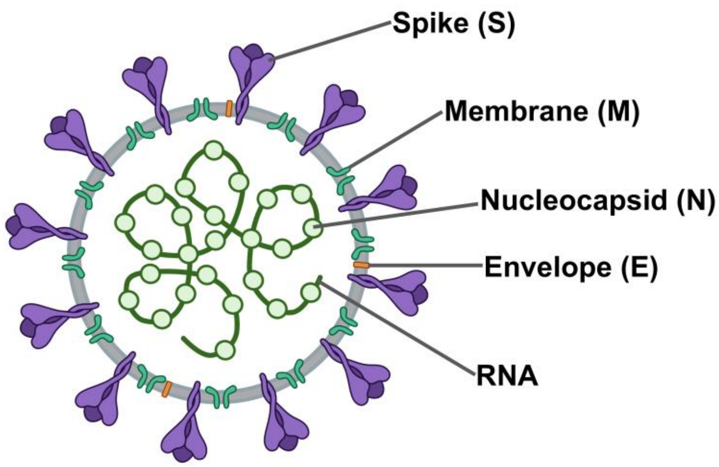

Figure 1.9. SARS-CoV-2 structure.

The virus genome consists of a single-stranded, positive sense RNA that is approximately 30 kb in size. It involves untranslated regions (5' UTR and 3' UTR) and two open reading frames (ORF1a and ORF1b). The ORFs encode structural proteins (S, E, M and N), nonstructural proteins, and accessory proteins. The viral envelope is a lipid bilayer containing three major structural proteins: S, M, and E. The S protein initiates viral entry, the M protein stabilizes the structure of the virion, and the E protein aids in viral release. The figure was created with BioRender.com.

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Chapter 2 - Point of Care Diagnostics

Introduction

Transboundary animal diseases (TADs), such as FMD, ASF, and CSF, present significant threats to global livestock health, food security and trade. TADs spread across many countries in a short period, and rapid detection and actions are required to mitigate their impacts. Traditional laboratory based diagnostic methods are highly specific and sensitive, but they often tend to be slow, resource-intensive, and difficult to use in remote or resource-limited areas. In this context, POC diagnostic tools offer a transformative solution by enabling rapid, on-site detection of pathogens at farm or field level. These portable and easy-to-use technologies, including lateral flow assays (LFA), isothermal amplification methods (LAMP, RPA, RCA, SDA, etc.), and portable PCR and gene sequencing systems, deliver timely results that are essential for early outbreak control and on-site decision making. Integrating POC diagnostics into veterinary surveillance systems improves real-time disease monitoring and strengthens the global response to TADs and endemic diseases.

POC tests (POCTs) can be used for various pathogens and analytes. They are commonly used in veterinary diagnostics to detect nucleic acids, antibodies, and antigens. POCTs are a rather new approach in veterinary diagnostics as they provide portable and quick results, allowing immediate response and animal health management. The convenience and portability of POCTs make them especially valuable in field settings where traditional laboratory facilities are not available (Vashist 2017).

During the COVID-19 pandemic, researchers worldwide developed and commercialized SARS-CoV-2-specific antigen POCTs, which allowed individuals to test themselves during the pandemic. This familiarized the public with POCTs and simplified the diagnosis of SARS-CoV-

2 infection. Several at-home COVID-19 tests have been given Emergency Use Authorization (EUA) from the Food and Drug Administration (FDA), and most of them are sold over-the-counter (OTC) tests without a prescription. The COVID POCTs revolutionized the field of OTC POCTs, providing consumers with rapid results without the wait (Kalia, Kaila et al. 2022).

The use of POCTs is expected to increase in the coming years. An increase of POCTs at a compound annual growth rate (CAGR) of 9.4% is likely, ie from USD 49.7 billion to 77.8 billion projected from 2023 to 2027, due to factors such as the high occurrence of human respiratory and infectious diseases, and the increasing availability of POCTs in the market (Markets 2023).

This chapter discusses the development of LFA-based tests for the detection of ASFV as well as portable sequencing of ASFV, CSFV and FMDV using the portable MinION sequencing platform, to enhance the early detection and management of high-consequence transboundary animal diseases.

2.1 Monoclonal antibody generation for ASFV antigen detection LFA

Introduction

ASFV affects both domestic and wild pigs. The virus spreads directly or indirectly between pigs through infected ticks or via human activities. ASFV can lead to severe illness in pigs that results in up to 100% mortality among infected pigs. In 2007, a virulent isolate emerged in Georgia, spreading rapidly throughout Europe and Russia. In 2018, ASFV was first identified in China and has since expanded to Eastern Asia, Western Europe, and the Caribbean.

Antigen detection plays a crucial role in the early diagnosis and rapid field screening of ASFV, especially during acute outbreaks. The antigen tests identify ASFV proteins (such as p30 or p72) in clinical samples, including blood, serum, spleen, or lymph nodes. ASFV p30 is the earliest antigen detected in the host among structural proteins, as it is abundantly expressed during the initial phases of viral infection (Gomez-Puertas, Rodriguez et al. 1998, Gaudreault, Madden et al. 2020). Antigen tests, including ELISA, LFA, Immunohistochemistry (IHC), and Direct Fluorescent Antibody Tests (FAT), are designed to be rapid, user-friendly, and, in some cases, field-deployable, with results presented within minutes to a few hours, depending on the test format. Among these antigen tests, the LFA is the most user-friendly and portable test format for diagnosing ASF in the field.

The ASFV antigen detection LFA is typically used during the acute infection, when viral load is high level. It can identify ASFV proteins in blood, serum, or tissue homogenates. LFAs often provide results within 10–20 minutes and it is well suited for use on farms or in quarantine zones. Even though LFAs are fast and simple, they are generally less sensitive than laboratory based techniques like ELISA or qPCR. Their portability, low cost, and minimal training requirements make them suitable diagnostic tools for resource-limited or field environments.

Recent evaluations of commercially available antigen-detecting ASF LFA kits show that the sensitivity ranges from 70% to 90%, and the specificity ranges from 95% to 98%. This depends on the viral load, sample quality, and the stage of infection or quality of monoclonal antibodies (mAbs) used in the LFA. The mAbs are the most crucial component in developing a reliable antigen-detecting LFA. Therefore, we developed mAbs targeting the ASFV-specific p30 protein to create a LFA for detecting the ASFV p30 antigen. The LFA demonstrated robust sensitivity (mainly depending on clinical signs and viral load) and specificity when using a panel of blood samples from naïve or experimentally ASFV infected pigs (Madden, Sunwoo et al. 2022).

Materials and Methods

Ethics statement. The animal research was conducted in accordance with protocols that were approved by the Institutional Animal Care and Use Committee (IACUC, Protocol #4402) and in compliance with the Animal Welfare Act and the Kansas State University (KSU) Institutional Biosafety Committee (IBC, Protocol #1460). All animal and laboratory work was performed in biosafety level-2+ and level-2Ag laboratories and facilities at the Comparative Medicine Group (CMG) at KSU in Manhattan, KS, USA.

Detection of ASFV p30 antibodies by indirect ELISA. An in-house developed iELISA was used to detect mouse antibodies against the ASFV p30 during the screening of clones. Each well of Maxisorp plate (ThermoFisher, MA, USA) was coated with 100 ng of ASFV p30 in 100 µl of coating buffer (Carbonate–bicarbonate buffer, Sigma-Aldrich, St. Louis, MO, USA), then incubated overnight at 4°C. The following day, the plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked using 200 µl of casein blocking buffer (Sigma-Aldrich, MO, USA) per well. The plates were incubated at room temperature for 1 h. The plates were then washed three times with PBS-T, and 100 µl of cell supernatants per well was added to the ELISA plate and incubated for 1 h at room temperature. The wells were washed three times with PBS-T. Then, 100 µl of HRP-labelled goat anti-mouse IgG (H + L) or goat anti-pig IgG (H + L) secondary antibody (Jackson ImmunoResearch, PA, USA), diluted 1:10,000 in blocking buffer, was added to each well and incubated for 1 h at room temperature. After 1 h, the plates were washed five times with PBS-T, followed by the adding 100 µl of TMB ELISA Substrate Solution (Abcam, MA, USA) to each well. After incubating at room temperature for 5 minutes, the reaction was stopped by adding 100 µl of Stop Solution for TMB Substrate (Abcam, MA, USA) to each well. The optical density (OD) of the ELISA plates was measured at 450 nm using an ELx808 BioTek plate reader (BioTek, Winooski, VT, USA). An OD above 2.0 was considered positive.

Indirect immunofluorescence assay. An indirect immunofluorescence assay (IFA) was performed to evaluate the reactivity of mAbs against ASFV p30 in cell culture. Vero cells were seeded in 96-well plates and allowed to attach prior to ASFV infection at the desired multiplicity of infection (MOI). Infected cells were incubated at 37°C for 48 h. Following infection, culture medium was removed, and cells were washed once with phosphate-buffered saline (PBS, pH 7.4). The monolayers were fixed with 80% acetone for 10 minutes and subsequently washed twice with PBS. The wells containing fixed monolayers were incubated overnight at 4°C with monoclonal antibodies (hybridoma supernatant). The following day, cells were washed three times with PBS containing 0.05% Tween-20 (PBS-T). Then, the goat anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC), was added. The plates were incubated in the dark for 1 hour at room temperature. The cells were then washed three times with PBS-T, and 300 nM DAPI was added to each well. The cells were then incubated for 5 minutes to stain the nuclei. Then the plates were washed with PBS three times with PBS, and were sealed, protected from light, and analyzed using an EVOS fluorescence microscope. After imaging, all consumables and waste were disposed of in accordance with BSL-3 biosafety procedures, and the workspaces were decontaminated.

Testing ASFV Ag LFA. The LFA strip contains both test and control lines. The ASFV p30-specific mAb (detector) was immobilized on the nitrocellulose membrane, along with anti-mouse IgG for the control line, which were also applied to the membrane (Figure 2.2). For the test, 10 µl of the blood sample was gently mixed with 200 µl of tap water in the LFA reagent tubes using a pipette. The mixture was incubated for 5 minutes, and a test strip was placed in each tube, which allowed the sample mix to migrate up the strip. After 20 minutes, the strips were visually interpreted. A sample was considered positive if both the test and control lines were visible; if only the control line was visible, the sample was considered negative; if the control line was not visible, the test was invalid and had to be repeated. Infectious materials were processed under high-containment BSL-3 laboratory conditions.

Results

Recombinant protein generation. The open reading frame (ORF) sequence of CP204L (codes for the p30 antigen) was obtained from the Georgia 2007/1 ASFV genomic sequence (GenBank: FR682468.1) and synthesized into the pUC57 plasmid by a commercial manufacturer, GENEWIZ (South Plainfield, NJ, USA). Gene-specific primers were used to amplify the full-length CP204L ORF. The DNA amplified by PCR was inserted into the pET101/D-TOPO vector (Invitrogen, Waltham, MA, USA) and subsequently transformed into chemically competent *E. coli* One Shot™ TOP10 (Invitrogen). The CP204L sequence and orientations were confirmed through PCR and commercial Sanger sequencing (GENEWIZ) after purifying plasmid DNA with a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The purified plasmid DNA was subsequently transformed into chemically competent BL21 (DE3) *E. coli* cells (Invitrogen, Carlsbad, CA, USA). To express the recombinant proteins, transformed BL21 *E. coli* cells were grown in LB medium (Sigma-Aldrich, St. Louis, MO, USA) containing 100 µg/mL ampicillin (Sigma-Aldrich) and induced by adding 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG; Sigma-Aldrich) until the optical density (O.D.) reached 0.6. After 16 h IPTG induction, cells were harvested by centrifugation and were lysed with a lysis buffer containing 50 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole (Sigma Aldrich), 10% glycerol, and 400 U/g of cell pellets of benzonase nuclease (EMD Millipore, Burlington, MA, USA). Cellular debris was pelleted after centrifugation at 14,000 × g for 20 minutes, and the supernatant was collected. QIAGEN's Ni-NTA Superflow Resin was used to purify recombinant p30 from the supernatant. To exchange the buffer, purified recombinant p30 protein was dialyzed overnight against phosphate-buffered saline (PBS; Gibco) containing 150 mM NaCl, 4 mM EDTA, and 10% glycerol. The protein was then concentrated using Amicon Ultra-15 centrifuge filter units (EMD Millipore). Recombinant p30 protein expression and purity were evaluated with NuPage 12% bis-tris gels (Invitrogen) followed by Coomassie blue staining with the eStain L1 Protein Staining System (GenScript, Piscataway, NJ, USA). Following that, a western blot analysis was performed using an anti-His (C-term) HRP monoclonal antibody (Invitrogen, Carlsbad, CA, USA) to confirm the recombinant protein.

Mouse immunization with ASFV p30 recombinant protein. Five Balb/C mice, eight weeks of age, were obtained from Charles River Laboratories (MA, USA) and handled according to guidelines set by the IACUC at KSU. The mice were immunized with 20 µg of ASFV p30 recombinant protein in PBS emulsified with Freund's Incomplete Adjuvant (Sigma-Aldrich, MO, USA) via subcutaneous injection. Two booster doses of 20 µg of ASFV p30 recombinant protein were administered to the mice at 14- and 21-days post-vaccination (DPV). Mouse sera were collected five days after the final boost, and the ASFV p30-specific antibody titer was estimated in an indirect ELISA (iELISA) using a plate coated with the ASFV p30.

Monoclonal antibody generation. Sp2/0Ag14 myeloma cells (ATCC, VA, USA) were cultured in Hybridoma-SFM medium (ThermoFisher, NY, USA), supplemented with 10% FBS (R&D Systems, Minneapolis, MN, USA) and antibiotics/antimycotics (ThermoFisher Scientific, MA, USA) prior to fusion with splenocytes. A day before cell fusion, the myeloma cells were passaged into two T75 flasks. Cells were ready to be harvested and fused on the following day when they reached 70 to 80% confluence. The day before cell fusion, five 96-well tissue culture plates with feeder cells (mouse splenocytes) were prepared (100 µl/well, 1 x 10^5/ml). The feeder cell preparation involved homogenizing a mouse spleen and washing the splenocytes three times in DMEM (Corning, New York, NY, USA) with centrifugation at 1200 RPM for five minutes. Following this, the splenocytes were resuspended in 52 ml of HAT medium (Hybridoma medium supplemented with HAT, Sigma Aldrich, MO, USA, 20% FBS, and antibiotics/antimycotics) and plated at a density of 100 µl per well.

Cell fusion. Once the antibody titer against the ASFV p30 in mouse blood reached a high level, as indicated by an optical density (OD) above 2.0 in the iELISA at a serum dilution of 1:10,000 in PBS, the mouse was euthanized, and its spleen was collected for cell fusion. The mouse spleen was homogenized and washed twice in cold DMEM (Corning, NY, USA) by centrifugation at 1200 RPM for five minutes. As well, the myeloma cells were harvested from the T75 flasks and washed twice in cold DMEM (Corning, NY, USA). After washing, myeloma cells (~2 × 10^7) and splenocytes (~1 × 10^8) were combined in a 50 ml conical tube and centrifuged one final time. The supernatant was gently removed from the tube. Cell fusion was performed by adding prewarmed 1.5 ml polyethylene glycol (PEG) 1500 (Sigma Aldrich, Mannheim, Germany) dropwise onto the cell pellet for 5 minutes in 37°C in a water bath. Following the fusion, the mixture was washed by adding 10 ml of pre-warmed DMEM (Corning, NY, USA) and then centrifuged at 700 RPM for 7 minutes. Afterward, the supernatant was gently removed using a serological pipette. The cell pellet was then resuspended in 52 ml of HAT medium and transferred into the feeder cell-seeded plates at a volume of 100 µl per well.

Fused hybridoma cell maintenance. Twenty-four hours post-fusion, the medium was refreshed by gently adding 50 µl/well of HAT medium to avoid disturbing the cells.

Subsequently, the medium was partially refreshed every two days by removing 100 µl/well and replenishing with HAT medium until five days post-fusion. At seven days post-fusion, the medium was switched to HT medium (Hybridoma medium supplemented with HT, Sigma-Aldrich, MO, USA, 20% FBS, and antibiotics/antimycotics) and was partially refreshed every two days with HT medium until cell clusters were observed in the wells. Then, 100 µl of cell supernatant from the well with clustered cells was collected and tested by iELISA coated with ASFV p30. The positive clones were expanded into 24-well plates for subcloning.

Subcloning hybridomas. Subcloning was aimed at establishing monoclonal cell lines that produce ASFV p30–specific antibodies (monoclonal antibodies). Initially, the density of the mixed primary positive clones in each well of the 24-well plate was estimated using the Countess-3 cell counter (Thermo Fisher Scientific, MA, USA). Then, approximately one cell was seeded into each well of a 96-well tissue culture plate from each primary clone of the 24-well plate. Every two days, the cell medium was refreshed by removing 100 µl from each well and replacing it with an equal volume of warm HT medium. Once a visible cluster was observed, cell supernatant was collected and tested using the p30 iELISA. The positive clones were expanded in T25 flasks, and mAbs in the cell supernatants were confirmed by IFA on ASFV-infected Vero-E6 cells using a secondary antibody, Goat Anti-Mouse IgG H&L (FITC) (Abcam, MA, USA). Ultimately, three mAbs, 5C1, 2B8, and 1D8, which target the ASFV p30 protein, demonstrated high and specific reactivity with ASFV-infected cells. These mAbs were chosen as the basis for the ASFV antigen-detection LFA.

ASFV Ag LFA. We developed the ASFV Ag LFA in collaboration with Silver Lake Research Corporation (SLRC, Irwindale, CA). The ASFV Ag LFA to detect the ASFV antigen, utilizing the above-mentioned mAbs targeting the ASFV p30 protein as the capture and/or detection antibodies. The assay consists of a sample tube containing dried conjugate and a strip of paper stick where the sample migrates and develops color (Figure 2.1). The sensitivity of the ASFV antigen-detecting LFA was assessed using blood samples from experimentally infected pigs. The positive samples were verified with qPCR as positive. The LFA demonstrated slightly lower overall sensitivity (93.5%) in samples from pigs infected with the highly virulent Arm07 strain, detecting 29 of 31 qPCR-positive samples. However, in animals with severe disease manifestations, such as clinical scores >6, high fever scores (>105°F), or dead pigs, the LFA achieved 100% sensitivity, indicating strong performance when viral antigen levels are high. For the highly virulent E70 strain, the diagnostic method achieved 100% sensitivity (n = 5), regardless of clinical or fever score (Table 2.1). These results suggest that the ASFV antigen LFA offers a rapid and reliable detection of ASFV antigen, particularly effective in pigs with fever moderate to severe clinical signs; however, its sensitivity may be slightly reduced in early or mild infections compared to qPCR. Daniel Madden published the results in 2022 (Madden, Sunwoo et al. 2022).

Conclusions

The ASFV three genes CP204L, E183L, and B646L and their encoded protein products (p30, p54, and p72, respectively) are commonly used in ASF diagnosis (King, Reid et al. 2003, Zhu, Meng et al. 2021, Nah, Kwon et al. 2022, Huang, Cao et al. 2023). Consequently, p30 is an appropriate target for detecting ASFV antigens during viral infections, and thus we have chosen it as the target antigen for the development of our ASFV Ag LFA. This study evaluated the ASFV p30 lateral flow assay (LFA) and found it to be highly user-friendly in field settings, with high specificity and sensitivity depending on the infection status of the animal. Further testing in the field is needed to assess the performance of the p30 ASFV LFA as a pen-side diagnostic test, using samples from domestic and wild swine infected with different strains and genotypes of ASFV.

2.2 Lateral flow assay for ASFV antibody detection

Introduction

The causative agent of ASF, ASFV, is a complex double-stranded DNA virus with a genome ranging from 170 to 194 kbp, encoding 150 to 180 proteins (Alonso, Borca et al. 2018, Dixon, Sun et al. 2019). ASFV is the only member of the *Asfarviridae* family. It shares specific characteristics with large dsDNA viruses, such as Poxviruses and Iridoviruses, including genome structure and cytoplasmic replication. An infected pig may show mild fever and may survive, or a high fever with 100% mortality depending on the strain of ASFV (Blome, Gabriel et al. 2013). Virus strains with high virulence cause fever, hemorrhages, depression, and ataxia, eventually resulting in death within 4 to 15 days (Dixon, Sun et al. 2019). ASFV strains that are moderately virulent display similar clinical signs, but they are generally less severe. Among infected pigs, the mortality rate is 30% to 70%. ASFV infection with low-virulent strains causes intermittent fever, multifocal necrosis, and arthritis (Gallardo, Soler et al. 2015). Countries affected by ASF rely on early detection, quarantine, slaughter, and disinfection to control the disease. Early detection of ASF is regularly conducted using highly sensitive and specific molecular assays to identify ASFV genomic material in the laboratory. Serological detection is essential for regaining disease-free status after an acute outbreak or identifying pigs infected with low-virulent strains. In countries where ASF is endemic, many naturally attenuated ASFV strains have been recently detected (Gallardo, Soler et al. 2019, Sun, Huang et al. 2021, Zhenzhong, Chuanxiang et al. 2022, Avagyan, Hakobyan et al. 2024). These attenuated ASFV strains cause no or mild clinical signs and can remain undetected. The absence of or presence of mild clinical signs and low intermittent viremia in pigs infected with attenuated ASFV strains complicates detection using molecular assays. However, these low virulent viruses are likely detected during serological surveillance. Antibodies against ASFV appear early (about 7–10 days post-infection) and can persist for a long time, sometimes for several years (Gallardo, Nieto et al. 2015). As a result, anti-ASFV antibodies may indicate ongoing or past ASFV infections. ASFV-infected pigs produce robust antibodies against the highly conserved ASFV structural protein p54, encoded by the viral gene E183L. p54 is considered an excellent antigen for serological tests (Cubillos, Gomez-Sebastian et al. 2013, Cao, Han et al. 2021, Onyilagha, Quizon et al. 2024).

