CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Development and Evaluation of novel Loop-mediated Isothermal Amplification primers designed for specific Influenza B virus detection.

MASTER'S THESIS

Prague 2024

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Declaration

I, Panhavoan Ky, hereby declare that I have done this thesis entitled “Development and Evaluation of novel Loop-mediated Isothermal Amplification primers designed for specific Influenza B virus detection” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 20.04.2024

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Panhavoan Ky

Acknowledgements

Firstly, I would like to thank the Ministry of Education, Youth, and Sport of the Czech Republic for providing me with a scholarship in the program of support of foreign nationals' studies at Republic Institutions of Higher Education in Czech Republic. The scholarship not only helped me with financial support but also motivation and psychological satisfaction so that I could successfully complete my study and achieve this degree. Any of these wouldn't happen if it weren't for this scholarship program.

Secondly, I would like to thank my supervisor, Dr. Jiří Černý, PhD. for providing me everything I needed from the beginning, from choosing the topic until this thesis was successfully completed. Thank him for his all-wise advice, designing the protocol, sample collection, and all the material for the experiment in the laboratory.

Lastly, I would also like to thank all my colleagues and friends who have helped with advising, motivating, knowledge, information, or any other materials. With all my appreciation, I would like to thank Jignesh Italiya, who's been helping me a lot with his advice as well as the help in the laboratory.

The work on this diploma thesis was supported by Internal Grant Agency of the Faculty of Tropical Agrisciences, Czech University of Life Sciences Prague IGA 20233104 and by the Technology Agency of the Czech Republic program Gamma grant TP01010050 2020_11_02.

Abstract

The Influenza B virus (IBV) constitutes a significant threat within the realm of common flu viruses, resulting in substantial morbidity in humans due to its capacity to propagate as an airborne pathogen. It was also isolated from infected animals as seals. To facilitate viral identification and diagnosis, laboratory technicians have traditionally relied on methods employing molecular amplification and antigen detection. Among these techniques, Reverse Transcription Polymerase Chain Reaction (RT-PCR) stands out as the most widely utilized approach for IBV detection, owing to its superior sensitivity and specificity in comparison to alternative methods. However, the adoption of RT-PCR is impeded by the necessity for high-cost specialized equipment and the considerable time investment required, presenting challenges for laboratories with limited resources.

This study endeavors to address these challenges by focusing on the implementation of Loop-mediated Isothermal Amplification (LAMP) as a molecular-based detection method. LAMP offers a streamlined and rapid assay, representing a promising solution for point-of-care applications. By employing this innovative approach, the study aims to enhance the efficiency and accessibility of IBV detection, paving the way for more efficient and widespread diagnostic practices in the field of virology.

A set of primers was designed based on the most conserved region with the lowest mutation rate in the IBV Matrix 1 gene of segment VII. The combination of LAMP with colorimetric products eliminates the need for specific expensive instruments and time, which is suitable under field conditions for the detection of infectious pathogens. Both lineages of IBV (Victoria and Yamagata) were used as positive samples to evaluate the specificity and sensitivity of the primer at a specific constant temperature (65°C) in both colorimetric and fluorescent products. All experiments tested the primer's cross-reactivity using SARS-CoV-2 RNA.

The designed primers were successfully used to amplify on a specific target amplicon in the time range of 12–19 minutes for real-time fluorescent products, and colorimetric products were visualized by naked-eye color changes before 40 minutes

for both lineages of IBV. The assay was validated by using negative, cross-reactivity, and positive controls as well as samples from asymptomatic volunteers.

Keywords: Influenza B virus, Loop-mediated Isothermal Amplification, LAMP Primer construction.

Contents

List of tables

List of figures

List of the abbreviations used in the thesis.

aa	-	Amino Acid
cDNA	-	Complementary DNA
ELISA	-	Enzyme-linked immunosorbent assay
FAO	-	Food Agricultural Organization
GIBS	-	Global influenza B study
GISIAD	-	Global Initiative on Sharing All Influenza Data
H or HA	-	Hemagglutinin
HEF	-	Hemagglutinin-esterase-fusion
HI	-	Hemagglutination inhibition.
IAV	-	Influenza A virus
IBV	-	Influenza B virus
ICV	-	Influenza C virus
LAMP	-	Loop-mediated Isothermal Amplification
N or NA	-	Neuraminidase
NAI	-	Neuraminidase inhibitor
PCR	-	Polymerase chain reaction
RdRP	-	RNA dependent RNA polymerase
RT-PCR	-	Reverse transcription Polymerase chain reaction
RT-LAMP	-	Reverse transcription Loop-mediated Isothermal Amplification
SA	-	Sialic acid
ssRNA	-	Single-stranded RNA
WHO	-	World Health Organization

1. Introduction and Literature Review

1.1. Introduction

Since the influenza pandemic in Spain 1918-1920 (Trilla et al. 2008), seasonal flu remains a substantial matter of public health concern. Influenza viruses are the primary pathogens responsible for severe disease of lower respiratory tract (Paget et al. 2022). During an epidemic of acute respiratory disease in the 1940s, influenza B virus (IBV), which shares clinical symptoms with influenza A virus (IAV), was isolated and characterized. IBV was the term given to this new virus, which was serologically different from the IAV (Francis Jr 1940). Nowadays, we have the family Orthomyxoviridae divided into several genera; four of them are Aplha-, Beta-, Gamma-, and Delta-influenzavirus, which contain species of IAV, IBV, ICV, and IDV commonly caused disease in animals. However, only two of these viruses, IAV and IBV, are responsible for the regular outbreaks of illness in humans known as seasonal flu (WHO 1979). Unlike IAVs, which are known to have a variety of host species, IBV is believed to have a restricted host range and circulate mostly among the human population. However, IBVs were also found in other species, such as seals, dogs, horses, wild birds, non-human primates, etc. (Van De Sandt et al. 2015). This indicates that IBVs have the ability to infect animals other than humans, but yet, there are no reports that IBVs are able to circulate from animal to human or one animal species to another (Koutsakos et al. 2016).

In 2008, around 20 million lower respiratory illnesses were tested as influenza-associated globally; 1 million were severe cases, 28 000–111 000 were fatal, and most of those were children under 5 years old (Nair et al. 2011). 10 years later, in 2018, around 10 million lower respiratory infections were associated with influenza viruses and around 34,800 fatalities globally (Wang et al. 2020). The overwhelming majority of fatalities in children under the age of 5 and elders (>65 years of age) due to lower respiratory tract infections caused by influenza occur in countries with limited infrastructure. The first appearance of symptoms occurs between 1 and 4 days after infection; the symptoms are very similar to those caused by IAV infection and often last for one week (Zaraket et al. 2021). Interestingly, during some flu seasons, cases associated with IBV are even higher than those caused by human IAV subtypes H1N1 and H3N2 (Matias et al. 2014, Koutsakos et al. 2021). In the USA, for instance, an investigation on the fatality rates associated with influenza infection between the years 1976 and 1999 revealed that the IAV H3N2 subtype was associated with the highest morbidity, followed by IBV, and then by H1N1 (Thompson et al. 2009). Another epidemiological study in the United States in 1997/2009 revealed the same result as a total of influenza fatality, 29%

were responsible by the infection of IBV (Matias et al. 2014). According to this report, there were instances where the IBV infection resulted in a higher number of fatalities compared to the overall cases of influenza. For example, in the USA, IBV accounted for 95.38% (11303/11850) in 2000/2001, 78% in 2002/2003 (9376/12020), and 43.26% (11312/26148) in the 2008/2009 season of all influenza-associated fatalities (Matias et al. 2014). Additionally, during the seasonal flu outbreak in Australia in 2008, 54% of the influenza infections were reported to be IBV infections. IBV has shown the most activity in children between the ages of 5 and 14, according to the study that was conducted in Australia (Kaczmarek et al. 2010). This finding has demonstrated the same outcome as the influenza epidemic in England during the first half of 2012 and 2013 and the outbreak in Thailand between 2010 and 2014, which had the majority of activity in children between the ages of 5 and 19 (Koutsakos et al. 2016).

1.2. Taxonomy and classification of influenza viruses

Influenza viruses belong to the family of Orthomyxoviridae, a helical-shaped with genetic information packed in segmented of negative-sense single-stranded RNA. This family consists of 9 genera: Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus, Deltainfluenzavirus, Isavirus, Thogotovirus, Quaranjavirus, Sardinovirus, and Thogotovirus. The classification of influenza species is based on the antigenic variations in their nucleoprotein (NP) and matrix (M) proteins, resulting in four distinct types: influenza A, B, C, and D viruses. The IAV and IBV possess two primary surface glycoprotein antigens known as hemagglutinin (H or HA) and neuraminidase (N or NA) (Ma 2022).

Genus Alphainfluenzavirus is represented by a single species, Alphainfluenzavirus influenzae, routinely called Influenza A virus (IAV), an enveloped virus whose genome consists of 8 segments of negative-sense RNA (Cheung and Poon 2007). Further classification, a large number of subtypes based on their surface proteins, HA and NA, have been identified (Wille and Holmes 2020). Recently, there have been 18 different HA and 11 NA subtypes. IAV classified their subtypes by combination of HA and NA, making over 130 subtypes identified, with the majority source of sample coming from wild birds (Lin et al. 2023). Ecology and epidemiology of IAVs are very complicated while including a wide range of host species (avian and mammalian, including humans), a wide free-living range, captive-raised, and domestic, which led IAV the highest infection over all other members of the Orthomyxoviridae family (Suarez 2016).

The genus Betainfluenzavirus is composed of one species, Betainfluenzavirus influenzae, previously called Influenza B virus (IBV). Since the virus identification in the early 1940s, IBV has consistently circulated as one lineage along with IAV annually, spreading extensively over different locations and countries (Barr et al. 2006). The accumulation of mutations that cause amino acid (aa) to change the viral surface glycoprotein HA allows new stain variants to escape immunity produced by prior infection or vaccination (Both et al. 1983). During winter epidemics in the late 1980s, IBV was recognized as the genetic variant type of two highly distinct antigens by serological tests of hemagglutination inhibition (HI). These viruses were named after the place and time of identification, as B/Victoria/2/87 (Victoria87-like) was isolated in Victoria, Texas, United States of America (USA) during the epidemic of 1987, and B/Yamagata/16/88 (Yamagata88-like) was isolated during the flu epidemic in Yamagata, Japan. May 1988 (Rota et al. 1990).

Genus Gammainfluenzavirus is known to have one species, Gammainfluenzavirus influenzae, previously named Influenza C virus (ICV). The virus was identified in 1947 during the winter influenza survey in Venezuela (Taylor and Health 1949). Unlike IAV and IBV, ICV has only 7 RNA genomic segments that encode for 9 viral proteins. This virus does not have or rely on HA and NA binding sites for entry and escape from the host cell; segment 4 of the ICV encoded hemagglutinin-esterase-fusion (HEF) glycoprotein efficiently fulfills the functions of binding to the host receptor, breaking down sialic acid (SA), and membrane fusion (Sederdahl and Williams 2020). Epidemiologically and pathologically, ICV has been known to cause cold-like symptoms on the upper respiratory tract and occasionally on the lower respiratory tract, especially in children under 6 years old. Infected patients usually appear to have symptoms similar to those of IAV and IBV, such as fever, cough, and rhinorrhea (Matsuzaki et al. 2006).