Due to the rapid spread of ASFV, there has been an increasing demand for portable diagnostic tools. These tools are crucial for early detection and emergency response to ASF outbreaks, protecting swine populations and maintaining the stability of pork supply chains. Here we developed an LFA to detect ASFV p54-specific antibodies in pig serum as a portable and reliable diagnostic tool for identifying ASFV-infected pigs in the field.

Materials and Methods

Recombinant protein generation. The E183L open reading frame (ORF) sequence was derived from the genotype II Georgia 2007/1 ASFV genomic sequence (GenBank: FR682468.1). The E183L gene, encoding the ASFV p54 protein, was cloned into the pUC57 plasmid. In order to amplify the full length E183L, the gene specific primers were used. The PCR product was then inserted into the pET-32a (+) vectors and transformed into chemically competent *E. coli* One ShotTM TOP10. The E183L sequences and orientations were confirmed by PCR and commercial Sanger sequencing (GENEWIZ). After confirmation, plasmid DNA was purified using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), then it was transformed into chemically competent BL21 (DE3) *E. coli* cells (Invitrogen, Carlsbad, CA, USA). To express the recombinant protein, the transformed BL21 *E. coli* cells were grown in LB buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 100 µg/mL ampicillin (Sigma-Aldrich) and induced by adding 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG; Sigma-Aldrich) until the optical density (O.D.) reached 0.6. Cells were harvested 16 hours after IPTG induction, and the recombinant was purified using nickel affinity chromatography under native conditions from bacterial pellets. After the purification of recombinant p54, it was assessed by protein molecular mass via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), along with Western blot confirmation.

Samples from naïve and experimentally ASFV-infected pigs. Whole blood samples for serum isolation were collected from fifty naive outbred piglets aged 8 to 12 weeks. ASFV antibody-positive sera were obtained from seventeen outbred piglets within the same age range. These piglets were experimentally infected with the ASFV genotype II strain Armenia 2007. Fourteen days after the ASFV challenge, whole blood samples were collected via jugular venipuncture, and the sera were isolated and stored at -80°C until needed. All animal experiments were conducted in a high-containment BSL-3Ag environment at Biosecurity Research Institute of Kansas State University, following protocol approval and evaluation by KSU's Institutional Biosafety Committee (IBC) under protocol numbers 850, 1049, and 1379, as well as the Institutional Animal Care and Use Committee (IACUC) with protocol numbers 3513, 3758, and 4363.

Preparation and testing of Ab LFA. The LFA for detecting antibodies against the ASFV p54 was developed in collaboration with BioStone Animal Health (Southlake, TX).

Various components were assembled to produce the LFA strips, including an adhesive backing card, a sample pad, a nitrocellulose membrane, a glass-fiber conjugate pad, and a wicking pad. The LFA strip was designed to include both test and control lines. Protein G conjugated with gold nanoparticles was immobilized on the glass-fiber pad, and the ASFV p54 recombinant protein (for test line), alongside undiluted goat serum (for the control line), was applied to the nitrocellulose membrane (GE Healthcare #FF 120 HP, NJ, USA). The glass-fiber conjugate pad and the nitrocellulose membrane were dried at 37°C for one hour before being assembled onto an adhesive backing card with the sample pad and wicking pad. The assembly was cut into strips of 5 mm width using a micro cutter. In LFA, 20 µl of serum were diluted in 80 µl of running buffer (10 mM Tris + 1% Triton X-100), this solution was then applied to a sample pad, and the results were evaluated after 15 minutes (Figure 2.3). A sample was considered positive if both the test and control lines were visible; if only the control line was visible, the sample was deemed negative (Figure 2.4); if the control line was not visible, the test was invalid and had to be repeated.

Results

Test result of the ASF Ab LFA. Pig serum samples from fifty naïve pigs and seventeen ASFV challenged pigs were analyzed, yielding positive results (both duplicates showed positive results), negative results (both duplicates showed negative results), or suspect results (duplicate samples showed conflicting results). As with the LFA, 49 out of 50 naïve pig sera tested negative, indicating a 98% test specificity. All 17 ASFV antibody-positive serum samples, all derived from animals late after infection (14 DPC), were previously confirmed via IFA on ASFV-infected Vero E6 cells. These ASFV antibody-positive pig sera tested positive with the LFA, resulting in a total test sensitivity of 100% (Table 2.2).

Conclusions

There is currently no vaccine or treatment that is safe and effective for non-ASF endemic countries. Consequently, early detection and movement restrictions are crucial for controlling the spread of ASFV. As a diagnostic tool, amplifying genes or detecting antigens of ASFV can be helpful within the first few days of infection; however, they become less effective in later stages of the disease. In contrast, serological tests reliably detect ASFV-specific antibodies within 10 days to years after infection (Gallardo, Nieto et al. 2015). Additionally, the serological approach is widely employed for diagnosing ASF in endemic settings because of its simplicity, cost-effectiveness, efficiency, and precision (Cao, Han et al. 2021). Serological tests such as ELISA, IPT, IFAT, and IBT must be performed in a laboratory (WOAH 2021). Antibody detecting LFA, on the other hand, provide a rapid and field-deployable method of detecting ASFV-specific antibodies, which is especially helpful for identifying chronically infected pigs.

Studies have previously identified various immunogenic proteins of ASFV, including both structural and non-structural proteins(Reis, Parkhouse et al. 2007, Gaudreault, Madden et al. 2020). The p30, p54, and p72 ASFV proteins are the most commonly used ASFV proteins for serological diagnosis among more than 150 proteins (Neilan, Zsak et al. 2004, Tian, Liang et al. 2023). ASFV p54 is a membrane protein that is produced during the initial phases of ASFV infection. It shows strong antigenicity and immunogenicity and is quite conserved (Neilan, Zsak et al. 2004). The ASFV p54-based antibody detection ELISA showed the highest test sensitivity and specificity compared to other immunogenic antigen candidates used in ELISAs (Gallardo, Reis et al. 2009, Huang, Cao et al. 2023, Onyilagha, Quizon et al. 2024). Therefore, p54 is a suitable protein for LFA to detect ASFV antibodies.

This study found that the ASFV p54 Ab LFIA is highly sensitive, specific, and easy to use. It showed reliable sensitivity in detecting anti-ASFV-p54 antibodies in sera from pigs 14 days post-infection with genotype II ASFV isolates. Real-world conditions may differ from the "ideal" conditions under which serum samples were collected from experimentally infected animals. Also, due to limited availability, only a rather small number of ASFV-positive samples were used in this study. Further testing of the p54 LFA with a larger sample pool and field testing is necessary to assess the performance of the ASFV Ab LFA as a pen-side diagnostic test using samples from domestic and wild swine infected with various strains and genotypes of ASFV.

2.3 Rapid Identification of ASFV, CSFV and FMDV from Mongolian Outbreaks with MinION Short Amplicon Sequencing

The data in this chapter have been published as Bold D, Souza-Neto JA, Gombo-Ochir D, Gaudreault NN, Meekins DA, McDowell CD, Zayat B, Richt JA. Rapid Identification of ASFV, CSFV and FMDV from Mongolian Outbreaks with MinION Short Amplicon Sequencing. Pathogens. 2023 Mar 29;12(4):533. doi: 10.3390/pathogens12040533. PMID: 37111419; PMCID: PMC10140976.

Abstract

African swine fever virus (ASFV), classical swine fever virus (CSFV), and foot-and-mouth disease virus (FMDV) cause important transboundary animal diseases (TADs) that have a significant economic impact. The rapid and unequivocal identification of these pathogens and distinction from other animal diseases based on clinical symptoms in the field is difficult.

Nevertheless, early pathogen detection is critical in limiting their spread and impact as is the availability of a reliable, rapid, and cost-effective diagnostic test. The purpose of this study was to evaluate the feasibility to identify ASFV, CSFV, and FMDV in field samples using next generation sequencing of short PCR products as a point-of-care diagnostic. We isolated nucleic acids from tissue samples of animals in Mongolia that were infected with ASFV (2019), CSFV (2015), or FMDV (2018), and performed conventional (RT-) PCR using primers recommended by the Terrestrial Animal Health Code of the World Organization for Animal Health (WOAH). The (RT-) PCR products were then sequenced in Mongolia using the MinION nanopore portable sequencer. The resulting sequencing reads successfully identified the respective pathogens that exhibited 91–100% nucleic acid similarity to the reference strains. Phylogenetic analyses suggest that the Mongolian virus isolates are closely related to other isolates circulating in the same geographic region. Based on our results, sequencing short fragments derived by conventional (RT-) PCR is a reliable approach for rapid point-of-care diagnostics for ASFV, CSFV, and FMDV even in low-resource countries.

Introduction

Animals provide food and other essential resources to most of the world's inhabitants. Therefore, the World Organization for Animal Health (WOAH) and the Food and Agriculture Organization of the United Nations (FAO) have cooperated in the Global Framework for the Progressive Control of Transboundary Animal Diseases (GF-TADs) to mitigate the threat of such diseases to the global food supply, safe trade, and livelihoods (WOAH 2022). TADs are highly infectious and transmissible animal diseases that have the potential to cross borders and bring significantly negative socioeconomic and public health-associated consequences to the affected areas. In this context, identification of the factors contributing to disease pathogenesis and spread, with improved diagnostics and cost-effectiveness of preventive measures is crucial (Clemmons, Alfson et al. 2021). Outbreaks of several TADs including foot-and-mouth disease (FMD) (WRLFMD 2022), classical swine fever (CSF), and African swine fever (ASF), which resulted in significant economic losses, were recently reported in Mongolia (Enkhbold, Shatar et al. 2017, Ulziibat, Maygmarsuren et al. 2020, Ankhanbaatar, Sainnokhoi et al. 2021).

ASF is a highly fatal swine disease and no efficacious and safe vaccine is available. The causative agent of the disease, African swine fever virus (ASFV), is the sole member of the Asfarviridae family, genus Asfivirus. This enveloped virus has a large double-stranded DNA genome ranging in size from 170 to 190 kilobase pairs (kbp). ASFV is classified into 24 genotypes according to the sequence diversity of its major capsid protein p72, and into eight serotypes based on its CD2-like and C-type lectin proteins (Gaudreault, Madden et al. 2020). A highly virulent genotype II ASFV has recently emerged in many parts of Europe, Asia, and the Caribbean (Cwynar, Stojkov et al. 2019, Mazur-Panasiuk, Zmudzki et al. 2019, USDA 2021) since its introduction into Georgia in 2007 (Rowlands, Michaud et al. 2008, Le, Jeong et al. 2019, Vilem, Nurmoja et al. 2020, Ankhanbaatar, Sainnokhoi et al. 2021, Ito, Bosch et al. 2022, Shao, Li et al. 2022, Szymanska and Dziwulaki 2022).

CSF is one of most critical viral TADs affecting swine, caused by the classical swine fever virus (CSFV). CSFV is an enveloped, positive sense, single-stranded RNA virus with a 12.5 kb genome; it is a member of the Pestivirus genus in the Flaviviridae family (Edwards, Fukusho et al. 2000). CSF outbreaks have been reported around the world and recently in Colombia, Brazil, Russia, South Korea, and Japan (Wei, Liu et al. 2021).

FMD (WRLFMD 2022)is a highly contagious viral disease affecting cloven-hoofed animals, caused by the foot-and-mouth disease virus (FMDV). FMDV is a non-enveloped virus with a positive-sense RNA genome that is a member of the Aphthovirus genus in the Picornaviridae family. FMDV comes in seven genetically distinct serotypes (O, A, C, SAT-1, SAT-2, SAT-3, and Asia-1) and multiple topotypes. Despite its discovery in 1897 as the first vertebrate-infecting virus (Mahy 2005) and the availability of vaccines, FMDV outbreaks are still occurring worldwide. The virus is estimated to circulate in approximately three quarters of the global livestock population, mainly in Africa, the Middle East, and Asia, as well as in a limited area of South America (WOAH 2022). Approximately 2.5 billion doses of FMD vaccine are globally deployed each year in endemic countries, primarily in China and South America (Knight-Jones and Rushton 2013).

Standard TAD diagnostics are regulated by WOAH, and multiple specific assays may be available for a single disease, depending on its purpose (Torres-Velez, Havas et al. 2019). Currently, conventional PCR and real-time quantitative PCR (qPCR) are recommended for ASFV molecular detection, while serological tests such as ELISA, indirect immunoperoxidase test (IPT), and immunoblotting test (IBT) are routinely used for ASF diagnosis (WOAH 2021). The diagnosis of FMD is mainly performed by conventional RT-PCR and RT-qPCR, and both methods are officially recommended by WOAH. ELISA-based antigen and antibody detection tests are used to survey exposure to the virus or recovery from viral infection, respectively. For CSF, conventional RT-PCR or ELISA-based antigen and antibody detection tests are recommended for diagnosis (WOAH 2022), even though a number of validated RT-qPCR-based diagnostics are available (Risatti, Callahan et al. 2003, Eberling, Bieker-Stefanelli et al. 2011, Le, Jeong et al. 2019, Liu, Shi et al. 2022).

In most cases, TAD outbreaks occur in rural areas of the respective countries, and samples from suspected animals are collected by inspectors of local veterinary laboratories.

Testing is accomplished using basic diagnostic assays, including (RT-) PCR, ELISA, and point-of-care (POC) tests (Hobbs, Colling et al. 2021). Accurate and rapid diagnosis of TADs is critical during suspected outbreaks to allow for a fast and effective response, which can mitigate pathogen spread (Howson, Armson et al. 2018). Rapid diagnosis may be hindered in many cases by poor reliability of some assays or the lack of laboratory infrastructure in rural veterinary laboratories, which ultimately results in significant delays in pathogen identification (Knight-Jones, Robinson et al. 2016). Therefore, the development of fast, sensitive, specific, and reliable POC diagnostics is pivotal for early detection and mitigation of TAD outbreaks by the appropriate regulatory authorities.

Here, we used the MinION portable sequencer to identify ASFV, CSFV, and FMDV isolates collected during previous outbreaks in Mongolia. Our data show that all three pathogens can be quickly and reliably identified via amplicon sequencing with WOAH validated methods. This methodology is capable of providing additional important information such as genotype/serotype/strain differentiation in a relatively short turnaround time that other molecular or serological diagnostic tests may not offer.

Materials and Methods

Samples and Extraction of Nucleic Acids. The ASFV (Ankhanbaatar, Sainnokhoi et al. 2021), CSFV (Enkhbold, Shatar et al. 2017), and FMDV (WRLFMD 2022) samples were previously collected and identified at the State Central Veterinary Laboratory (SCVL) in Ulaanbaatar, Mongolia using OIE/WOAH-recommended methods; the results were confirmed by OIE/WOAH reference laboratories in the UK and Japan (Table 2.3).

Polymerase Chain Reaction (PCR) and Reverse-Transcription (RT-) PCR. PCR (ASFV) and RT-PCR (CSFV and FMDV) assays were performed at the SCVL in Mongolia, following the Terrestrial Animal Health Code (WOAH 2022) of the World Organization for Animal Health (WOAH; founded as OIE). Three primer sets (Table 2.3) were used to amplify fragments of the ASFV gene encoding VP72, the CSFV IRES, or the FMDV 5′ UTR, respectively, and the amplicons were analyzed using gel electrophoresis (Figure 2.5) (WOAH 2022). ASFV PCR reactions contained sample DNA (ASF samples) and DreamTaq Green PCR Master Mix (ThermoFisher, #K1082, CA, USA), whereas RT-PCR reactions contained sample RNA (either CSF or FMD samples) and SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Invitrogen, #12574035, CA, USA). The reactions were performed according to previously standardized conditions (WOAH 2021). Amplicons were purified with AMPure XP beads (Beckman Coulter, #A63881, IN, USA) according to the manufacturer's instructions prior to MinION library preparation.

Oxford Nanopore MinION Sequencing. Individual single-plex sequencing libraries were prepared with the Oxford Nanopore ligation sequencing kit (SQK-LSK109) for each virus according to the manufacturer's instructions. Briefly, 200 µL of bead-purified amplicons were added to 16 µL of library mix and incubated at 20 °C for 5 min and 65 °C for 5 min. Sequencing adapter ligation was prepared with 60 µL of library mix, 25 µL of Ligation Buffer 25, 10 µL of NEBNext Quick T4 DNA Ligase, and 5 µL of Adapter Mix with a final volume of 100 µL. The sample was gently mixed by flicking the tube, spun down, and then incubated for 10 min at room temperature. Next, the MinION flow cell (model R9.4.1) was primed with the MinION Priming Kit prior to loading the libraries following the manufacturer's instructions. Afterwards, 75 µL of a final mix (consisting of 37.5 µL of sequencing buffer (SQB), 25.5 µL of loading beads (LB), and 12 µL of amplicon library) was loaded dropwise onto the flow cell via the SpotON sample port for sequencing. Sequencing runs were controlled with the MinKNOW software following the instructions provided in the MinKNOW protocol. ASFV and CSFV amplicon libraries were sequenced individually using the same flow cell that was thoroughly washed between samples. About 10% (17,395) of the reads that were generated during CSFV library sequencing were assigned to ASFV likely due to carryover from one run to another, and, therefore, were disregarded from the analysis. The FMDV amplicon library was sequenced in a separate flow cell.

Sequencing Data Processing and Analysis. Sequencing reads were parsed into multiple FASTQ files with the MinKNOW control software. The data were initially analyzed using the cloud-based service EPI2ME (Metrichor). This analysis was performed using FASTQ WIMP (What's in My Pot) workflow, which relies on a method called Centrifuge, a microbial classification engine (EPI2ME workflow) (Nanopore 2017). The sequencing data was also analyzed with CLC Genomic Workbench 20.0 (Qiagen, Carlsbad, CA, USA). For this, MinION sequencing reads were filtered to remove reads with a q-score < 7 and lengths < 200 bp and >400 bp. FASTQs were imported into the CLC software and parsed into individual sample files. The filtered reads were mapped to the respective reference genomes (ASFV: FR682468.2, CSFV: KY290453, FMDV: LC438823) using the CLC Genomics Workbench Map to Reference Module with a 50% length and 80% similarity mapping parameters. The obtained consensus sequences were extracted and compared to the NCBI nucleotide database using the *blastn* tool within the CLC Genomic Workbench for taxonomic identification and the top 100 hits were downloaded for phylogenetic analysis.

Sequence Alignment and Phylogenetic Analysis. The consensuses sequences were aligned with other sequences of the respective pathogen (ASFV, CSFV, or FMDV) using ClustalW and phylogenetic analyses were performed with MEGA 11 (Molecular Evolutionary

Genetic Analysis) (Tamura and Nei 1993, Van Borm, Wang et al. 2016, Tamura, Stecher et al. 2021, McDowell, Bold et al. 2022). Phylogenetic trees were reconstructed with maximum likelihood and 1000 bootstrap replications.

Results

Using WOAH-recommended primer sets (WOAH 2021), we successfully amplified genomic fragments from ASFV p72, CSFV IRES, and FMDV 5′UTR, and further generated Oxford Nanopore MinION amplicon libraries that were individually sequenced. MinION sequencing generated approximately 1.4, 0.16, and 0.15 million reads for ASFV, CSFV, and FMDV libraries, respectively. The amplification and sequencing procedures, performed at the SCVL in Mongolia, were rapid and took as little as 1 h (CSFV and FMDV) and up to 6h (ASFV). Approximately 100%, 93%, and 91% of the total reads from each library were successfully mapped to their respective ASFV, CSFV, and FMDV genomic regions (Figure 2 .5a–c). A small proportion of reads from the CSFV and FMDV libraries (1% and 9%, respectively) matched those of other organisms, likely due to co-infections or environmental contamination at the time of sample collection and/or processing. A more detailed analysis of the sequenced fragments revealed that almost 100% of ASFV- and FMDV-matching reads were assigned to a unique pathogen strain (Figure 2.6 a, c). On the other hand, CSFV-matching reads were assigned primarily to CSFV Alfort/187, CSFV, and Pestivirus C (Figure 2.6 b). About 6% of the reads from the CSFV library were assigned to other pestiviruses (Figure 2.6 b). Considering the much lower taxonomic placement score of these reads compared to those classified as CSFV or Pestivirus C (Table 2.5), these reads either represent false-positive taxonomic assignments or infection of swine with other pestiviruses such as bovine viral diarrhea virus (Tao, Liao et al. 2013, de Oliveira, Mechler-Dreibi et al. 2020). Further analysis of the sequencing data with the CLC Genomics Workbench, where reads were mapped to their respective reference genomes, validated the initial genomic identification of the three transboundary pathogens. Using this pipeline, we obtained a 262 bp sequence with 1.3 × 106-fold coverage for ASFV, a 291 bp sequence with 1.11 × 105-fold coverage for CSFV, and a 330 bp sequence with 1.10 × 105-fold coverage for FMDV. We were, however, unable to differentiate between the three potential CSFV strains and other pestiviruses that were identified through the MinION analysis workflow as described above. Nonetheless, either approach successfully identified the targeted pathogens, demonstrating the suitability of this workflow for rapid and detailed identification of ASFV, CSFV, and FMDV. TADs including ASF, CSF, and FMD have been frequently reported in Mongolia in 2019, 2007–2015, and 2000–2022, respectively. The most predominant TAD in Mongolia is FMD, with serotypes O, A, and Asia-1 being present in livestock including camels (WRLFMD 2022). ASFV and CSFV were also identified and were caused by genotype II and sub-genotype 2.1b, respectively (Enkhbold, Shatar et al. 2017, Ankhanbaatar, Sainnokhoi et al. 2021). In order to determine the genetic proximity of the ASFV, CSFV, and FMDV isolates to those that were previously identified elsewhere, we conducted maximum likelihood (ML) phylogenetic analyses using the short genomic sequences obtained from these pathogens (Figure 2.7). The phylogenetic trees revealed that ASFV, CSFV, and FMDV are closely related with other viruses of the same genotype/serotype circulating in the same geographic area where they were isolated (Figure 2.7). The ASFV sequence (Mongolia, 2019) is part of a major genotype II cluster that is populated mainly by isolates from Eastern Asia, Eastern Europe, and Russia (Figure 2.7 a). The CSFV isolate (Mongolia, 2015) is part of a genotype 2.1 cluster mainly encompassing Eastern Asian CSFV isolates, and more closely associated to those that were identified in South Korea (2011), Mongolia (2014), Sri Lanka

(2011, 2016), and China (2013) (Figure 2.7 b). The FMDV sequence (Mongolia, 2018) is part of a phylogenetically related serotype O cluster represented mainly by Southern and Southeastern Asian, and Middle Eastern FMDV isolates (Figure 2.4 c).

Discussion

Next-generation sequencing (NGS) has become a valuable tool for the diagnosis of TADs and for the spatiotemporal tracking of pathogens (Van Borm, Wang et al. 2016); (RT-) PCR has been most widely used for routine diagnosis in veterinary laboratories. The advantage of using NGS together with (RT-) PCR for TAD diagnostics is that sequencing information may help to promptly identify detailed pathogen features that are not provided by other diagnostic tools. Hence, this work was performed as a proof-of-concept study to investigate the suitability of portable MinION sequencing for point-of-care (POC) diagnosis of ASF, CSF, and FMD in Mongolia, a low-resource country.

Using (RT-) PCR plus NGS on a portable sequencer such as the MinION for the rapid identification of ASFV, CSFV, and FMDV isolates from field samples can help improve POC diagnostics for TADs and guide authorities in the implementation of mitigation strategies. Currently available POC diagnostics typically rely on immunoassays for antigen and antibody detection, which present a number of limitations including cross reactivity, lower sensitivity and specificity (Sammin, Ryan et al. 2010). Sequencing-based POC diagnostics have the advantage of generating valuable information such as the rapid determination of pathogen classification and the identification of genotype/serotype/strain in a short period of time.

Although only a limited number of samples were analyzed as a proof-of-concept, our results demonstrate the suitability of short amplicon sequencing targeting critical genes of important transboundary pathogens on the MinION in a low-resource country as a potential POC diagnostic tool. This methodology is capable of providing pathogen genotype/serotype/strain identification in a fast turnaround time of approximately 8 h (Figure 2.8). Although diagnostic tests based on (RT-) PCR are faster, the data that were obtained from MinION sequencing can be used for assessing phylogeny and for improved diagnostic confidence, which is critical for further decision-making, for example executing rapid implementation of mitigation strategies during TAD outbreaks. The MinION in the current scenario provides almost real-time detection of specific viruses and their genotypes/serotypes and can be applied in the field. It is quite possible to further optimize the bioinformatic tools for real-time phylogenetic analysis in a field setting in the near future.

The limit of short amplicon sequencing is that it does not allow thorough molecular characterization of a particular pathogen compared to whole genome sequencing protocols that could also be employed on the MinION. However, the advantage here is that the short amplicon sequencing provides relatively rapid and reliable information when targeting critical genes/regions of pathogens that (RT-) PCR does not provide. Although the cost of this methodology compared to other POC diagnostic tests is higher, in the face of a TAD outbreak, the cost of the test becomes less of an issue since rapid (within 8 h) and accurate testing are the most critical parameters. Thus, our results show that sequencing short fragments of conventional (RT-) PCR products is a reliable approach for rapid POC diagnostics for ASFV, CSFV, and FMDV in low-resource countries.

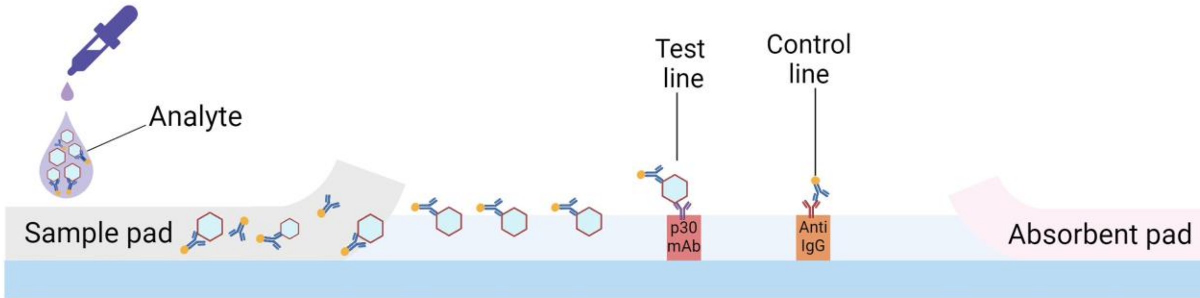

Figure 2.1. Schematic representation of LFA for ASFV antigen detection.

The ASFV sample antigen binds to a gold nanoparticle-conjugated detector ASFV p30 mAb in a test tube, and the analyte moves along the stick after placing it in the sample tube. Results showing as color development at the test (ASFV antigen binds to p30 capture mAbs if it is present in the sample) and control (the rest of the gold nanoparticle-conjugated mAbs bind to anti-mouse IgG antibodies) lines. The appearance of both the control and test lines indicates a positive result, while only the control line shows a negative result. A test is considered invalid if the control line is absent.

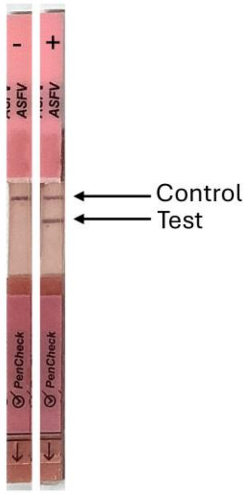

Figure 2.2. Example result of the LFA for ASFV Ag detection.

Each strip contains a control line (upper band) and a test line (lower band). The negative sample (−) displays only the control line, confirming proper analyte migration and assay validity but indicating the absence of detectable ASFV antigen. The positive sample (+) shows both the control and test lines, with the test line indicating successful capture of ASFV antigen by immobilized capture mAbs.

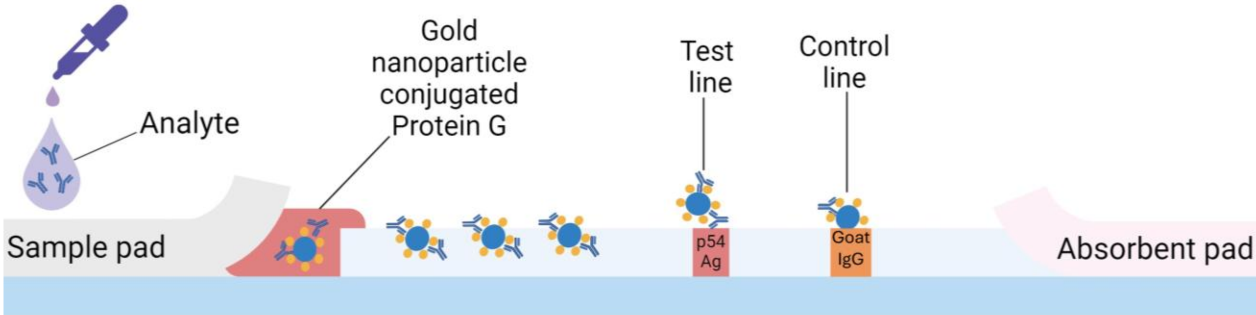

Figure 2.3. Schematic representation of LFA for ASFV antibody detection.

The ASFV p54-specific antibodies in the sample bind to a gold nanoparticle-conjugated protein G when the antibodies pass through the conjugate pad and move along the membrane. Results showing as color development at the test (ASFV p54–specific antibodies bind to p54 capture antigen if the ASFV antibodies are in the sample) and control (the rest of the gold nanoparticle-conjugated protein G bind to goat IgG) lines. The appearance of both the control and test lines indicates a positive result, while only the control line shows a negative result. A test is considered invalid if the control line is absent.

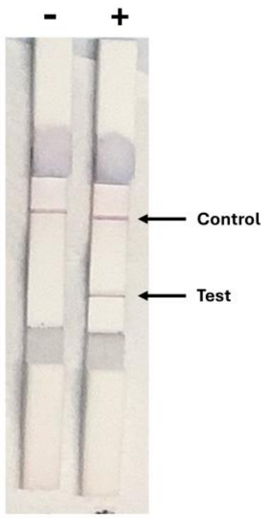

Figure 2.4. Example result of the LFA for ASFV Ab detection.

Each strip contains a control line (the upper band) and a test line (the lower band). The negative sample (−) displays only the control line, confirming proper analyte migration and assay validity but indicating the absence of detectable ASFV p54-specific antibodies. The positive sample (+) shows both the control and test lines, with the test line indicating successful capture of ASFV antibodies by immobilized ASFV p54 antigen.

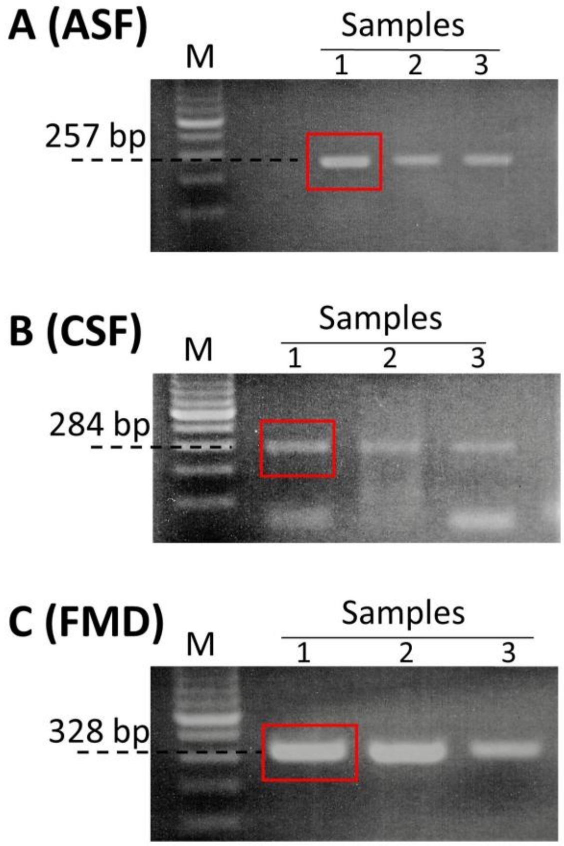

Figure 2.5. Amplified gene products of ASFV, CSFV, and FMDV in agarose gel.
Agarose gel electrophoresis (2% agarose) of (RT)-PCR amplified products using virus-specific primer sets. The amplicons used for the MinION sequencing are in the respective red squares for ASF (A), CSF (B), and FMD (C). Lane M, 100 bp DNA size marker.

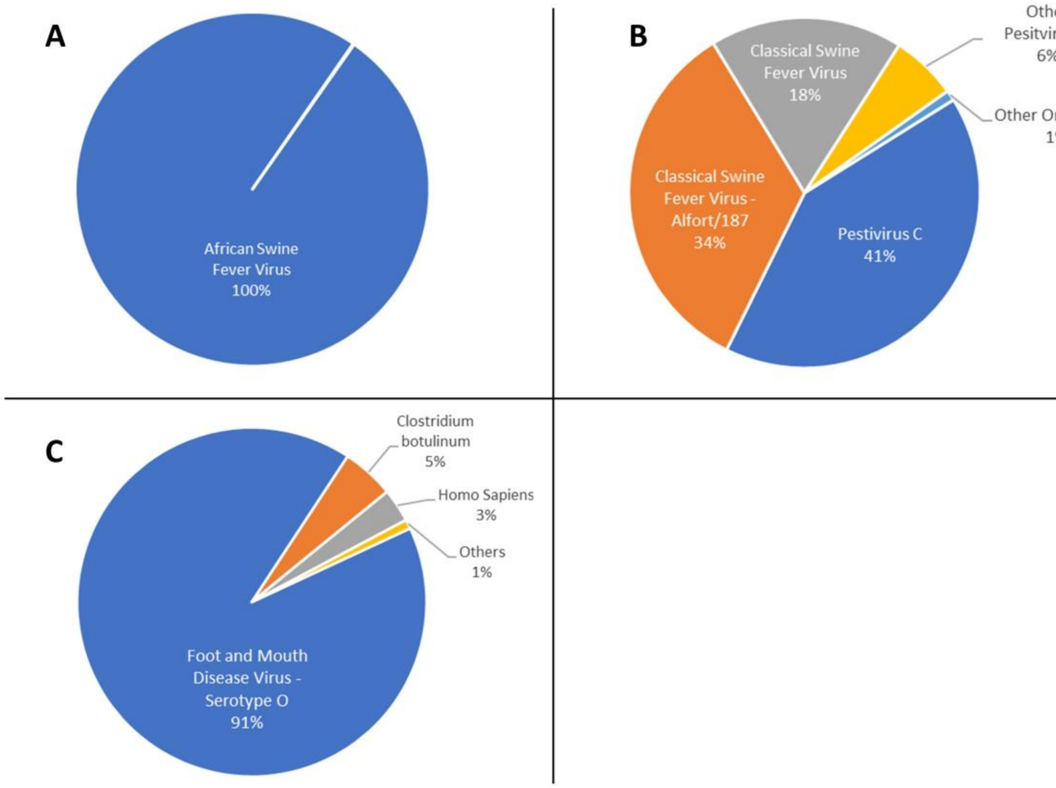

Figure 2.6. Relative distribution of sequence reads for PCR products of the pathogens. Relative distribution of reads in sequence data generated by nanopore sequencing of DNA from (RT-) PCR products of three pathogens: ASFV (A), CSFV (B), and FMDV (C). The pie-charts were created using Microsoft Office Excel 2021.

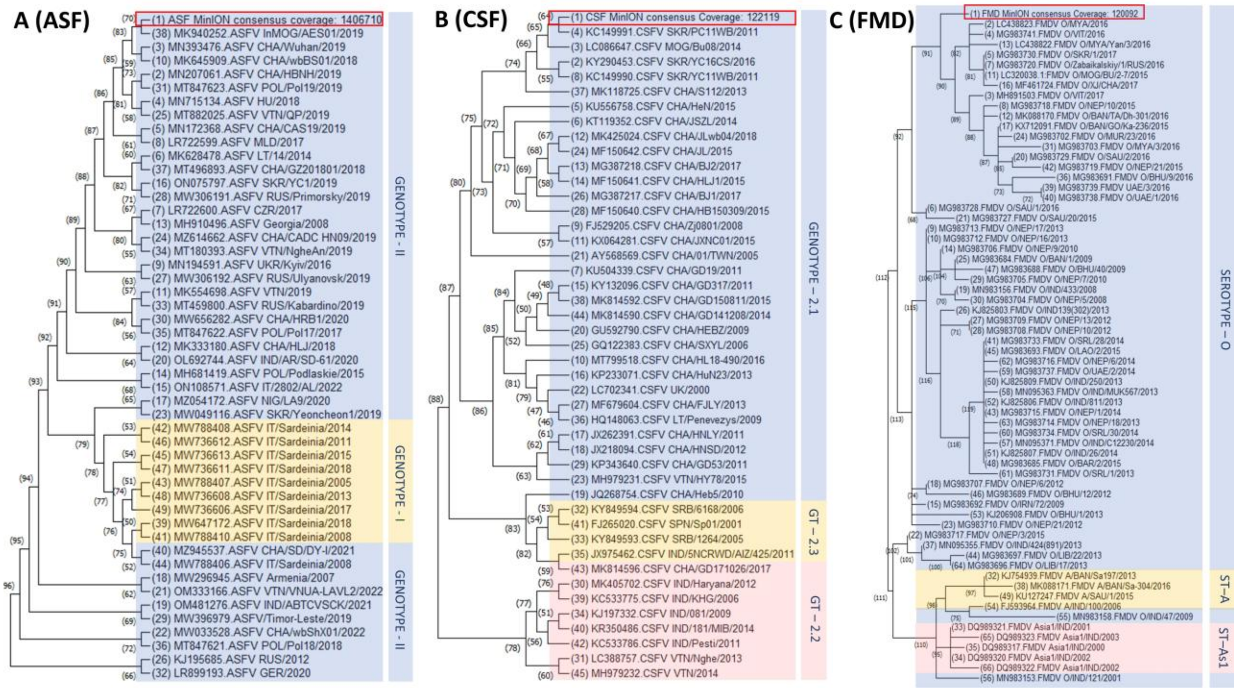

Figure 2.7. Phylogenetic analysis of the pathogens based on sequence data.

Phylogenetic trees of ASFV (a), CSFV (b), and FMDV (c) field isolates based on partial p72 (a), domain II of IRES (internal ribosomal entry site; (b)) and 5′ UTR (c), respectively. The Molecular Evolutionary Genetics Analysis (MEGA) Version 11.0.13 was used to create the phylogenetic trees. Genotype (GT) and serotype (ST) clusters are differentiated in each panel by color: (A) ASFV GT-I (yellow) and GT-II (blue); (B) CSF GT-2.1 (blue), GT-2.2 (pink) and GT-2.3 (yellow); (C) FMD ST-O (blue), ST-A (yellow) and ST-Asia-1 (As1; (pink).

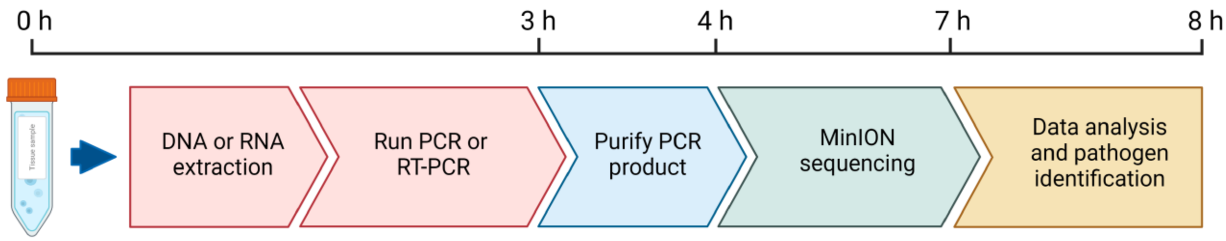

Figure 2.8. Timeline for pathogen identification.

Shown are the steps from tissue sample collection and preparation to sequence data analysis. The tools in biorender.com were used to make the figure.

Table 2.1. Sensitivity of the ASFV Ag LFA in ASFV-infected pig blood samples.

Challenge Strain	LFA			qPCR		
	Pos.	Neg.	Sen.	Pos.	Neg.	Sen.
Post-Arm07 (ASF#6)	29	2	93.5%	31	0	100.0%
CS > 6	10	0	100.0%	10	0	100.0%
Dead	3	0	100.0%	3	0	100.0%
F = 3	13	2	86.7%	15	0	100.0%
F = 4	3	0	100.0%	3	0	100.0%
Post-E70	5	0	100.0%	5	0	100.0%
CS > 6	2	0	100.0%	2	0	100.0%
F = 3	3	0	100.0%	3	0	100.0%

CS =clinical score, F = fever score, Pos. = positive result, Neg. = negative result, Sen. = sensitivity, Arm07 = Genotype II ASFV, E70 = Genotype I ASFV

Table 2.2. Positive serum samples for the ASFV Ab IFA and LFA post ASFV challenge.

Experiment	Animal ID	ASFV strain	LFA #1/#2	ASFV IFA	Samples tested (DPC)
#6	148	Arm07	+/+	>320	14
	146	Arm07	+/+	>1280	14
	13	Arm07	+/+	>320	14
#8	C2056	Arm07	+/+	>320	14
	C2057	Arm07	+/+	>640	14
	C2059	Arm07	+/+	>320	14
	C1866	Arm07	+/+	>320	14
	C7412	Arm07	+/+	>640	14
	C2114	Arm07	+/+	>320	14
	C2117	Arm07	+/+	>320	14
#9	784221	Arm07	+/+	>640	14
	784635	Arm07	+/+	>320	14
	783541	Arm07	+/+	>80	14
	783519	Arm07	+/+	>320	14
	784222	Arm07	+/+	>320	14
	783511	Arm07	+/+	>80	14
#13	296	Arm07	+/+	>160	14

Arm07 = Genotype II ASFV; DPC = days post challenge

Table 2.3. Sample information used for the MinION sequencing.

Pathogen	Virus species	Tissue	Collection date	Sample origin	Animal species
ASFV	Genotype 2	Spleen	January 2019	Bulgan province	Domestic pig
CSFV	Genotype 2.1b	Blood	April 2015	KUD, Ulaanbaatar	Domestic pig
FMDV	Serotype O	Tongue epithelium	January 2019	Dornod province	Cattle

Table 2.4. WOAH-recommended PCR primers for diagnosis of ASF, CSF, and FMD.

	Position	Forward primer	Reverse Primer	Length
ASF	VP73	5’-AGTTATGGGAAACCCGACCC-3’	5’-CCCTGAATCGGAGCATCCT-3’	257 bp
CSF	IRES	5'-ATGCCCACAGTAGGACTAGCA -3’	5'-TCAACTCCATGTGCCATGTAC -3'	284 bp
FMD	VP1	5’-GCCTGGTCTTTCCAGGTCT-3’	5’-CCAGTCCCCTTCTCAGATC-3’	330 bp

Table 2.5. Number of mapped reads with the CSFV sequence analysis.

Row Labels	Count of Reads	Average of score
Pestivirus C	42309	1010.36
Classical swine fever virus - Alfort/187	36259	997.24
Classical swine fever virus	18808	1087.15
Pestivirus	4612	312.34
Aydin-like pestivirus	518	241.08
Bovine viral diarrhea virus 1	242	430.90
Pestivirus giraffe-1 H138	77	336.71
Border disease virus	20	392.25
Bovine viral diarrhea virus 2	7	165.57
Bovine viral diarrhea virus 3 Th/04_KhonKaen	7	179.29
Pronghorn antelope pestivirus	1	169.00

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Chapter 3 - Serological Diagnostics

3.1 MAPIA for detection of ASFV-specific antibodies

Introduction

ASF is one of the most significant viral diseases of swine. The ASF outbreak in the country leads to significant economic consequences. The recent emergence of ASF in Europe, Southeast Asia, and the Caribbean has raised concerns about the potential further spread into North America. Therefore, it is critical to develop robust diagnostic methods for ASF preparedness. ASF is routinely diagnosed using molecular and serological tests. The long persistence of anti-ASFV antibodies in chronically infected animals makes serological assays critical for ASF diagnosis.