The genus Deltainfluenzavirus, consisting of one species, Deltainfluenzavirus influenzae, was named influenza D virus (IDV) before 2022, is a new member of the Orthomyxoviridae family. This pathogen has been isolated as influenza-like in Oklahoma, USA, in 2011. This newly classified virus has homology to ICV, which consisted of only 7 genomic segments exhibiting approximately 50% of the amino acid residues identified in ICV/human (Hause et al. 2013). This novel virus of the influenza family has been detected in several animals worldwide, including pigs, cattle, sheep, horses, and camels. It is believed to have the potential for zoonotic and interspecies transmission. Cattle are acknowledged as the main reservoir of IDV, although other small ruminants and humans have shown susceptibility to IDV infections under different conditions (Asha and Kumar 2019).

1.3. Epidemiologically of Influenza B virus

Contrary to IAV, IBV is recognized for its significantly restricted host range. There is insufficient evidence to support widespread transmission between animals and humans. Therefore, transmission mostly occurs through direct contact between humans (Chen and Holmes 2008). During some flu seasons, cases associated with IBVs are higher than the number of cases caused by human IAV subtypes H1N1 and H3N2, especially in children under 5 years of age and the elderly over 65 years of age (Soldevila et al. 2022). For example, in the USA between 1979 and 1999, where the morbidity caused by IBV was greater than by IAV subtype H3N2 (Koutsakos et al. 2016). According to the analysis of the Global Influenza B Study (GIBS) database, the incidence of IBV infection accounted for 23.4% of all reported cases of influenza-like illness annually from 2000 to 2018 (result from 31 countries). Additionally, IBVs are responsible for up to 52% of all influenza-associated fatalities in children (Zaraket et al. 2021). Interestingly, the circulation of influenza viruses has significantly declined during the first 2 years after the COVID-19 pandemic started. This pandemic was caused by the severe-acute-respiratory-syndrome-related coronavirus 2 virus (SARS-CoV-2) (de Jong et al. 2024). Yamagata lineage of the IBV probably extinctic during the COVID-19 pandemic as it was reported in the Global Initiative on Sharing All Influenza Data (GISAID) since May 2020 (Koutsakos et al. 2021).

1.4. Host range of IBV

As mentioned in the previous page, IBVs have been known as one of the primary influenza pathogens with a limited host range and have been mostly reported to have humans as a specific host. However, there are some reports of infection with human influenza-like disease in the harbor seal (*Phoca vitulina*) population in the Netherlands in 1995. The analysis of sequences and serological testing revealed that the B/Seal/Netherlands/1/99 virus is highly similar to strains that were prevalent in people around 4 to 5 years earlier (Osterhaus et al. 2000). Further evidence on the fur seal (*Arctocephalus australis*) in South America in 2004 has shown that serological tests HI revealed the presence of antibodies against IBV related to IBV/Beijing/184/93, IBV/Hong Kong/330/01, and IBV/Sichuan/379/99. Unfortunately, the viral genomic RNA was not isolated in this study (Blanc et al. 2009). Furthermore, a seroprevalence study conducted in the Netherland examined three primate species. The results revealed that 58.4% of gorillas, 75.4% of orangutans, and 26% of chimpanzees, out of a total of 561 ape sera, contained antibodies against IBVs. This study also indicated that IBVs had

circulated repeatedly within the primate colony between 1986 and 2000 (Buitendijk et al. 2014). Another serological investigation by the HI test during the flu-like outbreak in Hungary In 1979, the serological examination of zoo birds and migrating wild birds, pheasants, mallards, and domestic ducklings and chicks indicated that they had antibodies against IBV (Romvary et al. 1980).

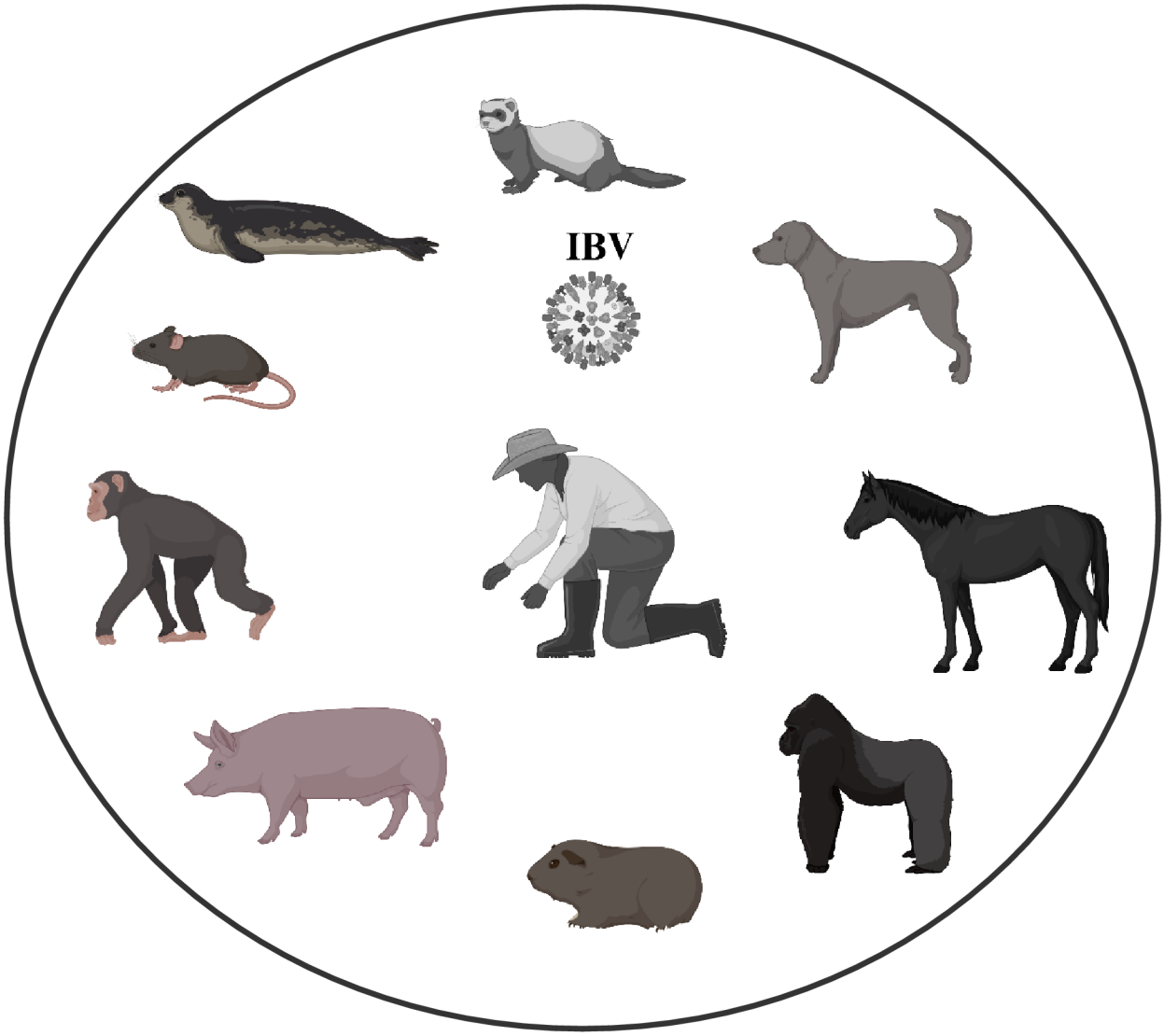


Figure 1: Host range of IBV.

Furthermore, a serology investigation conducted in Hokkaido, Japan, during the years 1976 and 1977 using the HI test to detect the presence of antibodies against influenza B and C viruses in animals revealed that 3.2% of the 504 horses tested were found to be positive for IBV antibodies (KAWANO et al. 1978). Additionally, IBV was isolated from dogs during the influenza epidemic in Taiwan in July 1971 (Chang et al. 1976). Similarly, a study conducted in

Taiwan between 2007 and 2017 has substantiated the favorable outcomes in swine, with 31 out of 15,983 pigs testing positive by serological investigation (Tsai et al. 2019). Evidence in pigs as susceptible hosts for IBV has also been conducted in the USA on swine farms across the country by using HI and detected with reverse-transcription polymerase chain reaction (RT-PCR) using NS segment as the standard marker primers (Ran et al. 2015). In Ecuador, antibodies against IBV have been found in livestock guinea pigs using the enzyme-linked immunosorbent test (ELISA) (Leyva-Grado et al. 2012). Additionally, guinea pig, ferret, and mouse are considered as the standard animal model of human IBV studies with efficiently viral replication, transmitting, and highly suitable for vaccine development (Pica et al. 2012, Elderfield et al. 2015, Do et al. 2024).

1.5. Genomic structure and function of Influenza B virus

Closely related to IAV, IBV contains genomic information in eight segmented of negative sense ssRNA. However, even though IBV and IAV share a common ancestry, there is no exchange of genetic information between these viruses. Reassortment and genetic drift have been reported to occur only within genera (RA 1995).

Not only are clinical symptoms indistinguishable, but IAV and IBV share morphological similarities under electron microscopy as well. The sphere-shaped, approximately 100nm-sized, and pin-like glycoprotein spikes of HA and NA on the surface membrane of the virus are alike (Negi et al. 2022). Similar to IAV, IBV organizes its genetic information into segmented units, with most of them encoding for a single viral protein (Chen et al. 2001).

1.5.1. Segments I, II, and III

On these segments, IBV encodes for polymerases proteins. Segment I encodes for polymerase acidic protein (PA), segment II encodes for polymerase basic protein 1 (PB1), and segment III encodes for polymerase basic protein 2 (PB2). The function of these polymerases is reported to have similar functional characteristics as IAV in viral replication process (RA 1995). During the viral replication process, PB1 serves as the central component of the RNA elongation activity of the RNA-dependent RNA polymerase (RdRP) complex. PB2 plays a crucial role in initiating RNA transcription as a cap-binding-like function, while PA acts as an enzyme breaking down complementary RNA (cRNA), exhibiting displacement activity exhibited during influenza viral replication (Deng et al. 2011).

1.5.2. Segment IV

Similar to segments I, II, and III, this segment only encodes for the protein hemagglutinin buildup for surface glycoprotein, which primarily facilitates the receptor attachment and fusion activities of virus particles into host cells. Influenza HA protein plays the initial role of the viral infection; the interaction of HA and host cell sialic acid (SA) receptors is the primary stage of viral invasion of host cells by influenza viruses (de Graaf and Fouchier 2014). In humans, HA of IBV also has the ability to bind with SA of both α-2,6 and α-2,3-linkage on ciliated and type I pneumocyte cells. The previous report on the crystal structure of IBV/HA indicated the differences from IVA/HA. In contrast to IAV/HA, which has Tyr-98 on the receptor-binding site, IBV/HA has Phe-95 instead (Ni et al. 2014). During the IAV/HA banding stage, three hydroxyl oxygen atoms on the side chain bond with the host receptor bonding site to stabilize the fusion activities. In IBV, the absence of these hydroxyl oxygens on the side chain bond of IBV/HA might be responsible for the limitation of host range in IBV (Wang et al. 2007, Wang et al. 2008).

1.5.3. Segment V

Segment V of IBV contains the genetic information necessary for the synthesis of nucleocapsid protein (NP). This protein is crucial for viral replication, transcription, and assembly, playing a central role in the lifecycle of the virus. It exhibits significant differences compared to the NP of the IAV. Notable distinctions include the absence of the two nuclear localization signals (NLSs) in IAV, which are located in the NP of IBV, which is about 150 nucleotides longer at the N-terminus. NP of IBV possesses a distinct set of 50aa at its N-terminus that is absent in NP of IAV (Stevens and Barclay 1998a). As reported by the study of Stevens and Barclay, 1998 (Stevens and Barclay 1998b), the N-terminal extension of B/NPs has shown a high degree of conservation in the amino acid sequence and might bear as the conserved function in IBV.

1.5.4. Segment VI

Similar to IAV, segment VI of IBV encodes Neuraminidase (NA) as a surface glycoprotein. Its main role is to facilitate the interaction with host SA by producing sialidase resulting in receptor destruction, which helps release viral progeny and promote the transmission of the infection to cells around it. This function is similar to the NA of IVA (Gottschalk 1957, Wagner et al. 2002). The functional form of IBV NA exists as a tetramer, comprising monomers, each adorned with two glycosylation sites (Asn283 and Asn143) and a

site for binding calcium ions. Asn283 is unique to IBV, whereas Asn143 remains consistent across various influenza viruses. The active site shared by NAs in both IAV and IBV consists of nineteen residues, highly preserved throughout their evolutionary divergence. The residues located in the catalytic site (R118, D151, R152, R224, E276, R292, R371, and Y406) have direct interactions with SA. Conversely, the foundational residues (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425) provide essential structural stability to the enzyme's active site. Notably, neuraminidase inhibitors (NAIs) employed in clinical therapy against IAV infections demonstrate reduced efficacy against IBV (Din et al. 2023).

1.5.5. Segment VII

In this segment, two proteins are encoded, known as matrix proteins 1 (BM1) and 2 (BM2). The specific function of BM1 in the virus life cycle remains unclear; however, it moves between the nucleus and cytoplasm, exhibiting nuclear export signals (NES) and a nuclear localization signal (NLS) (Wang et al. 2009, Koutsakos et al. 2016). BM1 has the highest degree of amino acid residue conservation among all viral proteins, with a length of 248 amino acid residues, and shares 30% of its viral genome with AM1 (Kordyukova et al. 2019, Wolff and Veit 2021). Because of their stability, BM1 and its IAV homologue are routinely chosen as the template targets for the detection of influenza viruses (Fouchier et al. 2000).

BM2 is a second protein encoded in this segment, its main function is as an ion channel, specifically a proton channel, which plays a significant role in viral replication and entry into host cells. The BM2 protein is synthesized during the later stages of viral infection in order to prevent premature fusion of the viral envelope and ensure the formation of viable virus particles (Imai et al. 2004). This protein is synthesized from a +2 open reading frame of 109 amino acid residues long. The translational approach employed is distinct due to the overlap between the starting codon of the BM2 and the stop codon of BM1, which is represented by the stop-start pentanucleotide UAA**A**UG (stop- and start-codon of BM1 and BM2) (Hatta et al. 2004).

1.5.6. Segment VIII

Segment VIII encodes two proteins: NS1 (NS1B) and NEP or NS2 (NS2B). NS1B is essential for the replication of the virus. The protein contains 281 amino acid residues, 51 amino acid residues longer than those in IAV/H1N1 (Bouvier and Palese 2008). It divides into three sections: N-terminus (residues 1–90), C-terminus (residues 120–281), and a corresponding region (residues 91–119) (Reich et al. 2014). NS1B is found in the host

cytoplasm in the later stage of infection; it interacts with importin α3 to transport vRNA from the host cell's cytoplasm to the nucleus by the function of residues 46–56 in the N-terminus; and it is localized in nuclear speckles containing the splicing factor SC35 (Din et al. 2023). Contrary to IAV, NS1B is unable to inhabit host mRNA exporting due to a lack of interaction of the NS1B C-terminus with host CPSF30 (Schneider et al. 2009). C-terminus of NS1B lacks the function to inhabit host mRNA, including antiviral mRNA. However, it is capable of recognizing variety aspects of Interferons (IFNs), initiating with inhibit IFN-αβ response by preventing activation and translocation of Interferon regulatory factor 3 (IRF3) (Donelan et al. 2004).

Segment VIII encodes NEP, or NS2B, as the second protein, which is a component of the virion viral ribonucleoprotein (vRNP). The N-terminus of NS2B has a nuclear export signal, and export activities are in the virion budding stage. Moreover, it arises in the latter phase of infection, initiated by the buildup of vRNA within the nucleus of the host cell. Subsequently, the vRNA is transported to the cytoplasm and ultimately concentrated within specific regions of the plasma membrane, where virions are assembled (Imai et al. 2003). However, the specific role of this protein remains unclear but is believed to be similar to that of NS2A (Din et al. 2023).

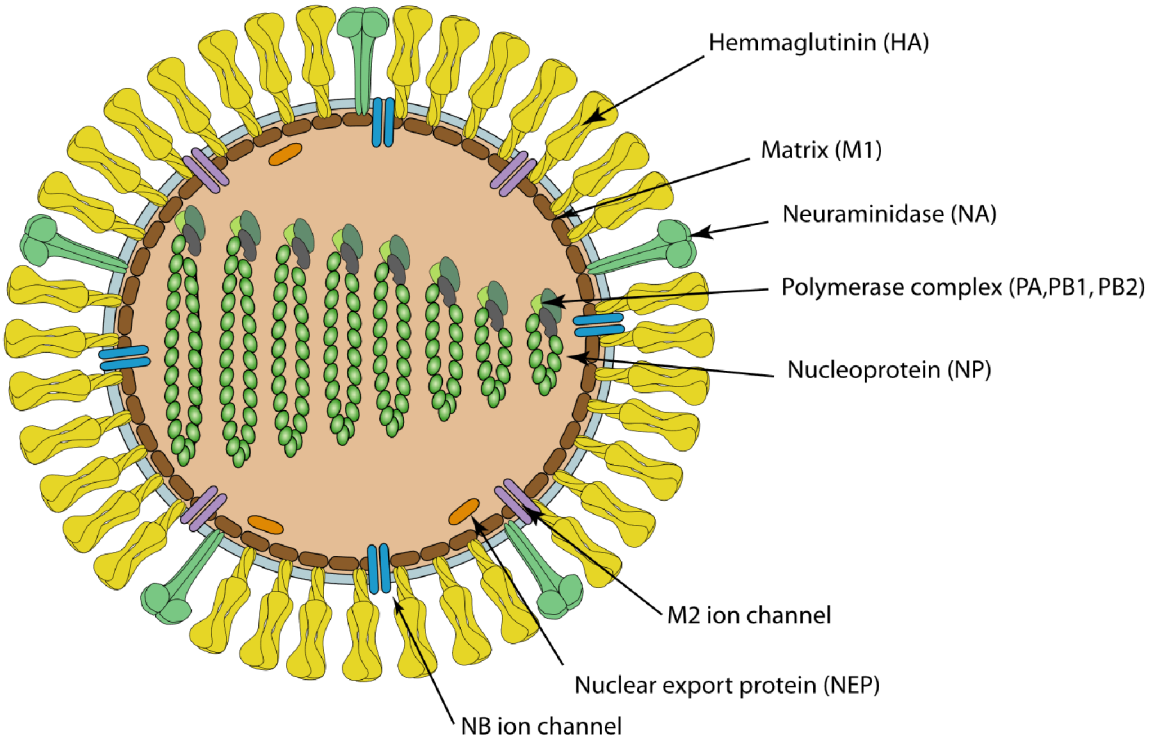


Figure 2: Structure of IBV particle and genome (ViralZone 2020).

1.6. Influenza virus detection

There are three types of influenza viruses (IAV, IBV, and ICV) known to cause respiratory infections in humans. IDV, a new influenza virus that emerged in cattle over a decade ago, may also have the potential to be zoonosis-infective (Asha and Kumar 2019, Liu et al. 2020). These viruses share similar clinical symptoms that are hard to distinguish and can easily lead to confusion in diagnosis. Even more confusion arises when other contagious respiratory diseases caused by other viruses or bacteria share similar clinical symptoms as influenza pathogens (Gavin et al. 2004, Dziąbowska et al. 2018). Generally, medical lab technicians often employ conventional methods such as serological assays and nucleic acid-based tests for the detection and identification of these pathogens. RT-PCR, ELISA, and HI are widely recognized as the most reliable methods for detecting viruses, including influenza viruses (Aoki et al. 2021). Other assays such as Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a nucleotide-based diagnostic approach that was developed in the early 2000s (Notomi et al. 2000). This method provides simplicity, specificity, sensitivity, and rapidity under isothermal conditions, leading LAMP assays to develop a significant and useful diagnostic test to identify bacteria, viruses, and parasites. This test can possibly be conducted in both field and high- or low-tech laboratory settings (Sun et al. 2014).

1.6.1. Serological Assay

A serological assay is a laboratory procedure used to detect and measure the presence of certain antibodies in an individual's biological serum samples. It serves the purpose of determining the existence and quantity of antibodies in the bloodstream. It is employed for the diagnosis and monitoring of infectious diseases. Antibodies are an important polypeptides; they are synthesized by the immune system in response to an infection. The immune system secretes these substances into the bloodstream and other tissues. They adhere to the specific pathogenic protein (Alhabbab 2018). The presence of antibodies against a pathogen in a tested organism therefore shows previous infection of this organism by this particular pathogen. Hemagglutination inhibition assay (HAI) and enzyme-linked immunosorbent assay (ELISA) are commonly employed as serological methods for detecting influenza (Li et al. 2017).

1.6.1.1. Hemagglutination inhibition assay

Hemagglutination Inhibition Assays (HI or HIA) is one of the most trustworthy and often employed assays for serologic surveys and seroepidemiologic studies. This method is frequently employed to detect the influenza virus by preventing the interaction between the

viral spike protein HA and the receptor on red blood cells (Dziąbowska et al. 2018). This method was developed by Hirst (1941), and first reported on influenza virus on the same year by McCLELLAND and Hare (1941) as a method measuring the concentration of antibodies against IAV. With its simple use, low cost, effectiveness, and rapid performance, HI has become commonly preferred for antibody analyses of influenza viruses. This method is based on the hemagglutination activity of the influenza HA protein and the capacity of HA-specific antibodies to identify the specific HA subtype of antibodies to the virus (Steinhauer 1999). However, the major issue with this method is non-specific HA and HA inhibitors, since other organisms also produce HA proteins (Spackman 2014).

1.6.1.2. Enzyme-linked immunosorbent assay

The ELISA test is acknowledged as the most useful and reliable serological diagnostic technique for influenza diagnosis (Newton et al. 2000), and has also been used to detect other infectious diseases (Leirs et al. 2016). It was originally and independently invented by two researchers at the same time by Engvall and Perlmann (1971). It comes with the primary advantages of high effectiveness, high throughput, ease of performance, and low cost, but the sensitivity and specificity are still limited when come to compared with molecular-based methods (Dziąbowska et al. 2018). Currently, there are 4 most commonly used types of ELISA, each possessing its own set of benefits.

I. Direct ELISA

This process, the biological sample (such as serum, blood, or saliva) is introduced into the microplate wells and attached to the bottom surface. Next, introduce the specific primary antibodies to the well, allows the binding if the antigens presence in the sample. In the final step, substrate and enzyme are added to interact with the attached antibodies-antigens, resulting in a color change if certain antigens are presence. The major disadvantage of this technique is that it works well only with a high concentration of antigens in the serum (Aydin 2015).

II. Indirect ELISA

This method was developed in 1978 by Lindström and Wager with the purpose of determining lower concentrations of antibodies than direct ELISA (Lindström and Wager 1978). This method has similarities to direct ELISA, starting with the same procedure by introducing the antigen into the wells, followed by the introduction of the serum sample. To

ascertain the outcome, a secondary antibody tailored to recognize the antibody in the serum is combined with an enzyme. Subsequently, the enzyme is supplied with a substrate to induce a color change, facilitating the determination of the concentration of the targeted antibodies (Aydin 2015). However, this method required enzyme-conjugated species-specific antibodies, which became the limitation of AIV surveillance investigations in non-model animal species (Song et al. 2009b), and the most available commercially iELISA tests for IAV in birds usually employ anti-chicken/turkey Ig secondary antibodies, which exhibit higher sensitivity and specifically in chickens but may not be as effective to test for IAV in natural reservoir wild-birds (Brown et al. 2009).