For the initial screening of ASFV-specific antibodies, ELISA-based tests are widely utilized, as recommended by the World Organization for Animal Health (WOAH 2024). However, false positive or false negative reactions in ELISA tests are common, especially when serum samples are in poor condition. The ambiguous results must be validated by confirmatory tests to enhance the accuracy of the findings as recommended by WOAH. Confirmatory methods for ASFV specific antibodies, including indirect fluorescent antibody tests (IFAT), immunoblotting tests (IB), and indirect immunoperoxidase tests (IPT) are commonly used. However, they have some limitations regarding their performance, interpretation, and fabrication. These test procedures are time consuming and labor-intensive and must be conducted in high-biocontainment facilities (BSL3) due to the use of infectious ASFV. Moreover, interpreting IB is often challenging due to a significant background signal from the whole ASFV proteome. The Multiantigen Print Immunoassay (MAPIA) is an innovative assay that addresses the limitations of existing confirmatory tests for ASFV-specific antibody detection. MAPIA is a membrane-based diagnostic test that consists of multiple recombinant target antigens immobilized on a transfer membrane, which captures specific antibodies. The MAPIA assay can serve as an acceptable reference test to verify inconclusive results obtained from the initial screening test using ELISAs. MAPIA has several potential advantages, including its reproducibility, high sensitivity and specificity, rapid results, straightforward interpretation, scale-up potential and usefulness in recognizing antibodies against multiple antigens without compromising test performance.

A variety of infectious diseases have been diagnosed using MAPIA, demonstrating numerous advantages over traditional serological methods (Lyashchenko, Singh et al. 2000). After ASFV-infection, antibodies against the virus can be detected as early as 7 days post infection and can persist for years in some pigs (Gallardo, Nieto et al. 2015). The successful development and implementation of the MAPIA test will greatly enhance current diagnostic methods for ASF, particularly in confirming seropositive results from initial screenings with ELISAs.

Materials and methods

Generation of recombinant antigens. The open reading frame (ORF) genomic sequences of KP177R, CP204L, E183L, and B646L were attained from the Georgia 2007/1 ASFV strain (GenBank: FR682468.1); they encode ASFV proteins p22, p30, p54, and p72, respectively.

The four recombinant ASFV proteins, p22, p30, p54, and p72, along with the VP1 protein of Epizootic hemorrhagic disease virus (EHDV) serotype-6 as an internal negative control, were used for the development of the ASFV MAPIA. The recombinant proteins were produced in *Spodoptera frugiperda 9* (Sf9) cells using the baculovirus expression system (p22, p54, and p72) and BL21 DE3 *E.coli* using the pET101 expression system (p30; details were provided in Chapter 2). GENEWIZ (South Plainfield, NJ, USA), a commercial manufacturer, synthesized the ORFs into the pUC57 plasmid. The KP177R, E183L, and B646L genes were amplified using PCR, cloned into the pGEM-T vector, and then subcloned into the pENTR/D-TOPO vector. PCR and Sanger sequencing (GENEWIZ, South Plainfield, NJ) were used to verify the orientation and insertion of the genes in the donor plasmid. The pENTR plasmid constructs were transformed into One Shot TOP10 chemically competent E.coli cells and propagated. Next, purification was performed using the Qiagen Midiprep kit. The constructs were subsequently incubated with Baculo-Direct linearized baculovirus DNA containing the Histidine Tag and LR clonase II to produce the recombinant baculovirus DNAs. The recombinant baculovirus DNAs were transfected into Sf9 cells using Cellfectin II reagent. The plasmid-transfected Sf9 cells were grown in SF900II medium with 100 μM ganciclovir to select recombinant baculoviruses. The success of the recombinant protein expression was assessed by Western blot analysis using infected Sf9 cell lysates, employing an anti-His monoclonal antibody conjugated with horseradish peroxidase (HRP) as well as polyclonal anti-ASFV pig serum. Upon confirmation of protein expression, histidine-tagged recombinant proteins were purified through affinity chromatography using Ni-NTA Superflow resin after cell lysis under native conditions. The cell pellets were resuspended in a buffer that contained 10 mM imidazole, 50 mM sodium phosphate, 500 mM sodium chloride, 10% glycerol, and 400 units of benzonase nuclease per gram pellet at pH 8.0. The suspensions were freeze-thawed twice and sonicated with five short (10 sec) cycles. The cell lysates were centrifuged at 12,000 x g for 20 minutes at 4°C, and protein purification was performed using the supernatants with Ni-NTA resin. The purified recombinant proteins were dialyzed against phosphate-buffered saline (PBS; pH 7.4, 4 mM

EDTA, 150 mM NaCl, 10% glycerol). The purities of the proteins were analyzed using Coomassie blue staining of SDS-PAGE gels and Western blots with ASFV- or HisTag-specific antibodies. Protein concentrations were measured using the Protein A280 application on the Nanodrop at an absorbance of 280 nm. The aliquots of recombinant protein were stored at -80 °C until they were used for MAPIA strip preparation.

Samples from naïve and experimentally ASFV-infected pigs. Included for testing were sera from 89 naïve pigs, aged 8 to 12 weeks, at Kansas State University (KSU), along with an additional 5 false ASFV-positive pig sera (which tested positive with the ASFV p72 iELISA and ASFV IB CISA, Spain but negative with the IFAT assay) from the annual infectious disease surveillance in Manitoba, Canada. To generate ASFV antibody-positive sera, 21 outbred piglets were obtained (two from the National Centre for Foreign Animal Disease [NCFAD] in Canada) and experimentally infected with ASFV at the Biosecurity Research Institute (BRI) at KSU or at the NCFAD Canada with the ASFV strains Armenia 2007 (genotype II) and OURT88/3 (genotype I), respectively. Fourteen days post-infection with ASFV, whole blood samples were collected via jugular venipuncture. The sera were stored at -80°C for later use. All animal experiments and procedures took place in high-containment BSL-3Ag facilities at the BRI of KSU and the NCFAD in Winnipeg, Canada, following protocols approved by the KSU Institutional Biosafety Committee (IBC) under protocol numbers 850, 1049, and 1379, as well as the Institutional Animal Care and Use Committee (IACUC) under protocol numbers 3513, 3758, and 4363, and protocols approved by Canadian Centre for Human and Animal Health (CCSHAH) Animal Care Committee under the animal use document numbers C-02-007 (2002), C-03-001 (2003), C-07-005 (2007), C-08-009 (2008), C-11-003 (2011) and C-15-001 (2015).

MAPIA strip preparation. The antigens were immobilized in a 0.45 μm pore-size nitrocellulose membrane (Cytiva, MA, USA) in the order of: internal positive (porcine serum, SigmaAldrich, MO, USA) and negative (VP1 protein of EHDV-6) controls, along with ASFV recombinant proteins p22, p30, p54, and p72, on MAPIA strips with parallel lanes spaced 10 mm apart, using the BioDot ZX 1010 dispensing system (BioDot CA, USA). The recombinant proteins, at a concentration of 150 μg/ml, as well as the negative internal control, were printed on nitrocellulose membranes at a speed of 50 mm/s, using a volume of 0.5 μl/mm. The pig serum, serving as an internal positive control (porcine serum, SigmaAldrich, MO, USA), was diluted in PBS at a ratio of 1:1000 and printed using the same parameters as the recombinant proteins. Afterward, the printed nitrocellulose membrane was dried at 37°C for one hour and then cut into strips that are 90 mm long and 3 mm wide.

MAPIA testing of pig serum samples. The in-house developed MAPIA strips for ASFV p22, p30, p54, and p72-specific antibody detection were used in the test, and each sample was tested in duplicate. The MAPIA testing started by blocking the strips with 1 ml of casein blocking buffer (Sigma Aldrich, MO, US) in mini-incubation trays (Bio-Rad, CA, US) on a rocking shaker for 1 h at RT. Next, the strips were incubated for 1 h at RT in 800 μl of blocking buffer containing pig serum diluted 1:400 while shaking. Following this, the strips were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and then incubated with 800 μl per strip of goat anti-pig IgG antibodies conjugated with peroxidase (Abcam, MA, USA), diluted 1:5,000 in casein blocking buffer, for 30 minutes on a rocking shaker at RT. Afterward, the strips were washed three more times as previously described and then incubated for five minutes on a rocking shaker with 400 μl of TMB liquid substrate (Sigma Aldrich, MO, USA). Then, strips were washed immediately with 800 µl of distilled water three times and assessed visually for the development of blue lines (Figure 3.1).

Test result interpretation. The test was evaluated visually by detecting blue lines on nitrocellulose membrane strips. Each strip has a head marker line that helps identify its top end and determine the order of the antigens. A sample was considered positive if at least three of the four ASFV antigens (p22, p30, p54, and p72) were detected in duplicate analytes. The strips that showed two visual ASFV antigens were regarded as suspicious, and sample tests with one or no visual bands were considered a negative test result. If the sample tested as suspicious, the test was repeated with a fresh sample. A test would be considered invalid when the positive control line was absent or when the negative control line was present.

Results

A total of 113 naïve pig sera, including 89 negative pig sera (Table 3.1) and five false positives (Table 3.2), along with 19 ASFV antibody-positive pig sera (Table 3.3), were used to evaluate the ASFV MAPIA. All ASFV antibody-positive serum samples had been previously confirmed via IFAT on ASFV-infected Vero E6 cells. All sera from naïve piglets were previously tested with the ASFV p30 iELISA. All 94 naïve pig sera tested negative with ASFV MAPIA, indicating 100% specificity of the test. However, we observed weak reactions with individual ASFV antigens, including two samples with p22, two with p30, one with p54, and two with p72, when tested with naïve pig sera (Table 3.1). Importantly, none of these samples showed reactivity with more than one ASFV antigen. Additionally, among the five false-positive pig sera (positive with the ASFV p72 iELISA) obtained from NCFAD Canada, all tested negative in the ASFV MAPIA (Table 3.2); however, one sample (serum #49) showed a reaction with the ASFV p72 antigen (Figure 3.2).

Out of 19 ASFV positive samples, two (783541, 783511) did not show a reaction with the p22 antigen, and one (783519) did not show a reaction with the p72 antigen (Table 3.3). Overall, the 19 ASFV samples showed a reaction with at least three antigens on the ASFV MAPIA strips (indicating positivity), resulting in a test sensitivity of 100% in this evaluation.

Discussion

Several confirmatory assays, including IFAT, IPT, and IB, are commonly used to validate serological tests for ASFV (WOAH 2024). However, these assays have certain limitations. In order to conduct confirmatory assays, it is necessary to work with live ASFV, which requires BSL-3 biocontainment conditions. These laboratory facilities are rarely available in low-resource areas. Also, the IB assay for serological confirmation has significant issues with false positive background staining, leading to ambiguous results, as happened with the false-positive pig sera from NCFAD, Canada (Figure 3.2).

ASFV MAPIA has the potential to overcome the limitations of current serological confirmatory assays for ASF, as it can be produced and handled under BSL-2 conditions. The ASFV MAPIA was evaluated using well-characterized ASFV antibody-positive and negative pig serum panels from the USA and Canada. To establish the ASF MAPIA, we expressed and purified the highly immunogenic ASFV structural proteins, p22, p30, p54, and p72, using baculovirus and *E.coli* expression systems. The selected ASFV structural proteins, p22, p30, p54, and p72, are also the major antigens used for ASFV antibody detection in ELISAs (Tesfagaber, Wang et al. 2021, Li, Zhang et al. 2022, Huang, Cao et al. 2023, Li, Jiao et al. 2023).

All naïve pig sera tested negative in the ASF MAPIA, indicating a test specificity of 100%. However, some naïve pig sera samples showed a reaction with a single ASFV antigen: two samples with p22, two samples with p30, one sample with p54, and another sample with p72

(Table 3.1). Therefore, this result shows that including and reacting with multiple antigens in the ASFV MAPIA test is essential to accurately determine if a serum is positive or negative, making it more specific than ELISAs that typically use only a single ASFV antigen. Additionally, five pig sera from Manitoba, Canada were included, which tested positive for ASFV p72 by iELISA. These false positive pig sera originated from industrial pig farms and had no history of movement or risk of ASFV infections. We tested these five pig sera with the ASFV MAPIA; all were negative, but one sample (pig number 49) showed a reaction with the p72 antigen on the ASFV MAPIA strip (Figure 3.2, Table 3.2). This finding confirms that this serum cross-reacts with the p72 antigen in the iELISA, the ASFV IB test and the ASF MAPIA. Importantly, the data presented here shows that the ASFV MAPIA test can be used as a serological confirmatory test in cases of inconclusive results.

The ASFV MAPIA demonstrated very high sensitivity, as all 19 ASFV- sera tested positive, indicating an overall test sensitivity of 100%. However, two samples did not exhibit reactions with the ASFV p22 antigen, and similarly one sample did not display a reaction for the p72 antigen. This suggests that the p22 and p72 ASFV antigens used in ELISAs for ASFV antibody detection may result in less sensitive tests than ELISAs utilizing the p30 and p54 ASFV antigens.

The present study demonstrates that the ASFV MAPIA seems to be more specific and sensitive than the ASFV IB and ASFV p72 iELISA. It can be used as a highly sensitive, specific and cost-effective confirmatory tool for detecting ASFV-specific antibodies in the serum of pigs, and has significant potential to support the serological diagnosis of ASF.

3.2. MAPIA for the detection of FMDV nonstructural protein-specific antibodies

Introduction

The causative agent of Foot-and-mouth disease (FMD) is the Foot-and-mouth disease virus (FMDV), an *Aphthovirus* in the *Picornaviridae* family. It is a highly contagious disease affecting cloven-hoofed animals, resulting in significant economic consequences. The virus has seven serotypes: O, A, C, Asia, SAT1, SAT2, and SAT3. There is no cross-protection among these serotypes. The viral genome encodes 12 proteins, including structural (1A-D) and non-structural (L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) proteins. The structural capsid proteins (1A-D) are cleavage products of a precursor protein (P1-2A) and are the antigenically critical components of FMDV. They are produced in infected animals and are the basis for FMDV vaccines. The non-structural proteins (NSPs) are only produced during active FMDV replication in animals, and commercial FMD vaccines do not contain NSPs (Clavijo, Wright et al. 2004).

Diagnosis of FMD involves observing clinical signs and confirming the diagnosis through laboratory testing of samples. Diagnosis of FMDV is based on the detection of whole virus, virus antigens, or viral genetic materials in clinical samples. The use of RT-qPCR, which employs FMDV-specific primers and probes, is routinely used in the early stages of the disease. However, serological tests are especially useful in late stages of the disease or for determining herd immunity. The use of serological assays, specifically virus neutralization assays and ELISAs, which detect antibodies against FMDV structural proteins and are specific for respective virus serotypes, is essential for determining the FMDV infection/vaccination status of a herd or a country. In contrast, antibodies against FMDV NSP proteins are serotype independent and they recognize all seven FMDV serotypes. Since they are only produced in animals with active FMDV replication and not in vaccinated animals, they are used to differentiate infected from vaccinated animals (DIVA).

Detecting antibodies against FMDV NSPs is essential for estimating the prevalence of FMD in endemic regions or demonstrating FMDV-free status in a country (IAEA 2007). DIVA tests are currently conducted using FMDV-specific NSP-based antibody detection tests. According to the OIE diagnostic manual, DIVA testing for FMD can be performed by ELISA using recombinant FMDV 3ABC NSPs. While various ELISAs utilizing different recombinant FMDV NSPs (e.g., 3AB, 3ABC) are commercially available, they have been successfully used for the initial screening of FMD prevalence. However, while the sensitivity and specificity of the commercially available tests are rather high, still some inconclusive results are obtained, since false positives and negatives are detected. Therefore, WOAH recommends using the Enzyme-linked immunoelectrotransfer blot (EITB) assay as a confirmatory test to follow up on inconclusive results from initial screening tests (Bergmann, Malirat et al. 1996, WOAH 2022). The EITB for FMD is mainly used in South America to follow up on inconclusive FMDV NSP antibody detection results. Several limitations exist with the EITB assay, including difficulty in interpretation, a lack of reproducibility, and the requirement that the assay be performed or used in BSL-3 biocontainment facilities. Detecting FMD NSP antibodies using MAPIA may represent an alternative to EITB. Therefore, we hypothesized that the FMDV MAPIA could be a robust confirmatory tool for detecting FMDV NSP-specific antibodies. Consequently, we established an FMDV MAPIA using the NSP proteins 2C, 3A, 3B, 3D, and 3ABC and evaluated the assay with a well-characterized serum panel of positive and negative samples.

Materials and methods

Recombinant protein generation. The FMDV RNA, that codes for nonstructural proteins (NSPs), including 2C, 3A, 3B, 3D, and 3ABC, was derived from the FMDV strain O1/Campos/Brazil/58 (GenBank: AJ320488.1). DNAs were amplified using SuperScript One-Step RT-PCR (ThermoFisher, CA, USA) with specific primers targeting the complete gene coding sequences for the NSPs 3ABC, 3A, and 3B, which include *Hind*III and *BamH*I restriction sites in the reverse and forward primers, respectively. The primers for 3D and 2C contained *EcoR*I and *Not*I restriction sites. The amplified DNAs were separately inserted into the pET30c cloning vector, which included a 6xHis tag (Clavijo, Hole et al. 2006). The plasmid DNAs 3A, 3B, 3D, 3ABC, and 2C were transformed into One Shot™ TOP10 Chemically Competent E. coli (ThermoFisher, CA, USA) using the method of heat-shock. After purifying plasmid DNAs with a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), the positive clones were screened using PCR, followed by restriction enzyme digestions and Sanger sequencing. Purified plasmid DNA was subsequently transformed into BL21 (DE3) *E.coli* cell lines (Invitrogen, Carlsbad, CA, USA). To express the recombinant proteins, transformed BL21 *E. coli* cells were grown in LB buffer (Sigma-Aldrich, MO, USA) containing 100 µg/ml ampicillin (Sigma-Aldrich, MO, USA) and induced by adding 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, MO, USA) until the optical density (O.D.) reached 0.7. After 3 h IPTG induction at 37 °C, cells were harvested by centrifugation, and the cell pellets were lysed with BugBuster reagent (25 U/ml; Novagen, Darmstadt, Germany) to extract the recombinant 6xHis-NSP. Benzonase nuclease (Novagen) was added, and the suspension was incubated at RT with gentle shaking for 20 min. Insoluble cellular debris was pelleted after centrifugation at 14,000 × g for 20 minutes, and the pellets were collected. The pellets were resuspended in 30 ml of 8 M urea, 0.1 M sodium phosphate buffer, and 0.01 M Tris-HCl, pH 8.0 per gram until the solution became transparent. Then, the solubilized supernatants containing NSPs were collected through additional centrifugation at 12,000 x g for 30 minutes at 4°C, as previously described (Clavijo, Zhou et al. 2004). Afterward, recombinant 2C, 3A, 3B, 3D, and 3ABC were purified from the supernatant using gel electrophoresis. The expression and purity of the recombinant proteins were evaluated using SDS-PAGE) with a MOPS SDS running buffer (Invitrogen) on NuPage 12% bis-tris gels (Invitrogen), followed by Coomassie blue staining with an eStain L1 Protein Staining System (GenScript, Piscataway, NJ, USA). Subsequently, the recombinant protein was confirmed through western blot analysis using an anti-His (C-term) HRP monoclonal antibody (Invitrogen).

Samples from naïve and experimentally FMDV-infected animals. Serum collected from 30 naïve pigs, aged 8 to 12 weeks, and 20 naïve calves aged 6 to 7 months at NCFAD Canada, along with an additional 6 false positive cattle sera (which tested positive with the FMDV Ab 3ABC cELISA) from the annual disease surveillance in Manitoba, Canada were used for MAPIA testing. FMDV NSP antibody-positive sera were obtained from 20 outbred piglets aged 8 to 12 weeks and three calves aged 6 to 8 months. These piglets and calves were experimentally infected with the FMDV strains O/UK/11/2001 and A24/Cruzerio, respectively, at the NCFAD in Canada. Whole blood samples were collected via jugular venipuncture 14-28 days post-infection with FMDV. The sera were then isolated and stored at -80°C until needed. All animal experiments and procedures took place in a high-containment BSL-3Ag setting at the NCFAD in Winnipeg, Canada, following protocols approved by the Canadian Centre for Human and Animal Health (CCSHAH) Animal Care Committee under the following animal use document numbers C-02-007 (2002), C-03-001 (2003), C-07-005 (2007), C-08-009 (2008), C-11-003 (2011) and C-15-001 (2015).

MAPIA strip preparation. The antigens were immobilized in a 0.45 µm pore-size nitrocellulose membrane (Cytiva, MA, USA) in the order of internal positive (Porcine and Bovine serum, SigmaAldrich, MO, USA) and negative (ASFV p30) controls, along with FMDV recombinant NSP proteins 2C, 3A, 3B, 3D, and 3ABC on MAPIA strips in parallel lanes spaced 10 mm apart, using the BioDot ZX 1010 dispensing system (BioDot CA, USA). Recombinant proteins, including the negative internal control, were printed at a concentration of 150 µg/ml in phosphate-buffered saline (PBS) on nitrocellulose membranes in 20 cm lines at a speed of 50 mm/second, using a volume of 0.5 µl/mm. The porcine and bovine serum mix (1:1), serving as an internal positive control (Porcine and bovine serum, SigmaAldrich, MO, USA), was diluted in PBS at a ratio of 1:1000 and printed using the same parameters as the proteins. Afterward, the printed nitrocellulose membrane was dried at 37°C for one hour and then cut into strips that were 90 mm long and 3 mm wide.

MAPIA testing of pig serum samples. The in-house developed MAPIA strips for detecting antibodies specific to FMDV NSPs 2C, 3A, 3B, 3D, and 3ABC were used for this study. The MAPIA testing started by blocking the strips with 1 ml of casein blocking buffer (Sigma Aldrich, MO, US) in mini-incubation trays (Bio-Rad, CA, US) on a rocking shaker for 1 h at RT. Next, the strips were incubated with pig or cattle serum diluted in blocking buffer at a 1:200 ratio for 1 h at RT while shaking. Following this, the strips were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and then incubated with 800 µl per strip of goat anti-pig IgG or goat anti-cow IgG antibodies conjugated with peroxidase (Abcam, MA, USA), diluted 1:5000 in casein blocking buffer, for 30 minutes on a rocking shaker at RT. Afterward, the strips were washed three more times as previously and then incubated for five minutes on a rocking shaker with 400 µl of TMB liquid substrate for membranes (Sigma Aldrich, MO, USA). Then, strips were washed immediately with 800 µl of distilled water three times and assessed visually; blue lines were presented (Figure 3.3).