III. Sandwich ELISA

This method was initially developed by KATO et al. (1977). In this process, captured antibodies are applied to the microplate in order to bind to the surface of the wells. Afterward, the samples are introduced and interact with the captured antibodies, promoting the attachment of antigens. Unbound antigens are removed by a washing step after incubation. Subsequently, secondary antibodies conjugated with the specific enzyme for the targeted antigen are introduced and subjected to incubation. If the targeted antigens are present and attached to enzyme-tagged antibodies, they cannot be removed during the following washing step. Subsequently, the enzyme substrate is introduced into the solution and determined by a colorimetric reaction. This technique is referred to as sandwich ELISA because the antigen of interest gets lodged between two antibody molecules. Previous research has indicated that sELISA displays a greater sensitivity by 2–5 times than other types of ELISA, also for detection of influenza viruses (Aydin 2015). Therefore, this method is suitable for identifying viral antigens at the initial phase of infection. Additionally, the sELISA is capable of differentiating between subtypes of influenza, which is essential for precise diagnosis and treatment (Chen et al. 2019).

IV. Competitive ELISA

Competitive ELISA (cELISA) is another type of ELISA. It was originally developed by Yorde et al. (1976). The setup of this method is similar to other types of ELISAs. It involves introducing the sample and enzyme-tagged antigen or antibody into wells coated with antigen-specific antibody or antibody-specific antigen (reference antigen or antibody). If the targeted antigen or antibody is present in the sample, they will compete with the reference antigen or antibody in the wells for binding. Following incubation and washing to remove unattached

molecules, an enzyme substrate is introduced afterwards. In contrast to other types, the results will be inverted. If the amount of antigen or antibody binding to the reference antigen or antibody is low, the higher concentration of the targeted antigen or antibody in the sample, which will result in a lower signal (Aydin 2015). In cases of influenza-related studies or diagnoses, this method is acknowledged as a useful approach for viral surveillance or the evaluation of vaccine effectiveness. The main benefit of cELISA is its high sensitivity and not required species-specific enzyme-conjugated antibodies as iELISA (Song et al. 2009a).

1.6.2. Molecular Amplification Assay

Molecular diagnostic assay is the method used for the detection and identification of a biological organism in the molecular level. In the field of molecular biology, amplification refers to the enzymatic replication of a nucleic acid molecule, resulting in the production of a new population of molecules that have the exact same sequence as the original one. PCR is the most often employed technique for amplification. An "amplicon" refers to the outcome of a PCR amplification of a specific RNA or DNA molecule (Lanciotti 2003).

1.6.2.1. Reverse-transcription polymerase chain reaction assay

The initial PCR-based test for identifying influenza virus was documented in 1991 (Zhang and Evans 1991). Theoretically, PCR can only be applied to DNA, in the case of single-stranded RNA, reverse-transcription is required to transcript RNA into cDNA by introducing an enzyme called reverse transcriptase (Artika et al. 2020). RT-PCR is a very effective method for detecting influenza vRNA in throat swab and is often considered the most effective test for influenza detection globally. The method employs specific primers for the identification and classification of influenza viruses (Gavin et al. 2004). In response to the flu-pandemic 2009, the Centers for Disease Control and Prevention (CDC) in USA advised use the one step RT-PCR technology, which specifically targets on the matrix gene of H1N1/swine/09 (Carr et al. 2009). The quality of primers is very important for achieving reliable results and prevents false positives caused by primer-dimers where the primers fold upon themselves, amplifying a new population of non-specific sequences (Garafutdinov et al. 2020).

Because it provides high sensitivity and specificity, RT-PCR has been widely considered the gold standard for viral detection and diagnosis. There are two methods available for performing real-time RT-PCR: one-step and two-step.

In one-step RT-PCR, RT and PCR are combined in this method. This procedure amplifies RNA in a single reaction by reverse transcribing it to cDNA. In comparison to serological and viral culture tests, the primary benefits of this method are its sensitivity, specificity, and minimized sample handling, which reduces the chance of sample contamination and erroneous pipetting (Gavin et al. 2004).

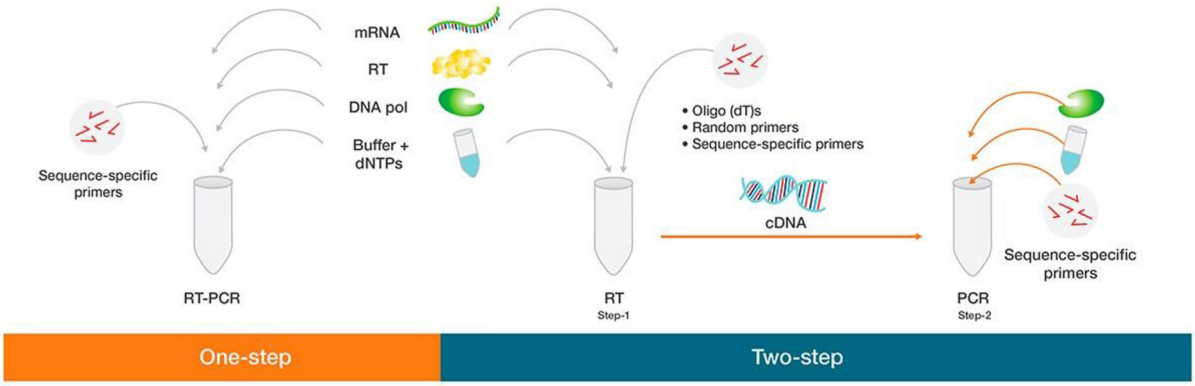


Figure 3: Differentiate between one-step and two-step RT-PCR (Thermofisher Inc, 2023).

In two-step RT-PCR, conventional PCR is used to amplify the cDNA after the RT reaction, which is performed independently from PCR to create cDNA. The main benefit of this approach is that the cDNA can potentially be conserved for use in future research on other genes of interest from the same virus sample (Wacker and Godard 2005). However, RT-PCR necessitates highly skilled technicians, specialized facilities, and expensive equipment, which might be a challenge for limited labs in developing countries and under field condition (Gavin et al. 2004, Chen et al. 2014).

1.6.2.2. Reverse transcription loop-mediated isothermal amplification

Molecular amplification-based method has been long relied on PCR test since 1980s, and almost the only option for nucleic acid-based diagnosis (Heilek 2016). PCR products work as displacement of the targeted sequences and re-initiation of a new cycle of DNA synthesis for each amplification, which required multiple temperature phases to achieve new DNA/RNA sequences (Notomi et al. 2000). Therefore, the PCR process required heat for DNA denaturation, followed by cooling down for the primers to attach in the annealing step, and increasing the temperature for the primers to extend synthesizing new sequences of the targeted template. The repeatedly multi-temperature steps in PCR products require expensive machines to perform these cycling (Balm et al. 2012). Alternative molecular-based diagnostic methods became increasingly important in purpose to reduce the cost and complex performance steps of PCR systems. LAMP is another nucleotide-based diagnosis method that can be used to

amplify DNA under isothermal temperature and was developed in the early 21st century by Notomi et al. (2000). In the LAMP process, the amplification initials from 5' to 3' by inner primers contained two distinct primer sequences of positive-sense and negative-sense, followed by denaturation by displacement DNA synthesis by outer primers, and other inner and outer primers employ the same process at the other end. This process continues until a loop-shape forms after phase 4, after which only the inner primers remain working (Figure 4) (Notomi et al. 2000). Loop primers have been developed by Notomi et al. (2004), which locates between inner primers (F2 and F1c or B2 and B1c) to increase the sensitivity and reduce the performance time of the LAMP products. This improvement has been succeeded by experiments on hepatitis B viruses, which indicated the possibility of determining the result in less than 30 minutes.

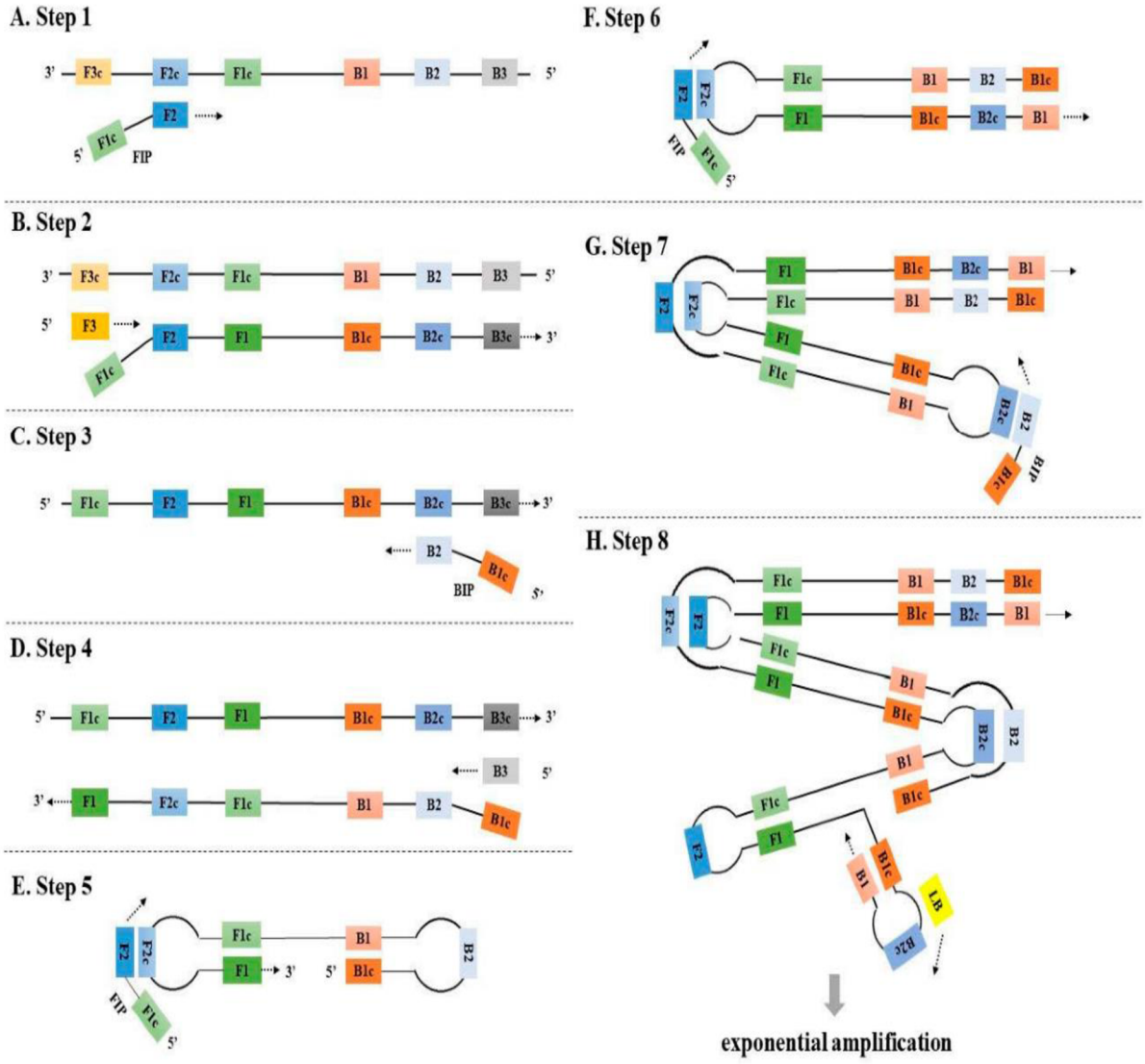


Figure 4: Structure of LAMP process (Zhang et al., 2023)

LAMP method provides simply use, low cost, specificity, sensitivity, and rapidity under isothermal conditions (Notomi et al. 2004). The LAMP technique has been reported to successfully detect many infectious pathogens (virus, bacteria, and parasite) (Parida et al. 2008, Xie et al. 2014), including the recent COVID-19 pandemic caused respiratory tract illness by the SARS-CoV-2 virus (Huang et al. 2022). Therefore, it becomes most commonly used overall for the isothermal molecular amplification tests by its great benefit of rapid and simple-used with the great potential of point-of-care by single temperature step performance (Zhang et al. 2014). In contrast, other isothermal diagnostic methods that required additional enzymes for amplification performance, LAMP required only DNA polymerase like PCR (Tanner 2019). For infectious RNA-virus, RT-LAMP products required one additional enzyme called RNA reverse transcriptase in order to synthesize cDNA according the RNA template (Poon et al. 2005).