Test result interpretation. The test was interpreted by visually detecting blue lines on nitrocellulose membrane strips. Each strip features a head marker line that helps identify the top and determines the order of the antigens. A sample was considered positive if at least three out of five FMDV NSP Ag (2C, 3A, 3B, 3D, and 3ABC) bands were present. The strips that show two visual FMDV NSP Ag lines are regarded as inconclusive, and one or no visual bands is considered a negative test result. The test should be repeated with additional serological testing if the sample was inconclusive. A test is considered invalid if the positive control line is absent or the negative control is positive.

Results

A total of 79 serum samples were used for FMDV MAPIA validation, including 30 FMDV NSP antibody-negative pig sera, 20 cattle sera (Table 3.4), six naïve cattle sera (Table 3.5) that tested positive for FMDV NSP 3ABC cELISA, and sera from 20 experimentally infected, NSP 3ABC cELISA-positive pigs and three cattle (Table 3.3). All FMDV NSP antibody-positive serum samples had been previously confirmed via FMDV 3ABC cELISA. All 50 sera from naïve 30 pigs and 20 cattle tested negative with FMDV MAPIA. However, two samples displayed reactions with FMDV NSP antigens, including samples p-12 and c-3 with antigen lines 2C and 3D, respectively (Table 3.4). None of those samples displayed more than one FMDV NSP antigen test line.

Additionally, six false-positive cattle sera from Manitoba, Canada, which were part of the annual infectious disease surveillance, all tested negative for FMDV MAPIA. However, two samples, c-62 and c-65, showed reactivity with FMDV 3A and 3D (Figure 3.4, Table 3.5).

Overall, all naïve pig and cattle sera tested negative with FMDV MAPIA, indicating 100% specificity of the test.

All 23 post-challenge samples, 20 pigs and 3 cattle, tested positive with FMDV MAPIA, resulting in a test sensitivity of 100% (Table 3.6). Five NSP antibody-positive samples did not react with all NSP antigens on the FMDV MAPIA: two sera (p1 and p9) were negative with 2C; one serum (p16) was negative with 3A; and two sera (p12 and p15) were negative with 3D. However, all positive sera showed reactivity with at least four antigens on the FMDV MAPIA strips and, therefore, were considered positive (Table 3.6).

Discussion

Several ELISA formats have been developed for FMDV NSP proteins, including indirect, competitive, and blocking ELISAs, along with various antigen options such as 3D, 3B, 3AB, and 3ABC. However, the 3ABC cELISA is the preferred assay system used worldwide to detect FMDV NSP-specific antibodies as an initial screening tool for DIVA purposes in countries using FMDV vaccines (De Diego, Brocchi et al. 1997, Mackay, Forsyth et al. 1998, Sorensen, Madsen et al. 1998, Bergmann, Malirat et al. 2000, Yang, Parida et al. 2015). The sensitivity of the NSP ELISAs ranges from 84.6% to 98.3% for cattle sera and 73.3% to 80.0% for pig sera. The specificity ranges from 99.0% to 100.0% (Seeyo, Choonnasard et al. 2024). This suggests that the NSP ELISAs may potentially lead to false negative and false positive results, and that inconclusive test results need to be followed up with an FMDV NSP-antibody confirmatory test, which may reduce the number of false results. The FMDV NSP MAPIA offers five different NSP antigens, and most sera from experimentally infected pigs and cattle reacted with all five antigens, except five pig sera, which didn’t react with individual NSP antigens (Table 3.6).

According to our FMDV NSP MAPIA criteria, they are still considered positive. Several sera from naïve pig and cattle as well as from the false NSP antibody-positive cattle showed reactions with a single NSP antigen on the FMDV MAPIA (Table 3.4; Table 3.5). According to our FMDV NSP MAPIA criteria, they are considered negative. Therefore, the overall test sensitivity and specificity of the FMDV MAPIA were both considered 100%.

The results obtained with the newly developed FMDV MAPIA indicate that this assay has significant potential for use as a confirmatory test for detecting FMDV NSP-specific antibodies. The assay has high specificity and sensitivity with the limited number of samples tested. Importantly, production, scale up and interpretation of results are considerably easier than for the EIBT test recommended by WOAH as the confirmatory test for FMDV NSP-specific antibody detection.

Furthermore, the FMDV MAPIA could also be a cost-effective diagnostic tool for detecting FMDV NSP-specific antibodies in pig and cattle sera, holding significant potential as an alternative test system to support the serological diagnosis of FMDV NSP-specific antibodies, thereby supporting DIVA diagnostics and enhancing epidemiological studies in FMDV-endemic countries.

3.3. Development of an iELISA for the Detection of SARS-CoV-2 Antibodies in Cats

The data in this chapter have been published as Bold D, Roman-Sosa G, Gaudreault NN, Zayat B, Pogranichniy RM, Richt JA. Development of an Indirect ELISA for the Detection of SARS-CoV-2 Antibodies in Cats. Frontiers Vet Sci. 2022 Jun 10;9:864884. doi: 10.3389/fvets.2022.864884. PMID: 35754530; PMCID: PMC9226769.

Abstract

Companion animals are susceptible to a variety of coronaviruses, and recent studies show that felines are highly susceptible to SARS-CoV-2 infection. RT-PCR diagnostic is currently the method of choice to detect the presence of SARS-CoV-2-specific viral nucleic acids in animal samples during an active infection; however, serological assays are critical to determine whether animals were exposed to the virus and to determine the seroprevalence of SARS-CoV-2-specific antibodies in a defined population. In this study, we utilized recombinant nucleocapsid (N) protein and the receptor-binding domain (RBD) of the spike protein of SARS-CoV-2 expressed in E. coli (N) and mammalian cells (N, RBD) to develop indirect ELISA (iELISA) tests using well-characterized SARS-CoV-2-positive and -negative cat serum panels from previous experimental cat challenge studies. The optimal conditions for the iELISA tests were established based on checkerboard dilutions of antigens and antibodies. The diagnostic sensitivity for the detection of feline antibodies specific for the N or RBD proteins of the iELISA tests was between 93.3 and 97.8%, respectively, and the diagnostic specificity 95.5%. The iELISAs developed here can be used for high-throughput screening of cat sera for both antigens. The presence of SARS-CoV-2-specific antibodies in a BSL-2 biocontainment environment, unlike virus neutralization tests with live virus which have to be performed in BSL-3 laboratories.

Introduction

The ongoing pandemic of coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a member of the Coronaviridae family in the betacoronavirus genus (Coronaviridae Study Group of the International Committee on Taxonomy of 2020). SARS-CoV-2 is an enveloped, single-stranded, positive-sense RNA virus with a large genome size of ~30 kilobases (kb). The family of Coronaviridae is divided into four genera: alphacoronavirus, betacoronavirus, deltacoronavirus, and gammacoronavirus (Coronaviridae Study Group of the International Committee on Taxonomy of 2020). Companion animals are susceptible to a variety of coronaviruses: felines can be infected by feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV or referred to here as FeCoV) and canines by canine coronavirus (CCoV) (Tennant, Gaskell et al. 1991, Hoskins 1993), with all these viruses belonging to the alphacoronavirus genus (Haake, Cook et al. 2020). Coronaviruses encode four structural proteins: three are membrane associated (the spike, envelope, and membrane proteins) and one, the nucleocapsid (N) protein, is associated with the viral RNA (Payne 2017). The spike (S) protein is the major glycoprotein that extends from the surface of the virion forming corona-like spikes (Payne 2017). The receptor-binding domain (RBD) of the S protein interacts with the cellular receptor angiotensin-converting enzyme II (ACE2) and, therefore, plays a critical role in virus attachment and entry into host cells (Payne 2017). The RBD is highly immunogenic and the major target of SARS-CoV-2 neutralizing antibodies (Premkumar, Segovia-Chumbez et al. 2020). The N protein packages genomic RNA into the ribonucleoprotein (RNP) complex of the virus; it interacts with the other viral structural proteins, is needed for virus assembly (Payne 2017), and N-specific antibodies can be detected as early as 8 days post-infection (Burbelo, Riedo et al. 2020).

SARS-CoV-2 was first reported in December 2019 in the city of Wuhan in China (Junejo, Ozaslan et al. 2020, Liu, Gayle et al. 2020) and since then spread quickly all over the world. The World Health Organization (WHO) officially declared SARS-CoV-2 a global pandemic on March 11, 2020 (Cucinotta and Vanelli 2020). According to the WHO, over 446 million human cases and 6 million deaths (as of March 8, 2022) have officially been reported thus far. Although the case fatality rate of SARS-CoV-2 is about approximately 2%, which is lower than for other human sarbecoviruses such as SARS-CoV and Middle East respiratory syndrome (MERS)-CoV, its global spread is causing massive numbers of human cases and deaths and significant economic losses (Liu, Gayle et al. 2020).

An accurate diagnosis for SARS-CoV-2 is essential to rapidly quarantine RNA/virus-positive people and to reduce potential virus transmission to naïve individuals (Cheng, Papenburg et al. 2020). RT-PCR is currently utilized as the method to diagnose COVID-19 as recommended by the WHO and CDC (WHO 2020, Zhu, Zhang et al. 2020). It is a highly sensitive and specific method, but inadequate sample collection and technical errors in RNA preparation may produce false-negative results, and cross-contamination can lead to false-positive results. However, shedding of virus or viral RNA is only transient and the RT-PCR test will only be positive during a certain window of time (Sethuraman, Jeremiah et al. 2020).

Serological assays are able to identify SARS-CoV-2-specific antibodies in clinical samples (such as plasma, serum, and saliva). SARS-CoV-2-specific antibodies can be detected using various methods, such as ELISA, virus neutralization assays, and lateral flow tests. The earliest detection of SARS-CoV-2-specific IgM or IgA isotype antibodies in humans is ~5 days post-infection, whereas IgG isotypes are found later around 10–14 days post-infection (Guo, Ren et al. 2020).

Serological assays can be used to investigate ongoing or retrospective assessments of COVID-19 outbreaks. Sero-surveillance can also be used to study seroconversion after infection or vaccination to determine herd immunity. As discussed above, serological assays should not be used as the method of choice to diagnose COVID-19 but in combination with other assays and methods (Meyer, Drosten et al. 2014).

Although serological assays are not “fit for purpose” for the diagnosis of SARS-CoV-2 during or early after infection, they are critical to determine the effectiveness of vaccine administrations and herd immunity of populations. They can also be a useful tool in combination with RT-PCR when applied, for example, at least 1 week after the onset of symptoms (Liu, Liu et al. 2020). A combination of a COVID-19 serological test with RT-PCR could improve the diagnostic sensitivity of COVID-19 diagnosis significantly (Guo, Ren et al. 2020, Wang 2020).

Recently, we and others have demonstrated that domestic cats are susceptible to SARS-CoV-2 by experimental infection and can readily transmit the virus to naïve cats (Bosco-Lauth, Hartwig et al. 2020, Gaudreault, Trujillo et al. 2020, Halfmann, Hatta et al. 2020). Cats inoculated via natural routes are readily infected and shed RNA/virus from nasal, oral, and rectal cavities starting from 1 up to 14 days, with peak RNA/virus shedding occurring within the first 7 days after infection (Bosco-Lauth, Hartwig et al. 2020, Gaudreault, Trujillo et al. 2020, Halfmann, Hatta et al. 2020, Shi, Wen et al. 2020). Experimentally infected cats also develop virus-specific and neutralizing antibody responses to SARS-CoV-2 (Bosco-Lauth, Hartwig et al. 2020, Gaudreault, Trujillo et al. 2020, Halfmann, Hatta et al. 2020, Shi, Wen et al. 2020, Zhang, Zhang et al. 2020, Fritz, Rosolen et al. 2021, Stevanovic, Vilibic-Cavlek et al. 2021). The aim of the present study was to develop an indirect ELISA (iELISA) test to detect SARS-CoV-2 N- and RBD specific antibodies in felines and analyze the cross-reactivity of recombinant SARS-CoV-2

N and RBD antigens with serum from cats positive for antibodies against feline infectious peritonitis coronavirus (FeCoV).

Materials and Methods

Recombinant N and RBD Protein Expression in Mammalian Cells. Nucleotide sequences encoding the nucleocapsid (N) protein and the receptor-binding domain (RBD) of the spike (S) protein of the SARS-CoV-2 isolate Wuhan-Hu-1, GenBank accession # MW036243 and # MT380725, respectively, were used for the establishment of the respective plasmids employed for protein expression. The N and RBD genes, plus two strep tags, were cloned into the mammalian expression vector pCAGGS; and the plasmids were purified using the Qiagen Plasmid Midi Kit (Qiagen, Germantown, MD, USA) (Table 3.7). The N and RBD recombinant proteins were produced in HEK (human embryo kidney) 293 cells after transfection of these cells with pCAGGS plasmid DNA. Transfected HEK-293 cells were cultured in Dulbecco's modified essential medium (DMEM; Fisher Scientific, Chicago, IL, USA). Supernatants from transfected cells were harvested on day 3 post-transfection by collection and centrifugation of the supernatant at 4,000g for 20 min. Recombinant proteins with strep tags were purified via affinity chromatography using Strep-Tactin® (IBA Lifesciences) after lysis of cells under native conditions. Recombinant proteins were dialyzed in a dialysis cassette against phosphate-buffered saline (PBS; pH 7.4, 150 mM NaCl, 4 mM EDTA, 10% glycerol; Dialysis Cassette—Thermo Fisher, Rockford, IL, USA). Each protein was concentrated in Pierce protein concentrators (Fisher Scientific, Rockford, IL, USA) and re-suspended in phosphate-buffered saline (PBS). The amino acid sequences of the N- and C-termini of both proteins are listed in Table 3.7.

Recombinant N Protein Expression in *E. coli*. The plasmid DNA encoding the N protein of SARS-CoV-2 (see below and Table 3.7) was amplified via PCR employing the N-containing pCAGGS plasmid using Phusion High-Fidelity PCR Master Mix Kit (Thermo Fisher Scientific, Rockford, IL, USA). To insert the N gene into the pETite N-His SUMO Vector (Lucigen, Middleton, WI, USA), flanking sequences identical to the vector sequence adjoining the insertion sites were added to the 5′-end of the primers as follows: forward primer 5′-CGCGAACAGATTGGAGGTTCCGATAACGGCCC-3′ and reverse primer 5′-GTGGCGGCCGCTCTATTAGGCCTGTGT AG-3′ (Integrated DNA Technologies IDT, IA, USA); the gene was then amplified by PCR. Briefly, the PCR mixture included 1 μL of the plasmid DNA, 3 μL of betaine solution (Sigma–Aldrich, St. Louis, MO, USA), primers at a final concentration of 0.2 μM each, and ddH2O in a total volume of 20 μL. PCR cycling conditions were as follows: initial denaturation step at 98°C for 30 s, 28 cycles with 30 s of denaturation at 98°C, 30 s annealing at 59°C, and 50 s extension at 72°C, followed by a final extension for 10 min at 72°C. Following the amplification of the N ORF by PCR, 1 μL of amplicon (~45 ng) and 2 μL of pETite N-His SUMO vector mix were transformed into chemical competent Top10 cells (Lucigen, Middleton, WI, USA). Colonies were screened for the presence of SARS-CoV-2 N-specific DNA by PCR, and positive colonies were selected and amplified; DNA was isolated using a plasmid isolation kit (Qiagen, Germantown, MD, Cat #: 27106).

The plasmid DNA was transformed into BL21(DE3) bacterial cells (Lucigen, Middleton, WI, USA), and PCR-positive clones were grown in LB buffer with 50 μg/mL of kanamycin (GoldBio, St. Louis, MO, USA). Protein expression was induced with 1 mM IPTG (GoldBio, St. Louis, MO, USA) when cell density reached an OD of 0.8, and then the cells were incubated for 3 h at 37°C on a bacterial shaker. Afterward, the IPTG-induced bacteria were harvested by centrifugation at 6,500 × g for 10 mins. The bacterial cells were lysed by BugBuster protein extraction reagent (Millipore Sigma, Burlington, MA, USA), and soluble protein was isolated by centrifugation of the lysate at 13,000 × g for 20 mins. Protein purification was performed using nickel resin, but the protein yield was low. Therefore, the insoluble recombinant protein was solubilized with serial dialysis of 1.0 M to 0.25 M NaCl in PBS.

SDS–PAGE. Recombinant proteins were analyzed by SDS–PAGE analysis to determine recombinant protein size and integrity. The recombinant protein (15 μg) was mixed with Tris–glycine SDS sample buffer (Thermo Fisher, Rockford, IL, USA), heated at 70°C for 10 min, and then loaded onto a NuPAGE protein gel (Thermo Fisher, Rockford, IL, USA). Staining and de-staining of the gel were performed with eStain L1C protein staining system (GenScript, Piscataway, NJ, USA).

Indirect ELISA. Plate wells were coated with 100 ng of the respective protein in 100 μL per well coating buffer (carbonate–bicarbonate buffer, catalog number C3041, Sigma–Aldrich, St. Louis, MO, USA), then covered, and incubated overnight at 4°C. The next day, the plates were washed two times with phosphate-buffered saline [PBS (pH = 7.2–7.6); catalog number P4417, Sigma–Aldrich], blocked with 200 μL per well casein blocking buffer (Sigma–Aldrich, catalog number B6429), and incubated for 1 h at RT. The plates were then washed three times with PBS Tween-20 (PBS-T; 0.5% Tween-20 in PBS). Serum samples were pre-diluted 1:400 in casein blocking buffer; then, 100 μL per well was added to the ELISA plate and incubated for 1 h at RT. The wells were washed three times with PBS-T, and then, 100 μL of HRP-labeled goat anti-feline IgG (H + L) secondary antibody (Thermo Fisher, catalog number A18757), diluted 1:2,500, was added to each well and incubated for 1 h at RT.

After 1 h incubation at RT, plates were washed five times with PBS-T, and 100 μL of TMB ELISA Substrate Solution (Abcam, catalog number ab171525, Cambridge, MA, USA) was added to all wells of the plate. Following incubation at RT for 5 min, the reaction was stopped by adding 100 μL Stop Solution for TMB Substrate (Abcam, catalog number ab171529) to all wells. The OD of the ELISA plates was read at 450 nm on an ELx808 BioTek plate reader (BioTek, Winooski, VT, USA). FeCoV-negative and SARS-CoV-2-negative cat sera were used as negative controls in the iELISAs to determine the cutoff value for negative cat sera. The average OD 450 nm (OD450) value of the negative control cat sera plus 3 × the standard deviation (SD) was used to calculate cutoff values for each assay. Everything above this cutoff was considered positive (Classen, Morningstar et al. 1987).

Indirect Immunofluorescence Tests for SARS-CoV-2 and FeCoV. A commercial immunofluorescence assay (IFA) for detection of antibodies to FeCoV was used in this study (VMRD, #SLD-IFA-FIP2) following the manufacturer's instructions. For SARS-CoV-2 IFA, Vero E6 cells were plated on 96-well tissue culture plates and infected by adding 200 TCID50/well of SARS-CoV-2 virus. The cells were incubated at 37°C and 5% CO2 for 48 h, then fixed by adding 100 μL of 80% acetone to each well, and incubated for 10 min at room temperature RT. Afterward, each well was washed with 100 μL of PBS one time, and plates were dried for 15 min in a BSC. The serum samples were diluted in PBS in two-fold serial dilutions from 1:40 to 1:5,120 in a 96-well plate and incubated for 1 h at 37°C. The monoclonal antibodies were tested using only one dilution of 1:200. FITC-labeled secondary antibodies, anti-cat IgG or goat anti-mouse IgG H&L (Jackson Immuno Research Laboratories, Inc., PA, USA; and Abcam Inc., Cambridge, MA, USA), respectively were added into each well of the plate. After incubation of the plate for 30 mins at 37°C, the plate was washed three times with PBS and evaluated by microscopy.

Virus Neutralization Test. SARS-CoV-2 and FeCoV neutralizing antibodies in sera were determined using microneutralization assays as previously described (Gaudreault, Trujillo et al. 2020). Briefly, heat-inactivated serum samples were subjected to two-fold serial dilutions starting at 1:20 and tested in duplicate. Then, 100 TCID50 of SARS-CoV-2 or FeCoV virus in 100 μL DMEM culture media was added 1:1 to 100 μL of the sera dilutions and incubated for 1 h at 37°C. The mixture was subsequently cultured on Vero E6 or Vero-TMPRSS2 cells (SARS-CoV-2) or Crandell Feline Kidney cells (FeCoV) in 96-well plates. The neutralizing antibody titer was recorded as the highest serum dilution at which at least 50% of wells showed virus neutralization based on the appearance of CPE observed under a microscope at 48–72 h post-infection.

Serum Panel and Monoclonal Antibodies. Known SARS-CoV-2-positive cat sera used in this study were obtained during experimental SARS-CoV-2 challenge studies of cats (Gaudreault, Trujillo et al. 2020, Gaudreault, Carossino et al. 2021); sera positivity was confirmed using a classical virus neutralization (VN) test employing Vero E6 cells as described above. The SARS-CoV-2-infected cats were antibody profile defined/specific pathogen-free (APD/SPF) animals with no detectable antibody titers to feline herpesvirus (rhinotracheitis), feline calicivirus, feline panleukopenia virus, feline coronaviruses, feline immunodeficiency virus, Chlamydia felis, and Toxoplasma gondii (obtained from Marshall BioResources, North Rose, NY, USA). A total of 45 SARS-CoV-2-positive and 22 SARS-CoV-2-negative cat sera and 13 FeCoV-positive and 6 FeCoV-negative cat sera were utilized in this study (Table 3.8). FeCoV-positive and negative cat sera were provided by the Veterinary Diagnostic Laboratory (VDL) at KSU. The FeCoV-positive serum samples used in this study were collected before 2019, and therefore, cats from which the samples were derived were not exposed to SARS-CoV-2 virus. In addition, four in-house developed monoclonal antibodies (mAbs) specific for the RBD of the SARS-CoV-2 spike protein (Balaraman, Drolet et al. 2021, Balaraman, Drolet et al.

2021) and a FeCoV N protein-specific mAb (Thermo Fisher, #MA182189, IL, USA) were employed for the tests in this study (Tables 3.12 and 3.13). For in-house utilized developed SARS-CoV-2 mAbs, 7-week-old Balb/c mice were subcutaneously inoculated with 50 µg of recombinant RBD protein and Freund's incomplete adjuvant and boosted subcutaneously 14 days later. Antibody titers against the RBD antigen were tested within 1 week after the first booster. The mice with the highest antibody titers were selected for additional subcutaneous boosters at 21 days, and an intraperitoneal injection at 28 days. The mice were euthanized 3 days after the last booster, and hybridomas were generated following the fusion of the mouse splenocytes and Sp2/0-Ag14 myeloma cells (ATCC #CRL-1581, Rockville, MD, USA). Screening of desired hybridomas was performed using the recombinant RBD protein using an indirect ELISA, and specificity was confirmed by IFA on SARS-CoV-2-infected cells.

Results

Establishment of Indirect ELISA (iELISA) Tests. To determine the optimal amounts of antigen and antibody concentrations in the iELISAs, checkerboard titrations were performed using a range of concentrations (25–400 ng) of the recombinant proteins and two-fold dilutions (starting at 1:25) of a known SARS-CoV-2-positive and negative cat serum [cat serum #026 obtained at−1 day post-challenge (DPC) and at 21 DPC of an experimental SARS-CoV-2 challenge study] (Gaudreault, Trujillo et al. 2020). We found that coating the wells with 100 ng antigen and using a serum dilution of 1:400 was the optimal combination for the three recombinant antigens tested, based on the best ratio of positive vs. negative serum OD values. The optimal dilution of HRP-labeled secondary anti-cat antibody was estimated with two-fold serial dilutions starting with 1:1,250 up to 1:10,000; the best ratio of OD values for positive and negative cat serum was at a 1:2,500 dilution of the secondary antibody for the three recombinant antigens.

FeCoV-negative and SARS-CoV-2-negative cat sera were used as negative controls in the iELISAs to determine the cutoff value for negative cat sera. For the bacteria-expressed N protein (NE), the highest OD450 with the negative cat sera was 0.251 and the lowest OD450 was 0.195. The cutoff value of the E. coli-expressed N protein iELISA test was determined to be OD450 0.31; therefore, samples with an OD450 value below the cutoff of 0.31 were considered negative. For the mammalian-expressed N protein (NM), the highest OD450 for negative control sera was 0.198 and the lowest OD450 0.108, resulting in a cutoff value of 0.26. The highest and lowest OD450 of the negative cat sera for the mammalian-expressed RBD protein was 0.3 and 0.069, respectively. This resulted in the cutoff value of 0.36 for the RBD iELISA.

Determination of Diagnostic Sensitivity and Specificity of the iELISA Tests. A total of 45 SARS-CoV-2-positive and 22 SARS-CoV-2-negative cat sera were used to evaluate the diagnostic sensitivity and specificity of the iELISAs employing the three different SARS-CoV-2 antigens, namely, the N protein expressed in E. coli and the RBD and N proteins expressed in a mammalian cell expression system. The iELISA using the recombinant bacteria-expressed N protein and well-characterized cat sera from experimentally SARS-CoV-2-infected cats revealed that 42 out of 45 SARS-CoV-2-positive sera were positive and 21 out of 22 SARS-CoV-2-negative sera were negative. This indicates a diagnostic sensitivity and specificity of the N-specific iELISA of 93.3 and 95.5%, respectively (Table 3.9; Figure 3.5).

The iELISA using the recombinant mammalian-expressed N protein and well-characterized cat sera from experimentally SARS-CoV-2 virus-infected cats revealed that 21 out of 22 SARS-CoV-2-negative sera were negative and 44 out of 45 SARS-CoV-2-positive sera were positive. This indicates a diagnostic sensitivity and specificity for the mammalian cell-expressed N-specific iELISA of 97.8 and 95.5%, respectively (Table 3.10; Figure 3.6).

The iELISA using the recombinant mammalian-expressed RBD protein and well-characterized cat sera from experimentally SARS-CoV-2 virus-infected cats revealed that 21 out of 22 SARS-CoV-2-negative sera were negative and 43 out of 45 SARS-CoV-2-positive sera tested positive. Therefore, the RBD-specific iELISA has an estimated diagnostic sensitivity and specificity of 95.6 and 95.5%, respectively (Table 3.11; Figure 3.7).

Two sera that were considered negative based on the VNT tested positive on the N or RBD iELISAs. Specifically, one negative serum (#026, 5 DPC) tested positive on both N iELISAs, and another negative serum (#328, 5 DPC) tested positive with the RBD iELISA. These sera were collected from experimentally infected cats at 5 DPC; therefore, it is possible that these animals started to seroconvert at 5 DPC. The high sensitivity of the iELISAs likely allowed for earlier detection of virus-specific antibodies in these samples compared with the VNT reference test.

It is noteworthy to mention that there is a clear correlation between the RBD ELISA titers and the neutralization antibody titers as shown by the linear regression analysis in Figure 3.8. This analysis indicates that RBD iELISA titers correlate well with virus-neutralizing antibody titers (Figure 3.8A), whereas the N iELISAs show has no correlation (Figure 3.8B) with virus neutralization antibodies.

Cross-Reactivity Between SARS-CoV-2 and Feline Coronaviruses. FeCoV-positive and FeCoV-negative cat sera were analyzed to investigate the cross-reactivity of feline coronavirus-specific antibodies with SARS-CoV-2 antigens. A total of 19 cat sera (Table 3.8) representing 13 FeCoV-positive and 6 FeCoV-negative sera were tested using the three iELISAs coated with recombinant SARS-CoV-2 RBD and N proteins. The FeCoV-positive sera were clearly positive with the FeCoV-specific IFA test (titers >1:3,200), whereas the FeCoV-negative sera were negative in this test (Table 3.12). Nine out of 13 FeCoV-positive cat sera were also positive with at least one of the two N-specific iELISAs, and the N-iELISA-positive sera were also positive on SARS-CoV-2-infected cells (titers ranging from 1:80 to >1:2,400; Table 3.12). Importantly, these FeCoV-specific cross-reacting antibodies (SARS-CoV-2 N- and FeCoV-positive) did not react with the SARS-CoV-2 RBD region of the spike protein in the RBD-specific iELISA, and were also negative in a classical virus neutralization test with SARS-CoV-2. All FeCoV-negative serum samples were negative with the SARS-CoV-2 N and RBD antigens or in the SARS-CoV-2 IFA (Table 3.12; Figure 3.9).

Additional investigations into the cross-reactivity between FeCoV and SARS-CoV-2 were performed using: (i) a FeCoV N protein-specific monoclonal antibody (mAb), (ii) SARS-CoV-2 RBD-specific mAbs, and (iii) high titer SARS-CoV-2-positive cat sera. The FeCoV N-specific mAb was negative when used on SARS-CoV-2-infected cells by IFA and also negative in the virus neutralization test with SARS-CoV-2 (Table 3.12; Figure 3.9). The high-titer SARS-CoV-2 cat sera from the SARS-CoV-2 challenge study (Gaudreault, Trujillo et al. 2020) and monoclonal antibodies against the RBD region of the SARS-CoV-2 spike protein were tested using the commercial FeCoV-specific IFA and by virus neutralization tests using infectious FeCoV. All four high-titer cat sera reacted positive in the FeCoV IFA, but negative in the virus neutralization test with FeCoV. In addition, the SARS-CoV-2 RBD-specific mAbs tested negative with the FeCoV IFA and in the virus neutralization test with FeCoV (Table 3.13). Both, the SARS-CoV-2-positive cat sera and the SARS-CoV-2 mAbs were positive in the SARS-CoV-2 IFA and the virus neutralization test (Table 3.13; Figure 3.9).

Discussion

The detection of SARS-CoV-2 virus RNA by RT-PCR is routinely used worldwide for the diagnosis of COVID-19 (WHO 2020). However, reliable high-throughput serological assays to detect SARS-CoV-2-specific antibodies are needed to determine immune responses of different animal species to SARS-CoV-2 antigens (Gaudreault, Trujillo et al. 2020); in addition, immune responses to vaccination or the presence of maternal antibodies in respective samples can also be measured (Ju, Zhang et al. 2020, Muruato, Fontes-Garfias et al. 2020, Shi, Wen et al. 2020). Serological tests indicate exposure to the virus or viral vaccine antigens (Coronaviridae Study Group of the International Committee on Taxonomy of 2020, Zhu, Zhang et al. 2020). Serological assays can facilitate prevalence studies or retrospective studies by detecting virus-specific antibodies in serum or other feasible samples.

The SARS-CoV-2 nucleocapsid (N) protein can be used as a target antigen due to the fact that it is an abundant, genetically highly conserved, and immunodominant protein of coronaviruses (Burbelo, Riedo et al. 2020). In humans, N protein-specific antibodies can be detected as early as 8–14 days after infection (Seo, Wang et al. 1997, Burbelo, Riedo et al. 2020) which is earlier than spike (S) protein-specific human antibodies (Burbelo, Riedo et al. 2020). Similarly, experimentally SARS-CoV-2-infected cats develop N-specific antibodies 5–7 days after virus challenge, but RBD-specific antibodies were detected only approximately 14 days after infection (Gaudreault, Trujillo et al. 2020).

The S protein of SARS-CoV-2 is the major target for neutralizing antibodies which are critical for protective immunity against SARS-CoV-2 infections. The S1 subunit contains the RBD which is known to lack cross-reactivity between SARS-CoV-2 and SARS-CoV (Anderson, Tan et al. 2020, Ju, Zhang et al. 2020, Lv, Wu et al. 2020, Wrapp, Wang et al. 2020, Song, He et al. 2021, Stevanovic, Vilibic-Cavlek et al. 2021). Consequently, in the present study, we generated SARS-CoV-2-specific recombinant RBD and N proteins and used these proteins to develop indirect ELISA tests to detect antibodies in serum samples from cats experimentally infected with SARS-CoV-2. In-house SARS-CoV-2 ELISA tests for analysis of human sera were developed by different research groups, with sensitivity and specificity between 73.7 and 99.3% and 91.7 and 100%, respectively (Rikhtegaran Tehrani, Saadat et al. 2020). Sensitivities of our in-house ELISA tests using the RBD (expressed in mammalian cells), NE (expressed in E. coli), and NM (expressed in mammalian cells) antigens were 95.6, 93.3, and 97.8% and the specificities were 95.5, 95.5, and 95.5%, respectively. The NM antigen-coated ELISA was more sensitive compared with the RBD iELISA in our study; these data correlate with results obtained by Burelo et al. (Burbelo, Riedo et al. 2020). Interestingly, both N antigen iELISAs, one based on the recombinant N protein expressed in E. coli and the other in mammalian cells, had similar sensitivities and specificities when tested with positive and negative cat sera (Tables 3, 4). This indicates that both prokaryotic and eukaryotic expression systems can be used to produce recombinant N protein as antigens for a SARS-CoV-2 ELISA. Our study also indicates that the NM and NE protein-based iELISAs is more sensitive than the RBD protein-based iELISA to detect SARS-CoV-2-specific IgG antibodies in cat serum. However, some FeCoV-positive cat sera cross-react with the SARS-CoV-2 N-based iELISAs as shown in this study (Table 6). Other studies also identified cross-reactivity between the N protein of SARS-CoV-2 and the N protein of other human betacoronaviruses (Macnaughton, Madge et al. 1981, Agnihothram, Gopal et al. 2014, Gao, Zhou et al. 2015, Trivedi, Miao et al. 2019), but none of these studies investigated cross-reactivity between the SARS-CoV-2 N protein and alphacoronavirus-specific antibodies as done here. In addition, SARS-CoV-2-positive cat sera (infected with a Wuhan-like virus) reacted with FeCoV-infected cells using an IFA-based commercial test system (Table 3.13). However, there was no cross-reactivity of FeCoV-positive cat sera with the SARS-CoV-2 RBD antigen (Table 3.13), and vice versa of SARS-CoV-2 RBD-specific mAb with FeCoV-infected cells in the IFA test (Table 3.12). Importantly, high titer SARS-CoV-2-specific cat sera did not neutralize FeCoV (Table 3.13), nor did FeCoV-positive cat sera neutralize SARS-CoV-2 (Table 3.12). Since FeCoV-positive sera reacted with SARS-CoV-2 N, but not RBD antigen (see Table 3.12), and SARS-CoV-2-specific hyperimmune sera, but not RBD-specific monoclonal antibodies cross-reacted with FeCoV (see Table 3.13), we conclude that the cross-activity is most likely based on the N and/or other SARS-CoV-2 antigens, but not on the SARS-CoV-2 RBD antigen.

In summary, iELISA tests based on recombinant N and RBD proteins of SARS-CoV-2 were developed and optimal conditions for the iELISAs were established. The diagnostic sensitivity for the detection of feline antibodies specific for the SARS-CoV-2 N or RBD proteins of the iELISA tests was between 93.3 and 97.8% vs. 95.6%, respectively, and the diagnostic specificity was 95% for all three tests. In addition, we found a clear correlation between SARS-CoV-2 RBD-specific antibody and virus-neutralizing antibody titers. In conclusion, the iELISAs described here can be used for high-throughput screening of cat sera for the presence of SARS-CoV-2-specific antibodies in a BSL-2 biocontainment environment, as opposed to virus neutralization tests with live virus which require a BSL-3 laboratory.

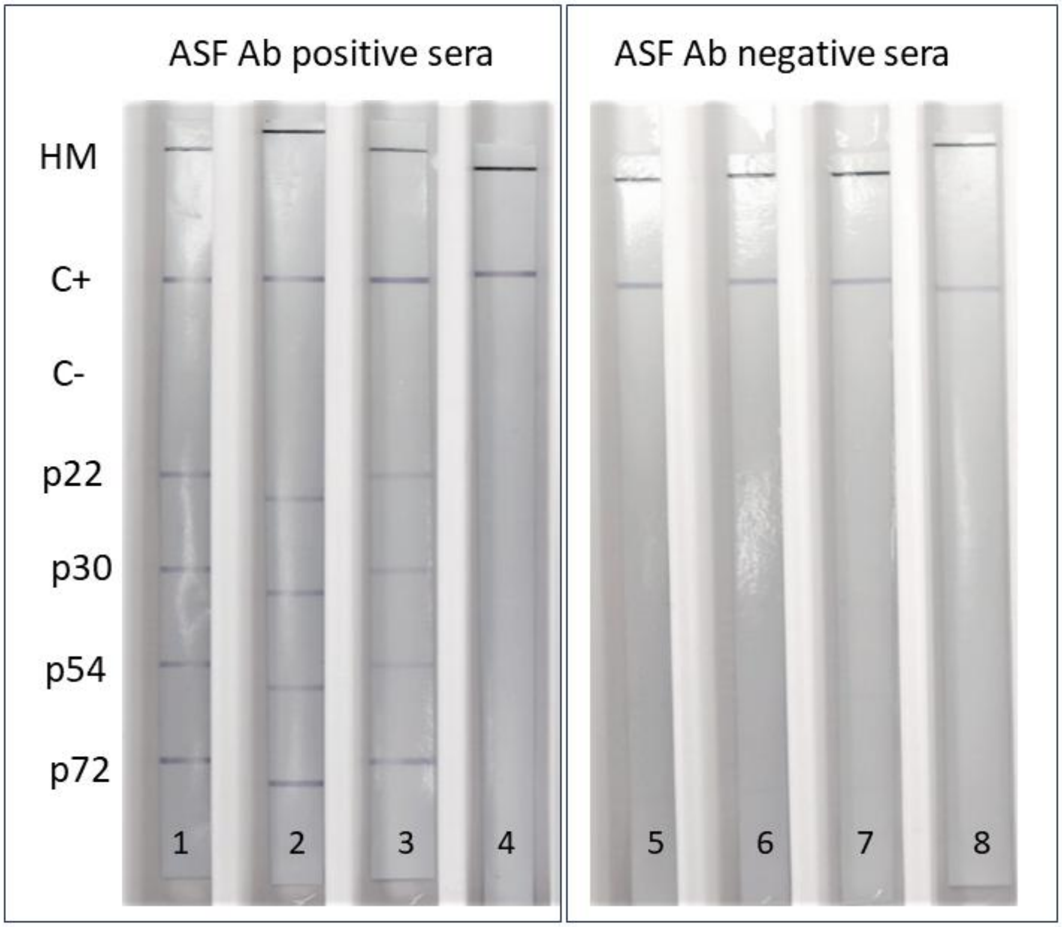

Figure 3.1 ASF MAPIA evaluation with positive and negative serum.
Each strip has a head marker (HM) to indicate the top of the strip, and an internal positive (C+, pig serum, 1:1000 dilution) and negative controls (C-, EHDV-6 VP1 recombinant protein, 150 µg/ml). ASFV antigens on the strip are p22, p30, p54, and p72, in the order from top to bottom. The samples were used for the validation: 1 – ASFV antibody positive pig sera (OURT88/3, DPI 14), 2 - Positive control of ASFV IB (ImmunoBlot, CISA, Spain) Kit, 3 – Weak positive (ImmunoBlot, CISA, Spain) Kit, 4 – Buffer Control (PBS), 5 – Naïve piglet serum, 6 - Commercial pig serum (SigmaAldrich, CA, USA), 7 - Negative control of ASF IB (ImmunoBlot, CISA, Spain) Kit, 8 – Sample diluent (Casein blocking buffer).

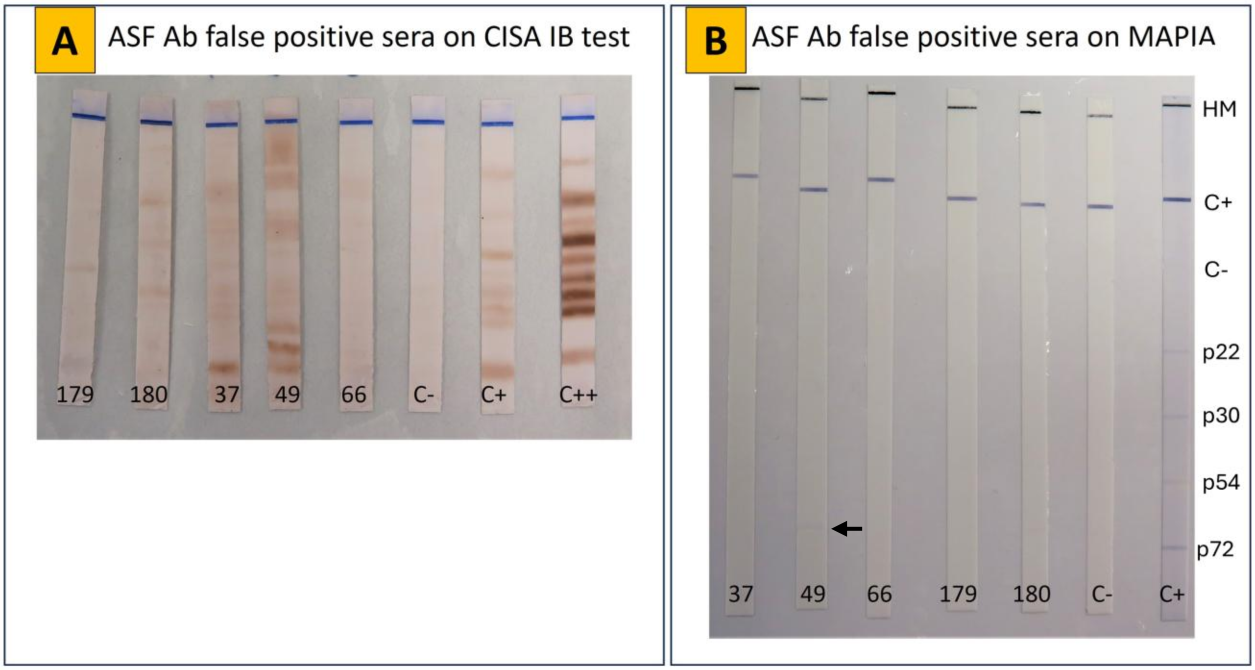

Figure 3.2 ASF MAPIA evaluation with false-positive pig sera.

Five pig sera obtained from annual surveillance tested positive with the p72 iELISA (37, 49, 66, 179, and 180) and controls (C- negative, C+ weak positive, and C++ strong positive control sera from the CISA ASFV IB test). The pig sera 37 and 49 presented an inconclusive staining pattern on the IB strips (Fig. 3.2A). The false positive pig sera and control (negative and weak positive) sera of the CISA ASFV IB test were used in the ASFV MAPIA test (Fig.3.2B), and only pig serum 49 presented a weak test line with ASFV p72 antigen (black arrow). Serum dilutions in the assays were 1:400 for both IB and MAPIA.