2. Aim of the thesis

The primary objective of this study is the development of a novel and effective LAMP diagnostic test against IBV. To do so I designed several sets of primers, determined the specificity and sensitivity of LAMP reaction using one selected set of these primers.

Primers are planned to be used in the future laboratory studies for the detection and identification of IBV for e4 research team in the Pathogen detection and characterization laboratory at the Faculty of Tropical AgriScience (FTZ), Czech University of Life Science Prague (CZU).

3. Method

3.1. Samples collection

This study divided the sample into 5 different groups: both lineages of IBV (Victoria and Yamagata), no-RNA temple control (N/C), asymptomatic personals, and cross-reaction test SARS-CoV-2. IBV and SARS-CoV-2 positive samples were provided by the National Reference Laboratory for Influenza and Other Respiratory Viruses of the National Institute of Public Health of the Czech Republic (NIPH). Saliva samples were collected from asymptomatic volunteer staff and students at FTZ, CZU.

No	Number of samples	Type of sample	Confirm by	Source
1	5	B/Yamagata	RT-PCR	NIPH
2	6	B/Victoria	RT-PCR	NIPH
3	1	SARS-CoV-2	RT-PCR	NIPH
4	12	asymptomatic personals	-	FTZ
5	-	N/C	-	-

Table 1: list of sample group.

3.2. Viral RNA purification

Viral RNA extraction was carried out using the GeneJET™ Viral DNA and RNA Purification Kit. The procedure in this step was followed according to the manufacturer's protocols of GeneJET^{TM}. The process was divided into five stages for the extraction of viral RNA from the samples: solution preparation, lysis, binding, washing, and sample elution. In advance of commencing the procedure, it is crucial to prepare the necessary equipment, including pipettes, nuclease-free pipette tips, a vortex, a microcentrifuge, a thermomixer, disposable gloves, a measuring cylinder, tubes, microcentrifuge tubes for sample lysis and elution, RNase-free tubes for RNA elution, and 96–100% ethanol.

I. Solution preparation:
    1. Wash Buffer: Washing buffer 1 (25mL of wash buffer and 15mL of Ethanol), Washing buffer 2 (11mL of wash buffer and 44mL of Ethanol).
    2. Mixture of lysis and carrier RNA: for mixture is required to be prepared in advance. According to manufacturer's protocols of geneJET^{TM}, 1 sample required 220 µL of Lysis solution and 5.5µL of carrier RNA.
    3. Spin column: Add 50µL of Column Preparation Liquid to the center of Spin Column membrane and keep at room temperature.

II. Lysis sample:
    1. Add 200µL of sample to an empty 1.5-mL lysis tube.
    2. Add 200µL of Lysis Solution that already contained Carrier RNA
    3. Add 50µL of Proteinase K and mix the contents using either a vortex or a pipette.
    4. Keep the mixture in the thermomixer for 15 minutes at 56°C and Centrifuge at maximum speed for 5 seconds.
III. Binding step:
    1. Add 300µL of ethanol and mix well and keep at room temperature for 3 minutes. Centrifuge for 3–5 seconds at full speed.
IV. Washing step:
    1. Transfer the contents into the Spin Column that has been previously prepared in the wash tube. Centrifuge for 1 minute at 6,000x.
    2. Remove the Wash Tube with the liquid that has passed through.
    3. Place the Spin Column into a new wash tube and add 700µL of washing buffer 1 to the Spin Column. Centrifuge for 1 minute at 6,000x.
    4. Change the spin column into a new washing tube and add 500µL of the washing buffer 2 to the Spin Column. Centrifuge for 1 minute at 6,000x.
    5. Change the spin column into a new washing tube. Centrifuge for 3 minutes at 16,000x.
V. Elute step:
    1. Transfer the spin column after centrifuge into a new elute tube.
    2. Add 50µL of Eluent solution to the center of spin column membrane and keep at room temperature for 3 minutes. Centrifuge the column for 1 minute at 13,000x. Note: Eluent solution needed to be 56°C at the time of use.
    3. The flow-through solution contains pure viral nucleic acids. Store at -20°C to -70°C.

3.3. Primer construction

Primer constructions were carried out on 74 randomly selected sequence samples of segment VII of European IBV isolates (submitted to GISAID database between January 2020 and February 2022). All sequences were aligned with BioEdit and analyzed for the identification of the most conserved region. Because genomic alignment results did not provide sufficient support for selecting a specific region for primer development in both IBV lineages,

B/Victoria was selected as the primary focus for primer development. This decision was made based on recent research indicating that B/Yamagata may have become extinct (Caini et al. 2023, Do et al. 2024). A 291-bp-long sequence with the lowest number of mutations of B/Victoria was used as the target for primer construction. Five sets of RT-LAMP primers were designed using Primerexplorer version 5 (https://primerexplorer.jp/e/v5_manual/index.html). One set of primers contains four primers: forward outer (F3), forward inner primer (FIP), backward outer (B3), and backward inner primer (BIP). Loop primers, forward loop primer (FL), and backward loop primer (BL), were designed separately using the Bio-Tools Oligonucleotide Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html).

3.4. Primer determination test

The main objective of constructing these primers were to develop a rapid, simple assay for IBV detection under isothermal conditions. The developed primers were evaluated for their capacity to detect IBV using two independent techniques: 1) Colorimetric LAMP and 2) Real-time florescent LAMP.

To set up the reaction for colorimetric LAMP, we used Warmstart® Colorimetric LAMP 2x Master Mix or Warmstart® LAMP Kit [New England Biolabs (NEB)] according to the manufacturer's protocols. The reaction was carried out by using the T100 thermal cycler (Bio-Rad) for colorimetric experiments and the CFX96 real-time PCR machine (Bio-Rad) for fluorescent experiments. The reactions were incubated at 65°C.

Primer's preparation: To simplify the setup of reactions and ensure the appropriate concentration of primers, we decided to utilize a 10x combination of 6 primers for this experiment.

Primer	Stock (100x)	Primer mixture
FIP	100 µM	16 µl
BIP	100 µM	16 µl
F3	100 µM	2 µl
B3	100 µM	2 µl
FL	100 µM	4 µl
BL	100 µM	4 µl
dH2O		56 µl

Final mixture (10x)		100 µl (16µM of FIP/BIP, 2µM of F3/B3, 4µM of FL/BL)

Table 2: Mixture of LAMP primers.

3.4.1. Colorimetric experiments:

The reactions were prepared according to the Warmstart® Colorimetric LAMP 2x Master Mix [DNA &RNA, New England Biolabs (NEB)] protocol. After 30 minutes of incubation at 65 °C, the reaction was observed at 5-minute intervals. In the event that no color changes after 40 minutes, an additional 10 minutes is recommended (Biolabs 2020). If the reaction started after 50 minutes of incubation, the result will be determined as a false-negative.

	IBV template	SARS	Asymptomatic volunteer	N/C
M1800 MM (2x)	12.5 µl	12.5 µl	12.5 µl	12.5 µl
Primer mix (10x)	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Template sample	1 µl	1 µl	1 µl	-
dH_2O	9 µl	9 µl	9 µl	10 µl
Final volume	25 µl	25 µl	25 µl	25 µl

Table 3: Mixture of colorimetric LAMP experiments.

3.4.2. Real-time fluorescent dye experiments:

The reactions were prepared according to the to the WarmStart LAMP Kit Protocol (NEB) by mixing 12,5 µl of 2x LAMP MM (NEB M1700S), 0,5 µl of 50x fluorescent dye (NEB B1700AA), 2,5 µl of 10x LAMP Primer Mix, 8,5 µl of dH2O, and 1 µl of template RNA (dH2O was added for no RNA negative control). The reactions ran at 65 °C for 68 minutes, and fluorescence was measured every 30 seconds. Samples were analyzed in triplicate. A sample was considered positive if at least two reactions in triplicate showed some DNA amplification before 50 minutes of incubation.

	IBV template	SARS	Asymptomatic volunteer	N/C
M1700S MM (2x)	12.5 µl	12.5 µl	12.5 µl	12.5 µl
B1700AA Fluorescent (50x)	0,5 µl	0,5 µl	0,5 µl	0,5 µl
Primer mix (10x)	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Template sample	1 µl	1 µl	1 µl	-
dH_2O	8.5 µl	8.5 µl	8.5 µl	9.5 µl
Final volume	25 µl	25 µl	25 µl	25 µl

Table 4: Mixture of LAMP florescent dye assay for one sample.

4. Result

4.1. Primer construction

The RT-LAMP primers were designed in the region with the lowest mutation rate in the IBV genome of segment VII. Five sets of primers were designed with the ability to detect distinct regions on BM1 gene. Only the set of primers showing the best parameters according to the Primerexplorer was finally used for the experiment based on the location of the inner primers that was most suitable for loop primers construction, according to the study of Notomi et al. (2004).

The primer set is composed of two outer primers (F3 and B3) and two inner primers, called the forward inner primer (FIP) and the backward inner primer (BIP). The FIP and BIP primers consist of reverse transcription F1c and B1c. Two loop primers (FL and BL) were manually constructed independently with the assistance of OligoCalc. The sequences of primers used further in LAMP assay are shown in Table 5. Sequences of the remaining primers sets are not shown in the thesis but can be provided upon the request.

Primer	Sequence (5'-3')
F3	CATTGATAGAAGATGGAGAAGG
B3	GATAAGGGCTCTGTGATGA
FIP (F1c-F2)	CAAGGCTGAGTCTAGGTCAAATTC-AGAACTAGCAGAAAAGTTACACT
BIP (B1c-B2)	ATACAAAAAGCACTAATTGGTGCC-TTTTTCTTTCCTGGTCTTTGG
FL	CTTTCCCACCAAACCAACA
BL	GCCTCTATATGCTTTTTAAAACCCA

Table 5: Primers sequences using as a template genome segment 7 of IBV coding matrix protein 1 (M1) and BM2 protein (BM2).