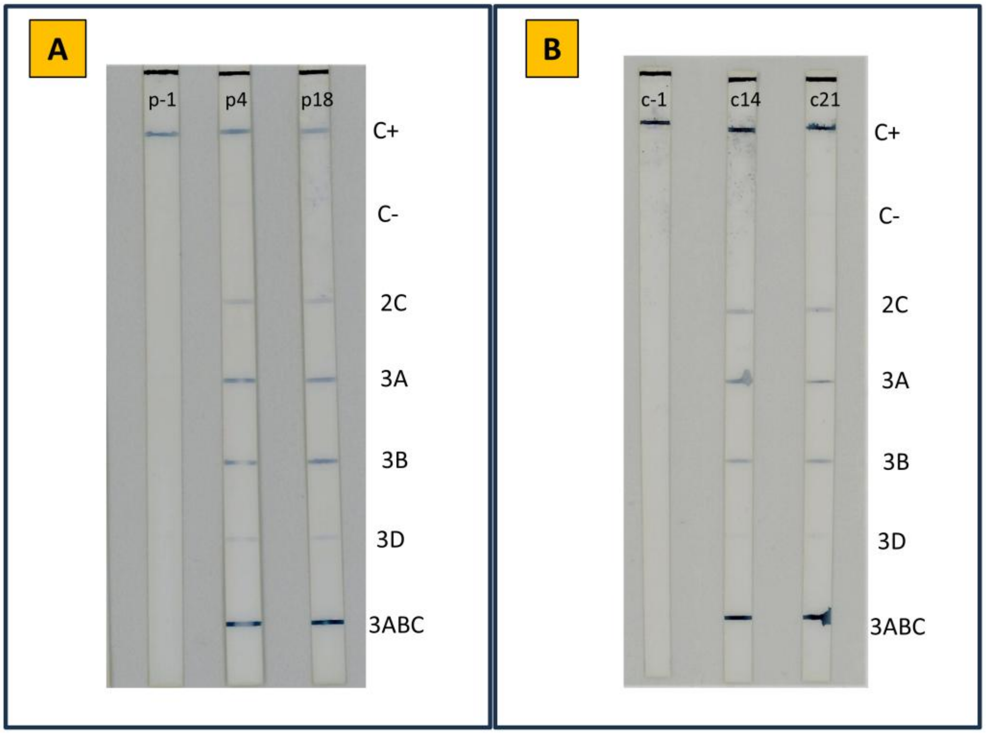

Figure 3.3 FMDV MAPIA evaluation with positive and negative sera.
A pig serum was obtained from a naïve piglet before the infection (p-1), and two pig sera (p4 and p18) were obtained 14 days post-infection of the FMDV O/UK/11/2001 strain (Figure 3.3A). A cattle serum was obtained from a naïve calf before the infection (c-1), and two sera (c14 and c21) were obtained 14 days post-infection of the FMDV strain A/24/Cruzerio (Figure 3.3B).

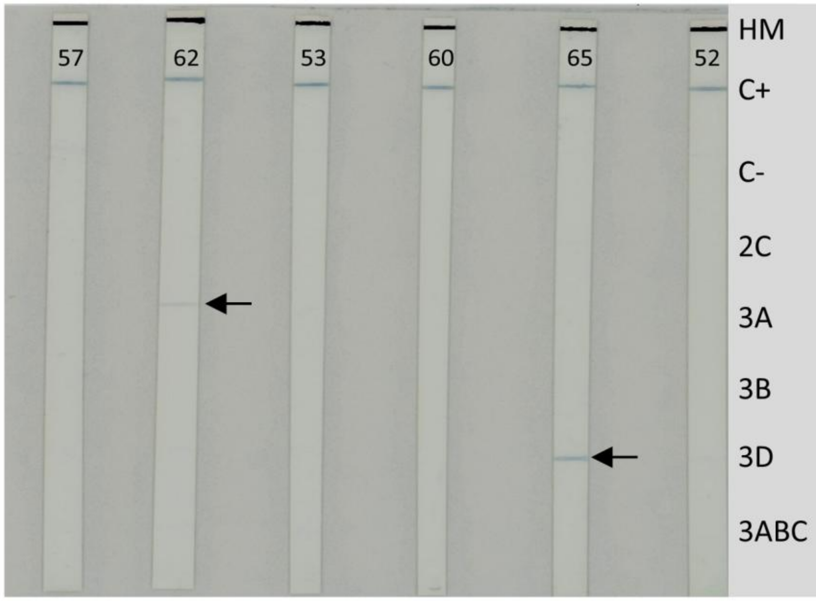

Figure 3.4 FMDV MAPIA evaluation with false-positive cattle sera.
Six cattle sera obtained from annual surveillance tested positive with FMDV NSP 3ABC cELISA (c-52, c-53, c-57, c-60, and c-65). The cattle sera c-62 and c-65 reacted with the individual NSP antigens 3A and 3D, respectively (black arrows).

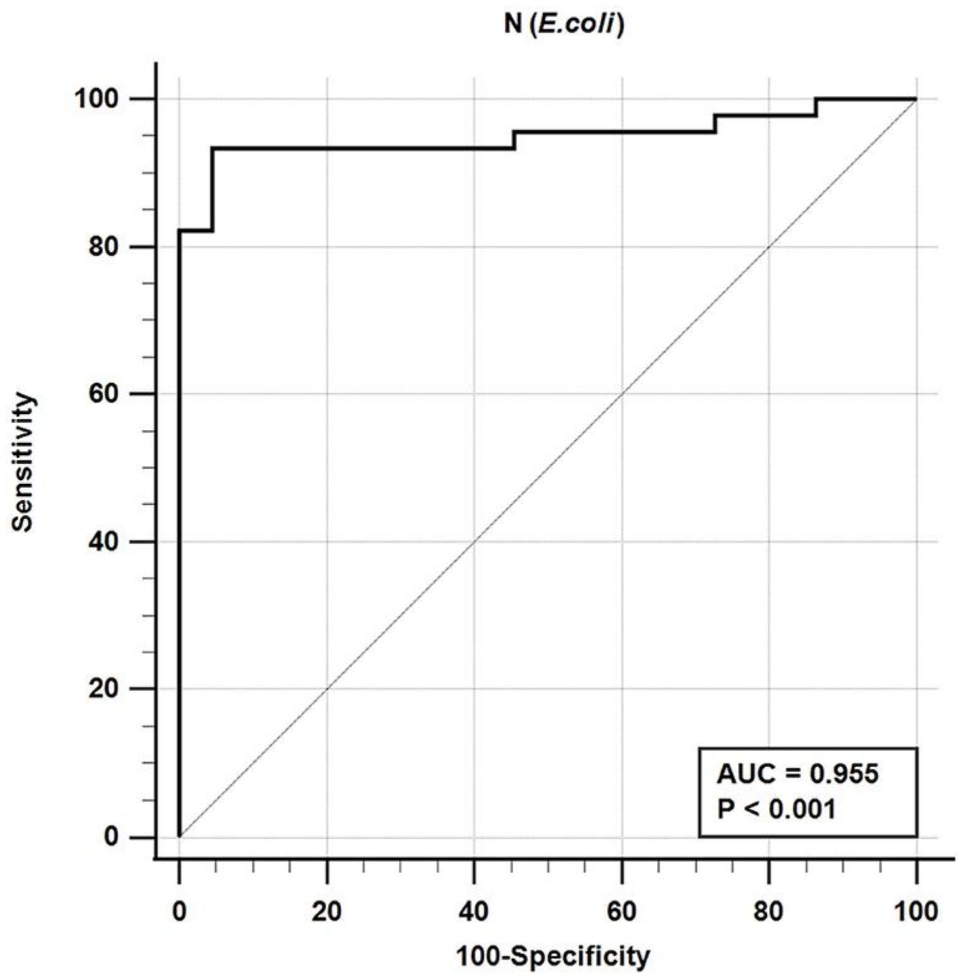

Figure 3.5 ROC curve analysis for the SARS-CoV-2 Np (*E.coli*) iELISA.
The line represents the 95% confidence interval, and the upper left corner is the highest Youden index indicating the best combined sensitivity and specificity of 93.3% and 95.5%, respectively. AUC, area under the curve.

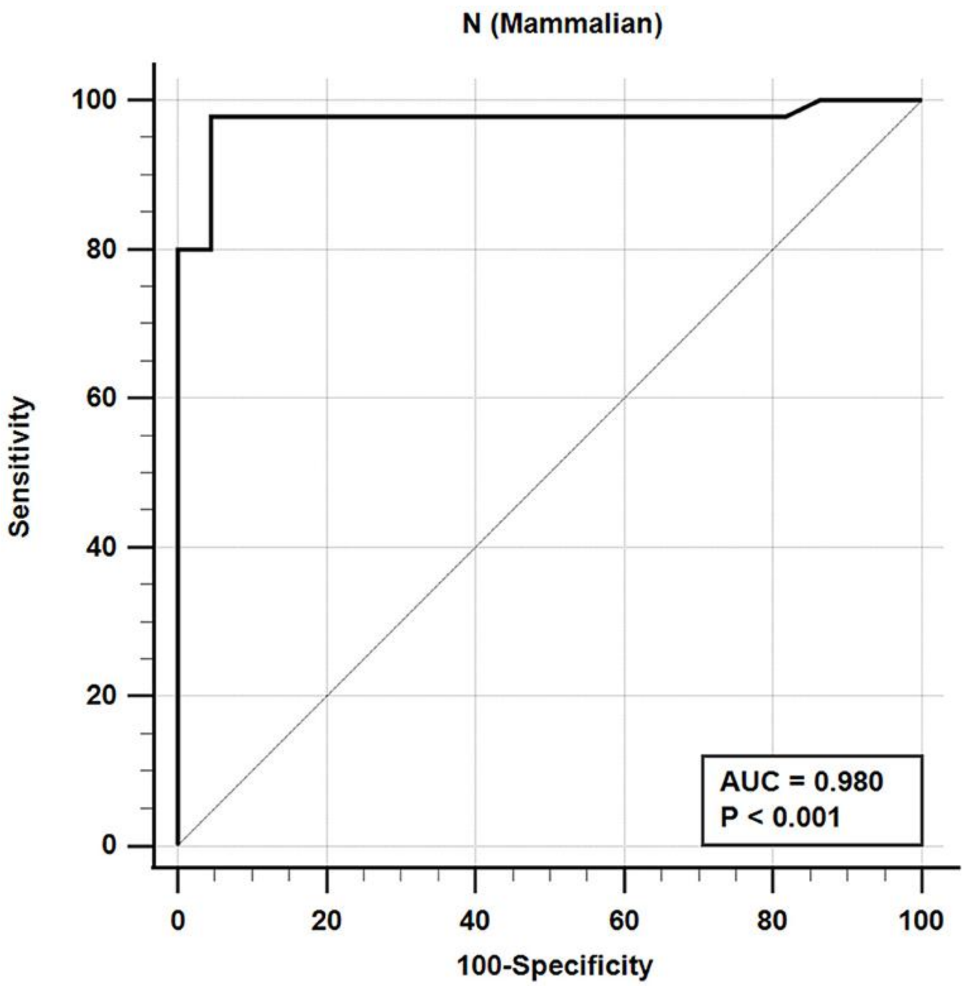

Figure 3.6 ROC curve analysis for the Np (mammalian) iELISA.

The line represents 95% confidence interval, and the upper left corner is the highest Youden index indicating the best combined sensitivity and specificity of 97.8% and 95.5%, respectively. AUC, area under the curve.

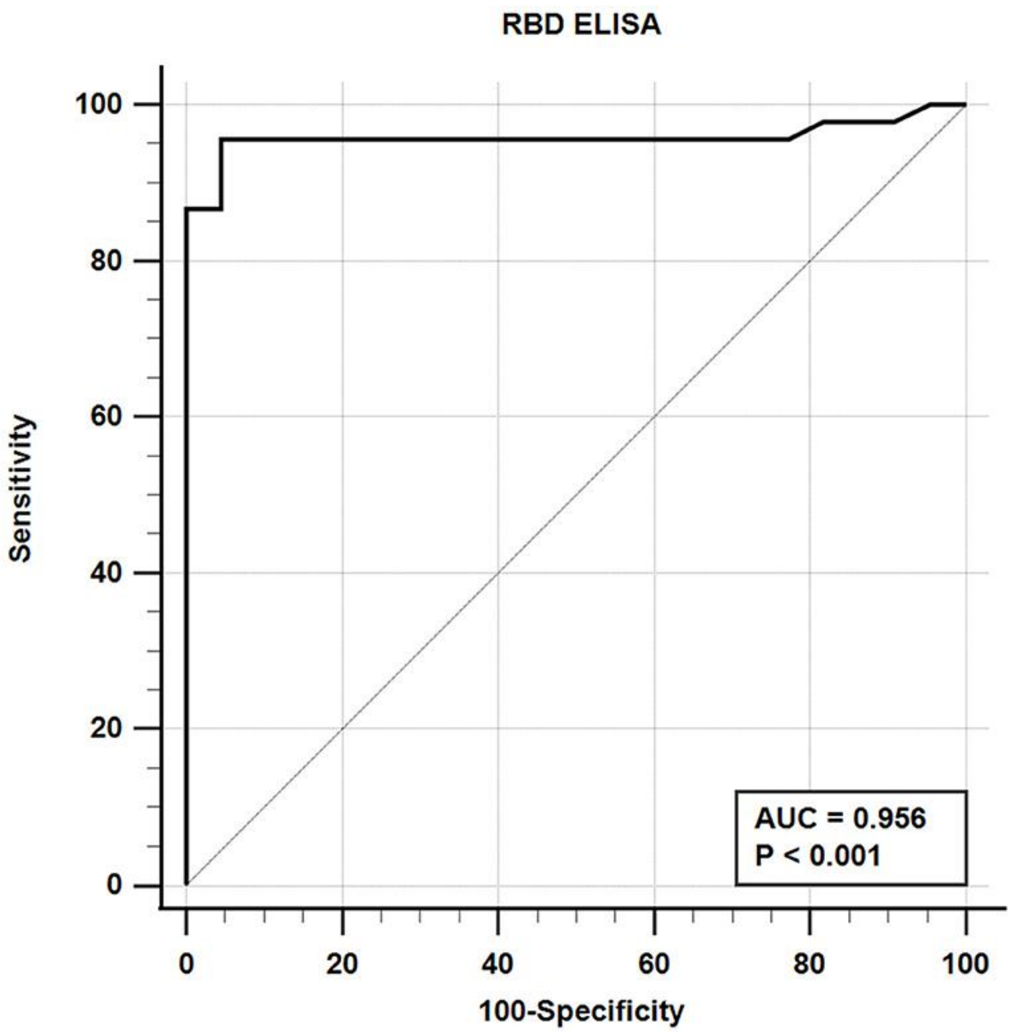

Figure 3.7 ROC curve analysis for the RBD protein iELISA.
The line represents the 95% confidence interval, and the upper left corner is the highest Youden index indicating the best combined sensitivity and specificity of 95.6% and 95.5%, respectively. AUC, area under the curve.

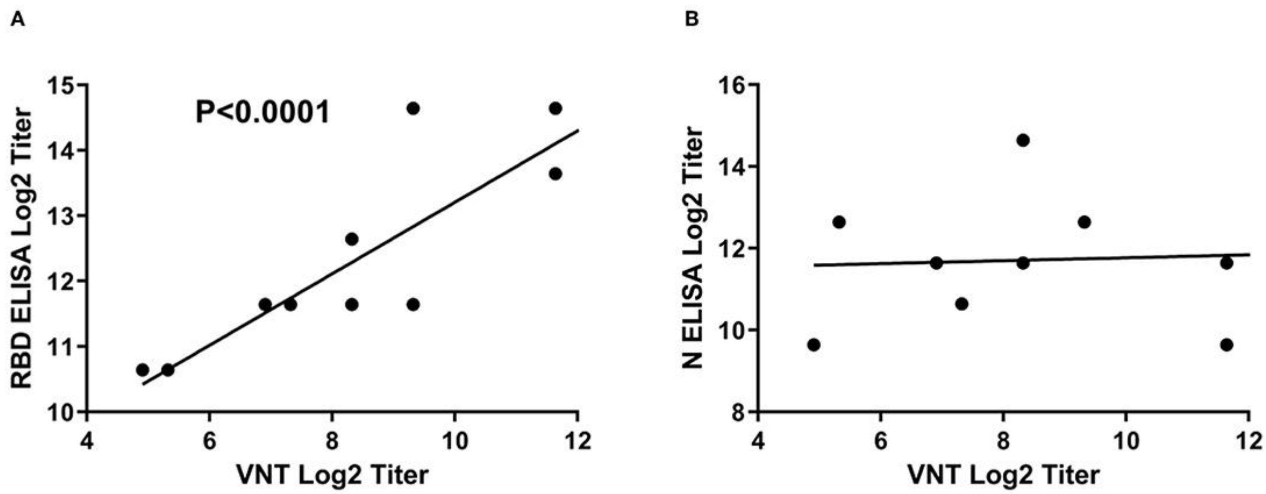

Figure 3.8 Correlation of antibody titers between SARS-CoV-2 iELISA and VNT.
Linear regression analysis of RBD (A) and N (B) iELISA antibody titers in relation to virus-neutralizing antibody titers using SARS-CoV-2-positive cat sera was performed using GraphPad Prism software; RBD r2 = 0.7924 and N r2 = 0.0041.

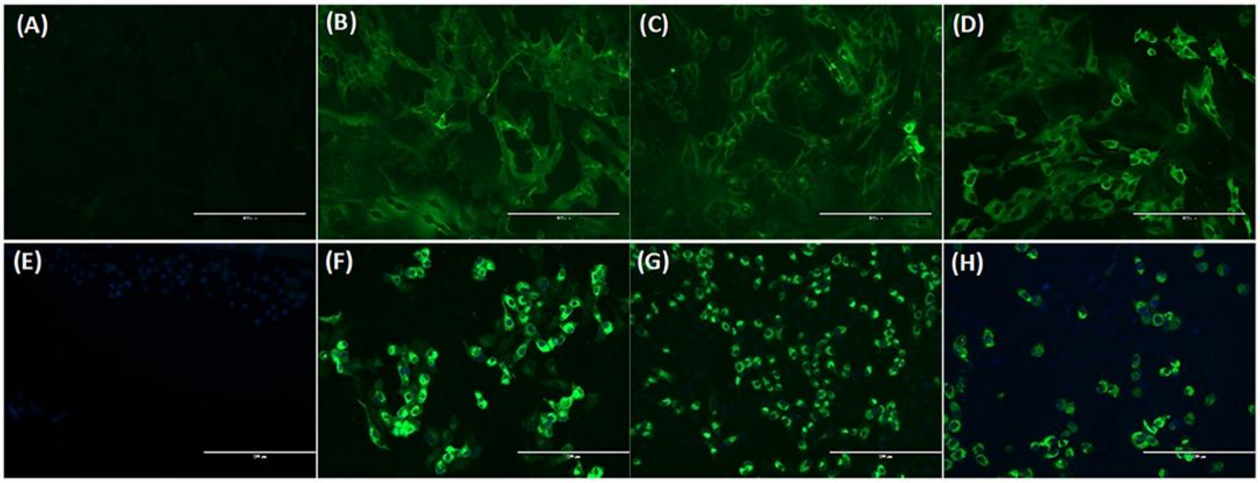

Figure 3.9 IFA analysis for FeCoV and SARS-CoV-2 – specific antibodies.

IFA results on Crandell feline kidney (CrFK) and Vero E6 cells infected with FeCoV virus (A–D) and SARS-CoV-2 (E–H), respectively. Results of FeCoV IFA (A–D) with negative control cat serum (A), FeCoV-positive cat serum #1 from Table 6 (B), SARS-CoV-2-specific antibody-positive cat serum #328 from Table 7 (C), and FeCoV N protein-specific monoclonal antibody (D). IFA for SARS-CoV-2 (E–H) with FeCoV antibody-negative cat serum #12 from Table 6 (E), SARS-CoV-2 antibody-positive cat serum #026 from Table 7 (F), FeCoV antibody-positive serum #1 from Table 6 (G), and SARS-CoV-2 RBD protein-specific monoclonal antibody #117A5 from Table 7 (H). The photographs were taken using a fluorescence microscope for both IFAs with a serum dilution of 1:160.

Table 3.1 ASF MAPIA evaluation using ASFV antibody-negative sera from naïve pigs. (n = 89)

Sample ID	p30 iELISA	Positive control	Negative control	ASFV p22	ASFV p30	ASFV p54	ASFV p72
1	−/−	+/+	−/−	−/−	−/−	−/−	−/−
2	−/−	+/+	−/−	−/−	−/−	−/−	−/−
3	−/−	+/+	−/−	−/−	−/−	−/−	−/−
4	−/−	+/+	−/−	−/−	−/−	−/−	−/−
5	−/−	+/+	−/−	−/−	−/−	+/+	−/−
6	−/−	+/+	−/−	−/−	−/−	−/−	−/−
7	−/−	+/+	−/−	−/−	−/−	−/−	−/−
8	−/−	+/+	−/−	−/−	−/−	−/−	−/−
9	−/−	+/+	−/−	−/−	−/−	−/−	−/−
10	−/−	+/+	−/−	−/−	−/−	−/−	−/−
11	−/−	+/+	−/−	−/−	−/−	−/−	−/−
12	−/−	+/+	−/−	−/−	−/−	−/−	−/−
13	−/−	+/+	−/−	−/−	−/−	−/−	−/−
14	−/−	+/+	−/−	−/−	−/−	−/−	−/−
15	−/−	+/+	−/−	−/−	+/+	−/−	−/−
16	−/−	+/+	−/−	−/−	−/−	−/−	−/−
17	−/−	+/+	−/−	−/−	−/−	−/−	−/−
18	−/−	+/+	−/−	−/−	−/−	−/−	−/−
19	−/−	+/+	−/−	−/−	−/−	−/−	−/−
20	−/−	+/+	−/−	−/−	−/−	−/−	−/−
21	−/−	+/+	−/−	−/−	−/−	−/−	−/−
22	−/−	+/+	−/−	−/−	−/−	−/−	−/−
23	−/−	+/+	−/−	+/+	−/−	−/−	−/−
24	−/−	+/+	−/−	−/−	−/−	−/−	−/−
25	−/−	+/+	−/−	−/−	−/−	−/−	+/+
26	−/−	+/+	−/−	−/−	−/−	−/−	−/−
27	−/−	+/+	−/−	−/−	−/−	−/−	−/−
28	−/−	+/+	−/−	−/−	−/−	−/−	−/−
29	−/−	+/+	−/−	−/−	−/−	−/−	−/−
30	−/−	+/+	−/−	−/−	−/−	−/−	−/−
31	−/−	+/+	−/−	−/−	−/−	−/−	−/−
32	−/−	+/+	−/−	−/−	−/−	−/−	−/−
33	−/−	+/+	−/−	−/−	−/−	−/−	−/−
34	−/−	+/+	−/−	−/−	−/−	−/−	−/−

35	−/−	+/+	−/−	−/−	−/−	−/−	−/−
36	−/−	+/+	−/−	−/−	−/−	−/−	−/−
37	−/−	+/+	−/−	−/−	−/−	−/−	−/−
38	−/−	+/+	−/−	−/−	−/−	−/−	−/−
39	−/−	+/+	−/−	−/−	−/−	−/−	−/−
40	−/−	+/+	−/−	−/−	−/−	−/−	−/−
41	−/−	+/+	−/−	−/−	−/−	−/−	−/−
42	−/−	+/+	−/−	−/−	−/−	−/−	−/−
43	−/−	+/+	−/−	−/−	−/−	−/−	−/−
44	−/−	+/+	−/−	−/−	−/−	−/−	−/−
45	−/−	+/+	−/−	−/−	−/−	−/−	−/−
46	−/−	+/+	−/−	−/−	−/−	−/−	−/−
47	−/−	+/+	−/−	−/−	−/−	−/−	−/−
48	−/−	+/+	−/−	−/−	−/−	−/−	−/−
49	−/−	+/+	−/−	−/−	−/−	−/−	−/−
50	−/−	+/+	−/−	−/−	−/−	−/−	−/−
51	−/−	+/+	−/−	−/−	−/−	−/−	−/−
52	−/−	+/+	−/−	−/−	−/−	−/−	−/−
53	−/−	+/+	−/−	−/−	−/−	−/−	−/−
54	−/−	+/+	−/−	−/−	−/−	−/−	−/−
55	−/−	+/+	−/−	−/−	−/−	−/−	−/−
56	−/−	+/+	−/−	−/−	−/−	−/−	−/−
57	−/−	+/+	−/−	−/−	−/−	−/−	−/−
58	−/−	+/+	−/−	−/−	−/−	−/−	−/−
59	−/−	+/+	−/−	−/−	−/−	−/−	−/−
60	−/−	+/+	−/−	+/+	−/−	−/−	−/−
61	−/−	+/+	−/−	−/−	−/−	−/−	−/−
62	−/−	+/+	−/−	−/−	−/−	−/−	−/−
63	−/−	+/+	−/−	−/−	−/−	−/−	−/−
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65	−/−	+/+	−/−	−/−	−/−	−/−	−/−
66	−/−	+/+	−/−	−/−	−/−	−/−	−/−
67	−/−	+/+	−/−	−/−	−/−	−/−	−/−
68	−/−	+/+	−/−	−/−	−/−	−/−	−/−
69	−/−	+/+	−/−	−/−	−/−	−/−	−/−
70	−/−	+/+	−/−	−/−	−/−	−/−	−/−
71	−/−	+/+	−/−	−/−	−/−	−/−	−/−
72	−/−	+/+	−/−	−/−	−/−	−/−	−/−
73	−/−	+/+	−/−	−/−	−/−	−/−	−/−

74	−/−	+/+	−/−	−/−	−/−	−/−	−/−
75	−/−	+/+	−/−	−/−	−/−	−/−	−/−
76	−/−	+/+	−/−	−/−	−/−	−/−	−/−
77	−/−	+/+	−/−	−/−	−/−	−/−	−/−
78	−/−	+/+	−/−	−/−	−/−	−/−	+/+
79	−/−	+/+	−/−	−/−	−/−	−/−	−/−
80	−/−	+/+	−/−	−/−	−/−	−/−	−/−
81	−/−	+/+	−/−	−/−	−/−	−/−	−/−
82	−/−	+/+	−/−	−/−	+/+	−/−	−/−
83	−/−	+/+	−/−	−/−	−/−	−/−	−/−
84	−/−	+/+	−/−	−/−	−/−	−/−	−/−
85	−/−	+/+	−/−	−/−	−/−	−/−	−/−
86	−/−	+/+	−/−	−/−	−/−	−/−	−/−
87	−/−	+/+	−/−	−/−	−/−	−/−	−/−
88	−/−	+/+	−/−	−/−	−/−	−/−	−/−
89	−/−	+/+	−/−	−/−	−/−	−/−	−/−

Table 3.2 ASFV MAPIA test results for false-positive pig sera.

The naïve pig sera tested positive with ASFV p72 iELISA (n = 5).

Sample ID	p72 iELISA	Positive control	Negative control	ASFV p22	ASFV p30	ASFV p54	ASFV p72
37	+/+	+/+	−/−	−/−	−/−	−/−	−/−
49	+/+	+/+	−/−	−/−	−/−	−/−	+/+
66	+/+	+/+	−/−	−/−	−/−	−/−	−/−
179	+/+	+/+	−/−	−/−	−/−	−/−	−/−
180	+/+	+/+	−/−	−/−	−/−	−/−	−/−

Table 3.3 ASF MAPIA evaluation using ASFV antibody-positive pig sera.

The samples were collected from piglets at 14 days post ASFV-infection (n = 19)

Sample ID	IFA	p30 iELISA	p72 iELISA	ASFV Strain	Positiv e control	Negative control	ASFV p22	ASFV p30	ASF V p54	ASFV p72
148	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
146	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
13	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
C2056	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
C2057	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
C2059	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
C1866	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
C7412	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
C2114	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
C2117	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
784221	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
784635	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
783541	+/+	+/+	NA	Arm 07	+/+	−/−	−/−	+/+	+/+	+/+
783519	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	−/−
784222	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
783511	+/+	+/+	NA	Arm 07	+/+	−/−	−/−	+/+	+/+	+/+
296	+/+	+/+	NA	Arm 07	+/+	−/−	+/+	+/+	+/+	+/+
1	NA	NA	+/+	OURT88/3	+/+	−/−	+/+	+/+	+/+	+/+
2	NA	NA	+/+	OURT88/3	+/+	−/−	+/+	+/+	+/+	+/+

Table 3.4 FMD MAPIA evaluation using FMD antibody-negative sera from naïve animals. (n = 50)

Sample ID	3ABC cELISA	Positive control	Negative control	FMDV 2C	FMDV 3A	FMDV 3B	FMDV 3D	FMDV 3ABC
p-1	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-2	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-3	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-4	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-5	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-6	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-7	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-8	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-9	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-10	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-11	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-12	−/−	+/+	−/−	+/+	−/−	−/−	−/−	−/−
p-13	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-14	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-15	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-16	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-17	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-18	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-19	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-20	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-21	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-22	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-23	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-24	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-25	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-26	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-27	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-28	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-29	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
p-30	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-1	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-2	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-3	−/−	+/+	−/−	−/−	−/−	−/−	+/+	−/−
c-4	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−

c-5	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-6	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-7	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-8	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-9	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-10	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-11	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-12	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-13	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-14	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-15	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-16	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-17	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-18	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-19	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-20	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−

p – pig; c - cattle

Table 3.5 FMD MAPIA test results for false antibody-positive sera from naïve cattle. (n = 6)

Sample ID	3ABC cELISA	Positive control	Negative control	FMDV 2C	FMDV 3A	FMDV 3B	FMDV 3D	FMDV 3ABC
c-52	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-53	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-57	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-60	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
c-62	+/+	+/+	−/−	−/−	+/+	−/−	−/−	−/−
c-65	+/+	+/+	−/−	−/−	−/−	−/−	+/+	−/−

Table 3.6 FMD MAPIA evaluation using FMDV antibody-positive sera.

The sera were collected from piglets and calves at 14 days post FMDV infection (n = 23)

Sample ID	Virus strain	3ABC cELISA	Positive control	Negative control	FMDV 2C	FMDV 3A	FMDV 3B	FMDV 3D	FMDV 3ABC
p1	O/UKG/11/2001	−/−	+/+	−/−	−/−	+/+	+/+	+/+	+/+
p2	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p3	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p4	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p5	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p6	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p7	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p8	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p9	O/UKG/11/2001	−/−	+/+	−/−	−/−	+/+	+/+	+/+	+/+
p10	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p11	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p12	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	−/−	+/+
p13	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p14	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p15	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	−/−	+/+
p16	O/UKG/11/2001	−/−	+/+	−/−	+/+	−/−	+/+	+/+	+/+
p17	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p18	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p19	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
p20	O/UKG/11/2001	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
c140-14	24 Cruzerio	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
c140-21	24 Cruzerio	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
c140-23	24 Cruzerio	−/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+

p – pig; c - cattle

Table 3.7 SARS-CoV-2 recombinant antigens used in this study.

Antigens	Location (start and end sequences)	Size (amino acid)	Expression system	Tag
RBD protein	PNITNLC .. VLSFELLHAP	192	Mammalian	Double strep tags, C-terminal
N protein	SDNGPQN..SADSTQA	418	Mammalian	Double strep tags, C-terminal
N protein	SDNGPQN..SADSTQA	418	*E.coli*	6xHis tag, N-terminal

Table 3.8 SARS-CoV-2 iELISA evaluation using cat sera and monoclonal antibodies.

Antibodies	Number of samples	Tests
SARS-CoV-2 positive cat sera	45	SARS-CoV-2, VNT
SARS-CoV-2 negative cat sera	22	SARS-CoV-2, VNT
FIP positive cat sera	13	IFA, FIPV
FIP negative cat sera	6	IFA, FIPV
SARS-CoV-2 RBD-specific mAbs	4	SARS-CoV-2, VNT
FIPV N-specific mAb	1	IFA, FIPV

Table 3.9 Sensitivity (Sn) and specificity (Sp) of the SARS-CoV-2 Np (*E.coli*) iELISA.

iELISA	Has Disease	No Disease	Total (Test)
Positive (Number)	42 **(Sn=93.3%)**	1	43
Negative (Number)	3	21 **(Sp=95.5%)**	24
Predictive value	97.70%	87.50%	

Table 3.10 Sensitivity (Sn) and specificity (Sp) of the SARS-CoV-2 Np (mam) iELISA.

iELISA	Has Disease	No Disease	Total (Test)
Positive (Number)	44 **(Sn=97.8%)**	1	45
Negative (Number)	1	21 **(Sp=95.5%)**	22
Predictive value	97.7%	95.5%	

Table 3.11 Sensitivity (Sn) and specificity (Sp) of the SARS-CoV-2 RBD iELISA.

iELISA	Has Disease	No Disease	Total (Test)
Positive (Number)	43 **(Sn=95.6%)**	1	44
Negative (Number)	2	21 **(Sp=95.5%)**	23
Predictive value	97.7%	91.3%	

Table 3.12 Cross-reactivity of FeCoV-specific antibodies with SARS-CoV-2 antigens.

Cat ID	RBD (mam)		N (mam)		N (*E.coli*)		VN	IFA (SARS-CoV-2)		IFA (FIP)	
FIP pos #1	0.21	neg	2.17	pos	2.86	pos	<1:20	>1:2400	pos	>1:6400	pos
FIP pos #2	0.15	neg	1.29	pos	1.32	pos	<1:20	1:80	pos	>1:6400	pos
FIP pos #0	0.18	neg	1.89	pos	1.53	pos	<1:20	1:80	pos	>1:6400	pos
FIP pos #3	0.09	neg	0.2	neg	0.28	neg	<1:20	<1:40	neg	>1:6400	pos
FIP pos #4	0.11	neg	0.12	neg	0.31	neg	<1:20	<1:40	neg	>1:6400	pos
FIP pos #5	0.31	neg	0.26	neg	0.37	pos	<1:20	<1:40	neg	1:3,200	pos
FIP pos #6	0.12	neg	0.19	neg	0.53	pos	<1:20	<1:40	neg	>1:6400	pos
FIP pos #7	0.05	neg	0.15	neg	0.16	neg	<1:20	<1:40	neg	1:3,200	pos
FIP pos #8	0.07	neg	0.15	neg	0.12	neg	<1:20	<1:40	neg	>1:6400	pos
FIP pos #27	0.09	neg	2.63	pos	1.74	pos	<1:20	1:320	pos	>1:6400	pos
FIP pos #28	0.28	neg	2.03	pos	2.41	pos	<1:20	1:320	pos	>1:6400	pos
FIP pos #29	0.07	neg	0.97	pos	1.29	pos	<1:20	1:80	pos	1:3,200	pos
FIP pos #30	0.22	neg	2.87	pos	2.26	pos	<1:20	>1:1280	pos	>1:6400	pos
FIP neg #9	0.23	neg	0.09	neg	0.55	neg	<1:20	<1:40	neg	<1:40	neg
FIP neg #10	0.07	neg	0.1	neg	0.21	neg	<1:20	<1:40	neg	<1:40	neg
FIP neg #11	0.27	neg	0.1	neg	0.24	neg	<1:20	<1:40	neg	<1:40	neg
FIP neg #12	0.14	neg	0.17	neg	0.41	neg	<1:20	<1:40	neg	<1:40	neg
FIP neg #14	0.09	neg	0.1	neg	0.1	neg	<1:20	<1:40	neg	<1:40	neg
FIP neg #15	0.1	neg	0.14	neg	0.17	neg	<1:20	<1:40	neg	<1:40	neg
FIP mAb	0.1	neg	0.27	neg	0.31	neg	<1:20	<1:40	neg	1:400	pos

Table 3.13 Cross-reactivity of antibodies to SARS-CoV-2 and FeCoV.

Serum ID	IFA (FeCoV)		VN (FeCoV)		IFA (SARS-CoV-2)		VN (SARS-CoV-2)	
#026 (21DPC)	>1:40	pos	>1:4	neg	1:5,120	pos	1:40	pos
#328 (4DP2C)	>1:320	pos	>1:4	neg	>1:5,120	pos	1:160	pos
#272 (4DP2C)	>1:320	pos	>1:4	neg	>1:5,120	pos	1:80	pos
#903 (4DP2C)	>1:320	pos	>1:4	neg	>1:5,120	pos	1:160	pos
mAb 91C2	<1:5	neg	>1:4	neg	1:1,280	pos	1:32	pos
mAb 91G12	<1:5	neg	>1:4	neg	1:2,560	pos	1:64	pos
mAb 117C1	<1:5	neg	>1:4	neg	1:640	pos	1:256	pos
mAb 117E2	<1:5	neg	>1:4	neg	1:2,560	pos	1:65	pos

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Chapter 4 - Conclusions

Transboundary animal diseases (TADs) such as ASF, CSF, and FMD continue to pose immense global challenges due to their rapid spread and severe socioeconomic consequences. At the same time, the COVID-19 pandemic has highlighted the importance of highly sensitive and specific serological diagnostic tools in monitoring animal infections and assessing herd immunity. The goal of this thesis was to address critical diagnostic gaps by developing, evaluating, and integrating a suite of novel or improved diagnostic assays that strengthen both field-level detection and laboratory confirmation of viral pathogens, through four major components, including ASFV antigen and antibody LFAs, portable MinION sequencing of AFV, CSFV and FMDV, ASFV and FMDV MAPIA serological confirmatory testing, and SARS-CoV-2 antibody detecting iELISAs. This research offers a comprehensive diagnostic advancement applicable to multiple high-impact animal and zoonotic viruses. The following sections synthesize the overarching findings and their broader implications.

Improving the rapid detection of ASFV antigen and antibodies in the field is a global priority due to the absence of vaccines in non-endemic countries and the devastating consequences of outbreaks. The findings of this thesis confirm that the ASFV structural proteins p30 and p54 remain among the most diagnostically valuable viral antigens. In this study, an ASFV p30-specific mAb was successfully developed and employed in an ASFV antigen-detecting LFA, which demonstrated notable sensitivity (93.5%) and specificity (99.4%) in clinically sick animals after ASFV infection. For diseases like ASF, which are highly contagious, usability for field applications is crucial, as timely diagnosis is essential for early containment and eradication strategies. The assay performed well in detecting ASFV antigen in animals with high fever and showing apparent symptoms of ASF. Its sensitivity and specificity support its utility for on-farm screening, especially during outbreaks or in areas lacking molecular diagnostic capacity.

In parallel, the p54-based antibody LFA filled an equally important diagnostic need by enabling the detection of past or ongoing ASFV infections during later stages of the disease. The p54 antigen stimulates strong and consistent antibody responses in the host, allowing the ASFV p54 antigen-based LFA to provide reliable detection of ASFV antibodies. This assay is particularly valuable for diagnosing chronic ASF infection in non-vaccinated domestic pigs, as well as for surveillance, epidemiological monitoring, and testing in wild boar populations, where sample integrity and timing often vary. The combined use of antigen (p30) and antibody (p54) LFAs highlights the strength of a complementary, multi-stage diagnostic approach. Together, these ASFV antigen and antibody assays establish a practical diagnostic pathway for ASF that facilitates rapid decision-making and supports large-scale surveillance efforts in the field.

Further validation using larger sample sets, multiple ASFV genotypes, and field-collected specimens from wild pigs is necessary to evaluate accurate analytical sensitivity and ensure applicability throughout diverse epidemiological contexts. It is possible, however, for this research to contribute substantially to the worldwide effort to develop accessible, effective and low-cost diagnostic tools.

A significant achievement of this thesis was demonstrating the possibility of combining PCR/RT-PCR with MinION nanopore sequencing for the rapid identification and genotypic confirmation of ASFV, CSFV, and FMDV in a low resource setting. This work addressed a critical gap in countries where laboratory infrastructure is limited or where rapid molecular confirmation is urgently required during TAD outbreaks.

MinION sequencing of (RT)-PCR amplicons produced results within eight hours, allowing for real-time decision-making. The MinION platform enabled the identification of pathogen species, genotypes, and serotypes, which are crucial for tracing the sources of outbreaks, understanding transmission pathways, and developing control strategies. Although short-amplicon sequencing does not replace full-genome sequencing, it nonetheless offers an efficient and affordable molecular approach that can be applied in resource-limited settings.

The findings from the MinION study emphasize the significant advantage of molecular confirmation over antigen- or antibody-based portable or laboratory tests. It does not require a host immune response and does not rely on large amounts of viral antigen in clinical samples. Therefore, (RT)-PCR amplification and subsequent sequencing can detect early infections with high sensitivity and specificity. These data also demonstrate that sequencing reduces diagnostic uncertainty in cases where immunoassays yield inconclusive or unexpected results. Despite the limited number of samples, the results provide essential proof-of-concept evidence for the broader adoption of portable sequencing technologies for the rapid identification of pathogens even in limited resource environments.

Future work should focus on expanding throughput, optimizing sample preparation protocols, and validating large-scale integration with national veterinary services. However, even at its current capacity, MinION sequencing represents a transformative diagnostic tool for regions prone to outbreaks.

The development and evaluation of the ASFV and FMDV MAPIA assays point to another significant contribution of this thesis to TAD diagnostics. Traditional confirmatory assays often require BSL-3 containment and complex and expensive procedures for their production and use, limiting access for many countries. MAPIA offers a safe, simple, and accurate alternative that can be performed under BSL-2 conditions for clinical samples.

The ASFV MAPIA greatly improved diagnostic specificity by utilizing multiple recombinant antigens (p22, p30, p54, and p72) to differentiate true positives from negative or false positive samples. Within the sample set used for this study, 100% sensitivity and specificity were achieved. A major advantage of the ASFV MAPIA is that it can resolve inconclusive or ambiguous ELISA results. Cases in which single-antigen ELISAs produced false positives, particularly with the ASFV p72 iELISA, were easily resolved through the ASFV MAPIA, confirming its value as a robust confirmatory tool for ASFV-specific antibody detection.

The FMDV MAPIA, utilizing a panel of NSP antigens (2C, 3A, 3B, 3D, and 3ABC), achieved equally high sensitivity and specificity (100%). The most substantial advantage of FMDV NSP-specific antibody detection is the ability to differentiate between infected and vaccinated animals (DIVA). For this purpose, 3ABC antigen-based ELISAs are commonly used; however, the unsatisfactory specificity of the 3ABC ELISAs results in false-positive samples. To follow up these results and ensure negativity with confirmatory tests is critical in FMD-free countries or those seeking FMDV-free status. Also, the FMDV MAPIA's compatibility with BSL-2 conditions and ease of interpretation could make it a valuable improvement over the WOAH-recommended EIBT test.

Together, the MAPIA assays developed in this thesis significantly enhance confirmatory serodiagnostic capacity for ASFV and FMDV. They represent practical and scalable solutions that enhance diagnostic accuracy and support international disease control standards.

The final component of this research extended the thesis beyond TADs into a zoonotic pathogen by developing and validating indirect ELISAs for the detection of SARS-CoV-2-

specific antibodies. The emergence of COVID-19 raised concerns about animal susceptibility, and cats were identified as one of the most susceptible domestic species. This study developed iELISAs using the recombinant RBD of S, and N proteins to detect SARS-CoV-2-specific antibodies in experimentally infected cats. The S and N proteins were expressed in a mammalian expression system, while an additional N protein was expressed in an *E. coli* expression system for comparison.

The iELISAs demonstrated high sensitivity (93.3-97.8%) and strong specificity (95.5%), confirming their suitability for serosurveillance. Importantly, the RBD-based ELISA did not cross-react with FeCoV-positive sera; and the test results directly correlated with the VNT results. In contrast, the N-based assays detected N-specific immune responses in a highly specific and sensitive manner but showed limited cross-reactivity with FeCoV-specific antibodies. This study confirms the utility of RBD ELISAs for confirming SARS-CoV-2 exposure and immunity in cats, while N-based assays may be useful as serological screening tools.

Given the need for efficient and BSL-2 compatible serological surveillance, these SARS-CoV-2 ELISAs provide a valuable diagnostic approach for monitoring potential animal reservoirs and understanding cross-species transmission dynamics. This work contributes to practical diagnostic advancement that supports broader *One Health* surveillance systems.

In summary, this thesis presents a comprehensive set of newly developed diagnostic tools that collectively enhance diagnostic and surveillance capacities for ASF, CSF, FMD, and SARS-CoV-2. By integrating rapid antigen and antibody detection, confirmatory MAPIA serology, and molecular sequencing, this work advances both field-level and laboratory-based diagnostic methods in meaningful ways. The assays developed here fill essential gaps in accessibility and practicality, especially for resource-limited regions. Future research should focus on larger validation studies and broader epidemiological applications. Still, the contributions of this thesis already represent essential progress toward improved global readiness against high-consequence animal and zoonotic viral diseases.