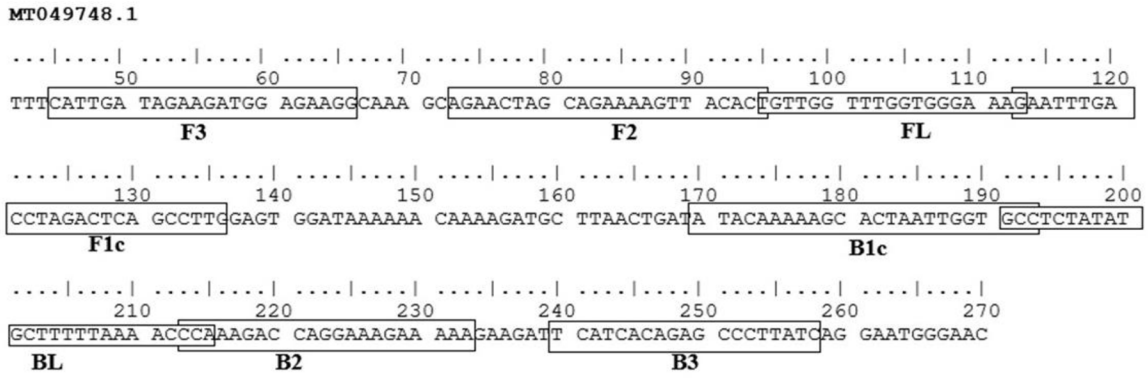


Figure 5: Position of primers on the M1 gene on IBV genome in NCBI (No. MT049748.1).

The primers were aligned with the segment VII of the reference B/Victoria strain virus isolate B/Texas/43/2019 genome sequence (ID MT049748.1) of the of the National Center for Biotechnology Information (NCBI) to see the precise location of the primer set (Figure 5).

4.2. Optimalization of RT-LAMP products

A colorimetric and fluorescent dye experiment was conducted using a set of designed primers for the RT-LAMP assay to detect against IBV in a specific region of M1, with the objective of determining the specificity and sensitivity of the reaction. The set of primers was evaluated through 11 clinical samples of both lineages of IBV (B/Victoria and B/Yamagata) that were isolated during the seasonal flu outbreak in the Czech Republic between 2017 and 2018 by the NIPH research group. Additionally, 12 nasal swab samples of asymptomatic volunteers were collected from healthy staff and students of FTZ, and one positive SARS-CoV-2 RNA confirmed by PCR during the COVID-19 pandemic was provided by the NIPH research group. The samples have been divided into four groups, which have been mentioned in the method.

4.2.1. Sensitivity and specificity determination

Colorimetric and fluorescent dye assays were used to evaluate the sensitivity and specificity of the chosen set of primers. For the colorimetric experiment, we observed the reaction of color changing from pink to yellow after 30 minutes of incubation at 65 °C and at 5-minute intervals, and the fluorescent dye was determined every 30 seconds for 68 minutes.

4.2.1.1. Colorimetric experiment

The color development of colorimetric products is evidenced by the presence of RNA amplification, as evidenced by the pH change in the solution. The result is determined by sight observation of a color reaction that changes from pink to yellow in a positive sample and remains pink in a negative sample (no RNA is amplified). The result of the RT-LAMP colorimetric products, which contained both B/Victoria and B/Yamagata RNA, SARS-CoV-2 RNA templets, and a no-RNA negative control, indicated that all the B/Victoria samples started to develop color reactions after 35 minutes (Figure 6), while only 2 B/Yamagata samples showed some reaction (Figure 7). Samples 2 and 3 of B/Yamagata started a clearly visible color change after 40 minutes, while all B/Vitoria samples finished the reaction. After 45 minutes of incubation, two samples of B/Yamagata completed the reaction, and the rest remained pink.

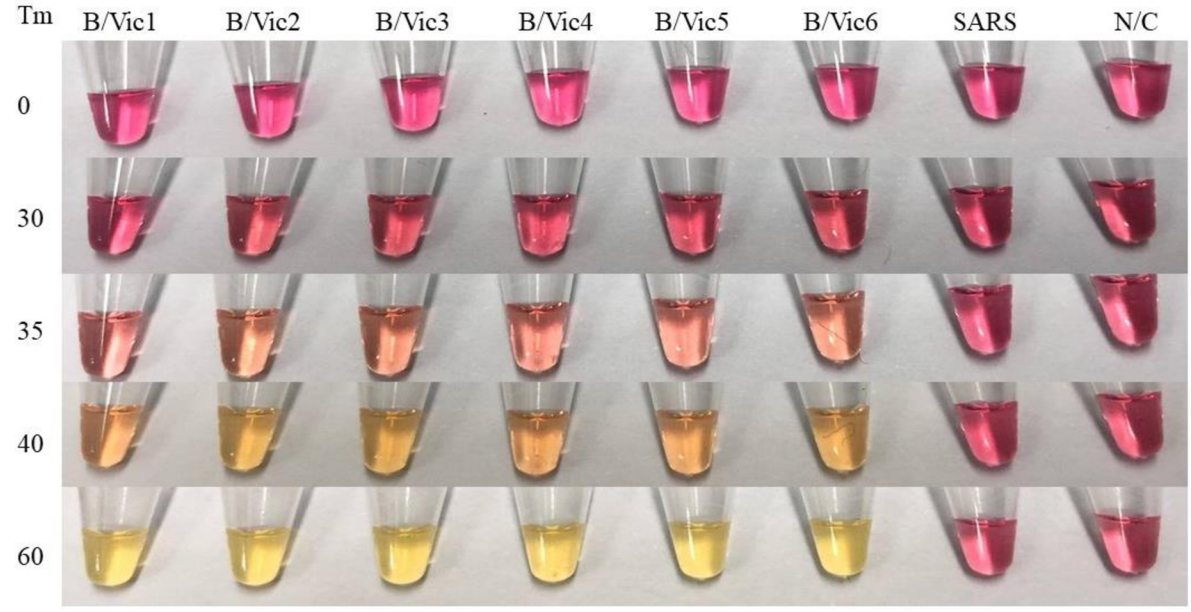


Figure 6: The reaction time of colorimetric test on B/Victoria, SARS-CoV-2, and N/C. Tm – time in minutes.

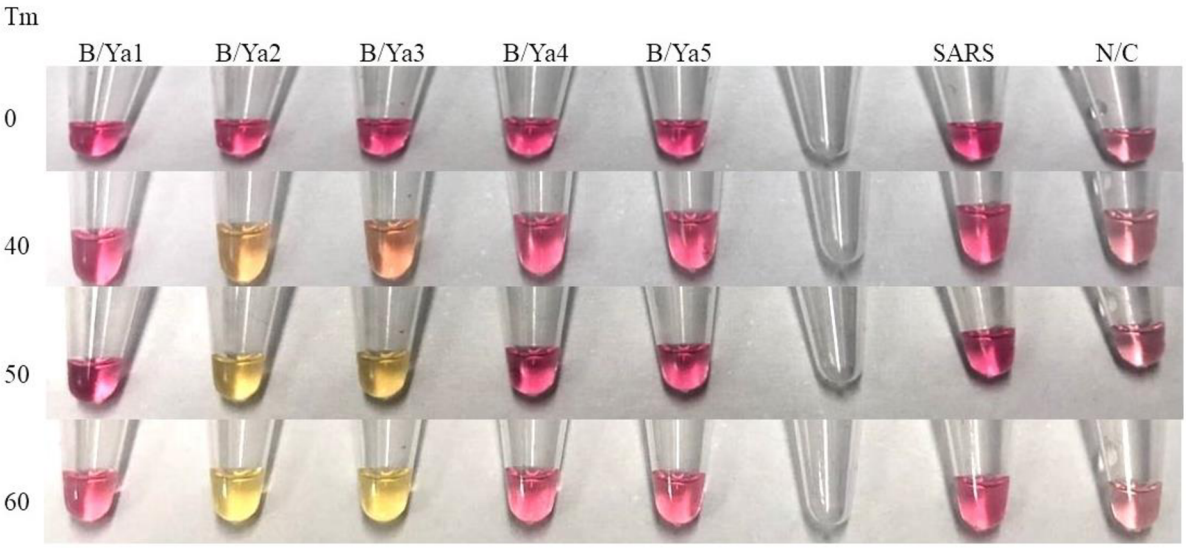


Figure 7: The reaction time of colorimetric test on B/Yamagata, SARS-CoV-2, and N/C. Tm – time in minutes.

Both experiments demonstrated the absence of any cross-reactions throughout the procedure. The findings indicated that all six samples harboring B/Victoria strains yielded positive results, while only two out of five samples containing B/Yamagata strains tested were positive. The false negative rate was determined to be 0% for B/Victoria strains, contrasting with a 60% false negative rate for B/Yamagata strains. Consequently, the sensitivity for B/Victoria strains was calculated at 100%, whereas for B/Yamagata strains, it was notably lower at 40%. The overall sensitivity across both strains was determined to be 72% (8 out of 11 samples). Experiments of cross-species reactions on SARS-CoV-2 and no-RNA templates

indicated no instances of false positives throughout the duration of the experiment (Figures 6 and 7).

The result of asymptomatic volunteer personnel and students from FTZ, CZU, have shown no RNA amplification by the evidence of no color changed (Figure 8), while the reference B/Vic1 P/C samples showed visible clear color change at 40 minutes. Furthermore, the experiment was extended to determine the specificity length of false-positive reactions caused by the appearance of the primer dimer (PD). Two samples from asymptomatic volunteers exhibited a partially visible color reaction at 70 minutes, reached completion 30 minutes later (at 100 minutes) (Figure 8), while one of N/C and most of the sample from asymptomatic volunteers (7/12) showed a false-positive reaction at 100 minutes and completed 20 minutes after. The outcome quantified the result: 0 samples out of 12 negative samples were positive; the false positivity ratio is 0%, which means specificity is 100%, and PD formation is even after 70 minutes.

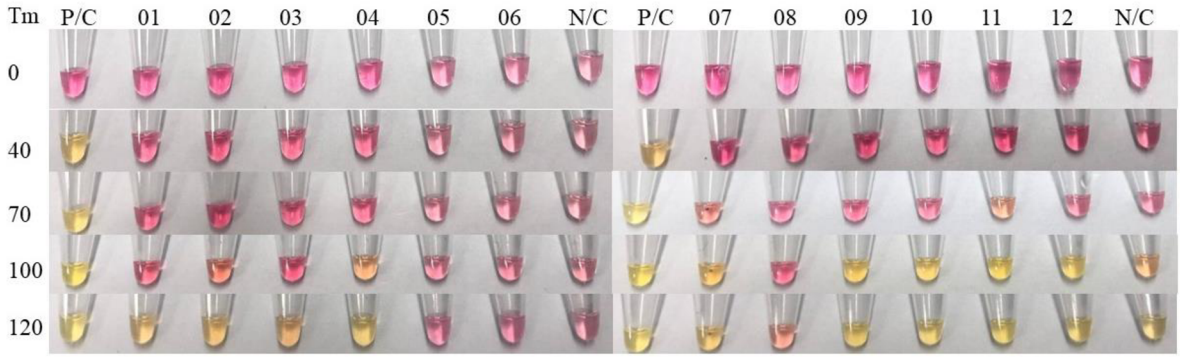


Figure 8: The reaction time of colorimetric test on asymptomatic volunteer. Tm – time in minutes.

4.2.1.2. Real-time RT-LAMP fluorescent dye experiment

In order to determine the quality of the primers, each sample of IBVs was employed in three distinct microtubes independently. The real-time RT-LAMP fluorescent dye yielded a quicker amplification onset, occurring at an average yielding point of 16.5 minutes (Figure 9) for B/Victoria and 27 minutes for B/Yamagata (Figure 10), similar outcome compared to colorimetric products (Figures 6 and 7). The amplification process for the Victoria strain was completed within an additional 5 to 10 minutes. On the contrary, only one sample of B/Yamagata had an early onset at an average yielded point of 17.66 minutes, while the others started after 30 minutes of reaction (Figure 10). Only one reaction coming from the B/Yamagata sample displayed a false negative result (no amplification of RNA). Additionally, there were

no false positives observed from N/C until 60 minutes, when one of the N/C samples showed a somewhat amplified curve. The fluorescent dye detection revealed that the designed primers specifically targeted the M1 gene, resulting in 100% sensitivity in both B/Victoria (n = 6*3) and B/Yamagata (n = 5*3), as presented in Figures 9 and 10.

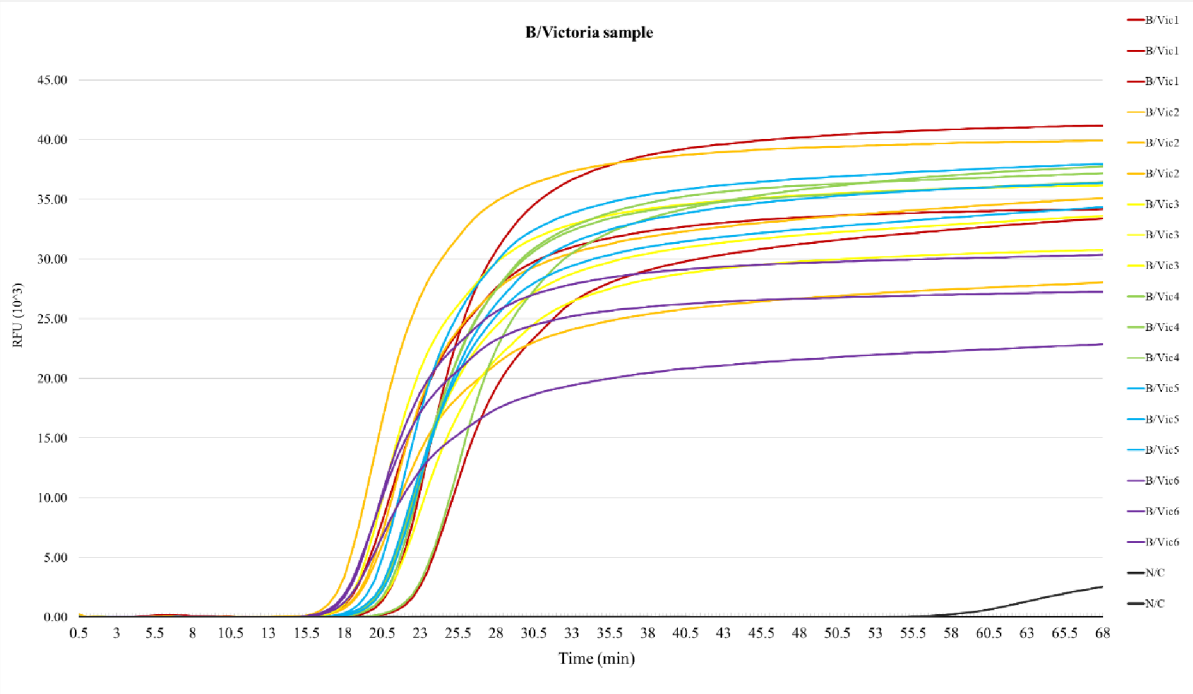


Figure 9: The reaction time of fluorescent dye test on B/Victoria. Each sample repeated in three independent tube-wells are represented by the same color of the curve.

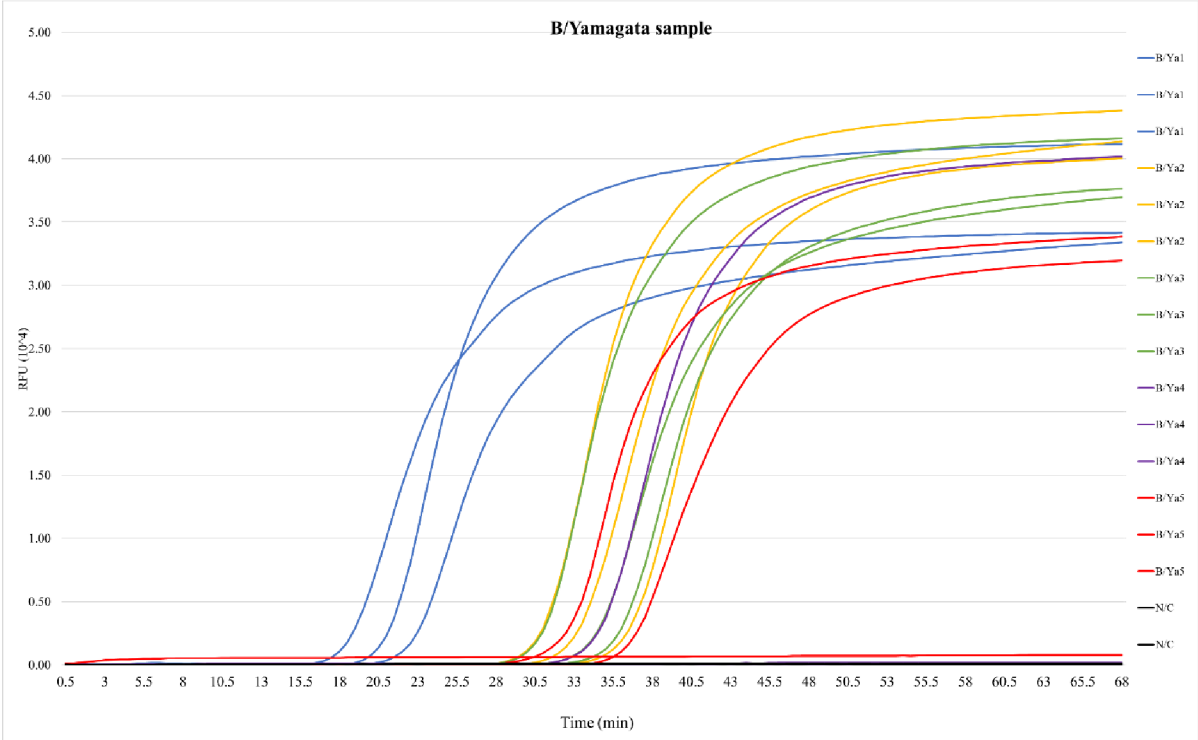


Figure 10: The reaction time of fluorescent dye test on B/Yamagata. Each sample repeated in three independent tube-wells are represented by the same color of the curve.

The result of asymptomatic volunteers' samples in real-time florescent dye indicated some amplification after 50 minutes of incubation and no cross reaction was observed in SARS-CoV-2 sample, while the reference positive control (B/Vic1) yielded a positive result at 20 minutes (Figure 11). Similar results in colorimetric experiment that showed some false-positive reaction after 70 minutes. Therefore, both results indicated that the sample from asymptomatic volunteers were absence of IBV and the rate of specificity was 100% in 0 out of 14 samples was false positive or cross species reaction.

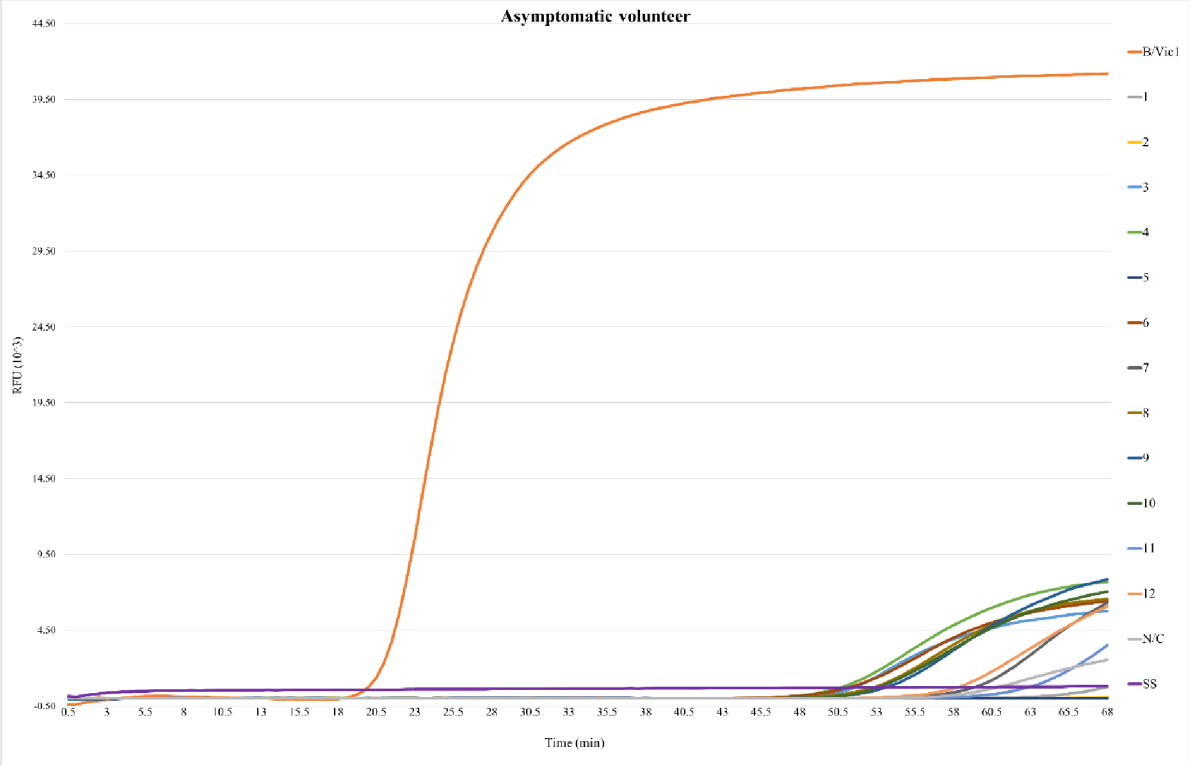


Figure 11: The reaction time of fluorescent dye test in asymptomatic personal samples and SARS-CoV-2. Each sample is presented by the color of the curve.

5. Discussion

The use of LAMP technique has been considered as a promising point-of-care diagnosis method for potential pandemic/epidemic infectious pathogens. This method provides significantly high sensitivity and specificity under isothermal conditions making it suitable for both laboratory and field conditions. The major benefit of this method lies on its rapidity and not required expensive equipment which can be employed on any instruments which capable of maintaining the required temperature (Xie et al. 2014, Strachan et al. 2023). Since this assay has been developed, it has attracted many researchers' attention for determining these approaches and achieved satisfaction success. For example, Chen et al. (2014) were successfully achieved 100% specificity over 171 samples tested against hepatitis E virus (HEV). Ohtsuka et al. (2005) also obtained high specificity and sensitivity by determining LAMP and PCR against salmonella over 110 liquid-egg samples shows only 10% positive by PCR and 100% by LAMP. Furthermore, two research team led by Mahony et al. (2013) and L'helgouach et al. (2020) used none-extracted RNA from clinical patients and obtained high specificities of 95.7% and 95.45% in the detection of influenza viruses and SARS-CoV-2, respectively.

The developed primer set for detection against IBV demonstrated to be a fully functional under isothermal conditions, 100% sensitivity for B/Victoria in both experiments, with a rapid yield of 40 minutes in colorimetric and Tm means of 16.46 minutes in real-time fluorescent. A lower specificity was observed in B/Yamagata in experiments with only 40% in colorimetric but 100% in real-time fluorescent. This outcome has been caused either by a low viral RNA load or the fact that the primer set are more effective against the B/Vitoria strain, as they were designed to detect this particular IBV strain. However, the reaction had 100% specificity, as demonstrated by the absence of false positives in both experiments on all sample groups (no RNA, healthy volunteers, and SARS-CoV-2 RNA).

The main limitation of the study is that only one strain of SARS-CoV-2 has been tested for cross-species reactivity and no other related pathogen has been tested, neither specimens of IAV. This was caused by the high workload of the national reference laboratory at NIPH, which could not provide us with more samples. This leads to some limitation in interpretation of the specificity level of the primers. We were also not provided by the c_t or other values quantifying amount of RNA in the samples provided by NIPH, which did not allow me to determine detection limit of the reaction.

In comparison with other results I would agree that the primers has lower sensitivity than those previous published in the study by Mahony et al. (2013) which achieved overall specificity and sensitivity against A/H1, A/H3, and IBV of 97.9% and 100% with Tm mean of 12 minutes over 108 samples in real-time fluorescent experiment. Jang et al. (2020) obtained a sensitivity and specificity of 94.62% and 100% against IAVs (AH1 and A/H3) and IBVs with Tm mean of 10 minutes.

The result of this study, in concordance with the result of other researchers (Mahony et al. 2013, Jang et al. 2020) showed that the colorimetric LAMP reaction has lower sensitivity, requires more time, and might also require a higher viral load (RT-qPCR Ct<30) than the real-time fluorescent LAMP reaction to show a reliable positive (Aoki et al. 2021). Due to these consequences, it is highly recommended to apply this method on patients who have visible clear symptoms of IBV. However, the colorimetric RT-LAMP method, which can be easily evaluated visually, can still be used as the first-defense line diagnostic by its advantages of rapidity, simple performance, and simple for result interpretation or in developing countries, as it is relatively cheap and does not require any expensive instrumentation to analyze the results.

Nevertheless, result determination in case of colorimetric LAMP should be analyzed carefully according to the manufacture's protocol. In case of unclear result (orange color), samples analysis should be repeated or confirmed by other reliable tests. It is important to note for colorimetric LAMP that the development of the positive result (yellow color) should take no longer than 50 minutes of incubation, in case of no color-changed (pink color) after 50 min should be considered as negative result. The extension of incubating time would not be beneficial useful due to unspecific primer-dimer formation followed by false-positive result (Biolabs 2020). This is caused by high sensitivity of the LAMP system and a large number of primers (usually 6 to 8, including loop primers) might result in the formation of hairpin structures, this increases the likelihood of the primers folding upon themselves and might cause a higher chance of primer dimerization compared to PCR products (Meagher et al. 2018, Garafutdinov et al. 2020). Nevertheless, these problems are present only in the too long incubated reactions, which is adequate to the situation we can observe in qPCR reactions run for too many cycles.

Despite the primers designed in this study have acceptable sensitivity and specificity for detection against IBV and that the assay is much faster and simpler than traditional PCR that require 90-120 min to obtain the results (Boivin et al. 2004). The assay published in the

study of Mahony et al. (2013) had better yields which might be the results of using higher concentration of RNA template (5 times larger). Nevertheless, due to that reason, I would recommend using rather that assay for future LAMP screens of influenza viruses in the e4 research group experiments.

6. Conclusion

I developed five sets of RT-LAMP primers against the highly conserved region of the IBV segment VII coding the viral matrix protein. Furthermore, I evaluated one set of these primers for IBV-specific detection. The assay was able to successfully identify B/Victoria in a short period of time at constant temperature in both the colorimetric and fluorescent LAMP reactions. The RT-LAMP assay has significant potential in terms of its ability to offer simple use, reduce the cost of expensive equipment, and facilitate rapid interpretation.

The advantage of LAMP assays tends to provide simple and reduced time consumption in comparison to conventional PCR and real-time PCR, which require complicated thermal cycler machines and long runs to perform the test. In this study, the designed set of primers can be used to detect the IBV at a constant temperature of 65°C. This method is more suitable for IBV detection under field conditions, such as during a virus circulation investigation, than RT-qPCR. On the other hand, I have to admit that there are other LAMP tests that are characterized by higher sensitivity than the assay developed in this diploma thesis. Nevertheless, even this reaction is comparable to RT-qPCR. The advantages of LAMP reaction are also apparent in the recommendation of using LAMP in the detection of some diseases by the OIE (Mansour et al. 2015), as well as other reports of successfully detection of other infectious pathogen (Wang et al. 2012, Peltzer et al. 2021).

Short of a final conclusion, the result data indicated that the newly developed set of primers has successfully worked in detection against B/Victoria for both diagnosis and point-of-care purposes. For the best, I would suggest not applying this set of primers on B/Yamagata, because, on the other hand, this lineage might already be extinct based on the previous reports (Do et al. 2024).

7. References

Alhabbab, R. Y. 2018. Basic Serological Testing. Springer.

Aoki, M. N., B. de Oliveira Coelho, L. G. B. Góes, P. Minoprio, E. L. Durigon, L. G. Morello, F. K. Marchini, I. N. Riediger, M. do Carmo Debur, and H. I. J. S. r. Nakaya. 2021. Colorimetric RT-LAMP SARS-CoV-2 diagnostic sensitivity relies on color interpretation and viral load. **11**:9026.

Artika, I. M., A. Wiyatno, C. N. J. I. Ma'roef, Genetics, and Evolution. 2020. Pathogenic viruses: Molecular detection and characterization. **81**:104215.

Asha, K., and B. J. J. o. C. M. Kumar. 2019. Emerging influenza D virus threat: what we know so far! **8**:192.

Aydin, S. J. P. 2015. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. **72**:4-15.

Balm, M. N., C. K. Lee, H. K. Lee, L. Chiu, E. S. Koay, and J. W. J. J. o. m. v. Tang. 2012. A diagnostic polymerase chain reaction assay for Zika virus. **84**:1501-1505.

Barr, I. G., N. Komadina, C. Durrant, H. Sjogren, A. C. Hurt, and R. P. J. C. d. i. Shaw. 2006. Circulation and antigenic drift in human influenza B viruses in SE Asia and Oceania since 2000. **30**:350.

Biolabs, N. E. 2020. WarmStart colorimetric LAMP 2x master mix typical LAMP protocol (M1800).

Blanc, A., D. Ruchansky, M. Clara, F. Achaval, A. Le Bas, and J. J. J. o. W. D. Arbiza. 2009. Serologic evidence of influenza A and B viruses in South American fur seals (Arctocephalus australis). **45**:519-521.

Boivin, G., S. Côté, P. Déry, G. De Serres, and M. G. J. J. o. c. M. Bergeron. 2004. Multiplex real-time PCR assay for detection of influenza and human respiratory syncytial viruses. **42**:45-51.

Both, G. W., M. Sleigh, N. Cox, and A. J. J. o. v. Kendal. 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. **48**:52-60.

Bouvier, N. M., and P. J. V. Palese. 2008. The biology of influenza viruses. **26**:D49-D53.

Brown, J. D., D. E. Stallknecht, R. D. Berghaus, M. P. Luttrell, K. Velek, W. Kistler, T. Costa, M. J. Yabsley, D. J. C. Swayne, and V. Immunology. 2009. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. **16**:824-829.

Buitendijk, H., Z. Fagrouch, H. Niphuis, W. M. Bogers, K. S. Warren, and E. J. J. V. Verschoor. 2014. Retrospective serology study of respiratory virus infections in captive great apes. **6**:1442-1453.

Caini, S., A. Meijer, M. C. Nunes, L. Henaff, M. Zounon, B. Boudewijns, M. Del Riccio, and J. J. m. Paget. 2023. Is influenza B/Yamagata extinct and what public health implications could this have? An updated literature review and comprehensive assessment of global surveillance databases.2023.2009. 2025.23296068.

Carr, M. J., R. Gunson, A. Maclean, S. Coughlan, M. Fitzgerald, M. Scully, B. O'Herlihy, J. Ryan, D. O'Flanagan, and J. J. J. o. C. V. Connell. 2009. Development of a real-time RT-PCR for the detection of swine-lineage influenza A (H1N1) virus infections. **45**:196-199.

Chang, C., A. New, J. Taylor, and H. J. I. J. Z. Chiang. 1976. Influenza virus isolations from dogs during a human epidemic in Taiwan. **3**:61-64.

Chen, L., F. Ruan, Y. Sun, H. Chen, M. Liu, J. Zhou, and K. J. J. o. M. V. Qin. 2019. Establishment of sandwich ELISA for detecting the H7 subtype influenza A virus. **91**:1168-1171.

Chen, Q., L. Yuan, J. Wan, Y. Chen, and C. J. J. o. V. M. Du. 2014. Colorimetric detection of hepatitis E virus based on reverse transcription loop mediated isothermal amplification (RT-LAMP) assay. **197**:29-33.

Chen, R., and E. C. J. J. o. m. e. Holmes. 2008. The evolutionary dynamics of human influenza B virus. **66**:655-663.

Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, and P. J. N. m. Palese. 2001. A novel influenza A virus mitochondrial protein that induces cell death. **7**:1306-1312.

Cheung, T. K., and L. L. J. A. o. t. N. Y. A. o. S. Poon. 2007. Biology of influenza a virus. **1102**:1-25.

de Graaf, M., and R. A. J. T. E. j. Fouchier. 2014. Role of receptor binding specificity in influenza A virus transmission and pathogenesis. **33**:823-841.

de Jong, S. P., Z. C. Felix Garza, J. C. Gibson, S. van Leeuwen, R. P. de Vries, G.-J. Boons, M. van Hoesel, K. de Haan, L. E. van Groeningen, and K. D. J. N. c. Hulme. 2024. Determinants of epidemic size and the impacts of lulls in seasonal influenza virus circulation. **15**:591.

Deng, Q., D. Wang, X. Xiang, X. Gao, P. R. Hardwidge, R. Kaushik, T. Wolff, S. Chakravarty, and F. J. V. r. Li. 2011. Nuclear localization of influenza B polymerase proteins and their binary complexes. **156**:49-53.

Din, G. U., K. Hasham, M. N. Amjad, and Y. J. C. I. i. M. B. Hu. 2023. Natural History of Influenza B Virus—Current Knowledge on Treatment, Resistance and Therapeutic Options. **46**:183-199.

Do, T. H. T., A. K. Wheatley, S. J. Kent, and M. J. E. R. o. V. Koutsakos. 2024. Influenza B virus neuraminidase: a potential target for next-generation vaccines? **23**:39-48.

Donelan, N. R., B. Dauber, X. Wang, C. F. Basler, T. Wolff, and A. J. J. o. v. García-Sastre. 2004. The N-and C-terminal domains of the NS1 protein of influenza B virus can independently inhibit IRF-3 and beta interferon promoter activation. **78**:11574-11582.

Dziąbowska, K., E. Czaczyk, and D. J. B. Nidzworski. 2018. Detection methods of human and animal influenza virus—current trends. **8**:94.

Elderfield, R. A., L. Parker, P. Stilwell, K. L. Roberts, S. Schepelmann, and W. S. J. J. o. G. V. Barclay. 2015. Ferret airway epithelial cell cultures support efficient replication of influenza B virus but not mumps virus. **96**:2092-2098.

Engvall, E., and P. J. I. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. **8**:871-874.

Fouchier, R. A., T. M. Bestebroer, S. Herfst, L. Van Der Kemp, G. F. Rimmelzwaan, and A. D. J. J. o. c. m. Osterhaus. 2000. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. **38**:4096-4101.

Francis Jr, T. J. S. 1940. A new type of virus from epidemic influenza. **92**:405-408.

Garafutdinov, R. R., A. A. Galimova, A. R. J. N. Sakhabutdinova, Nucleotides, and N. Acids. 2020. The influence of quality of primers on the formation of primer dimers in PCR. **39**:1251-1269.

Gavin, P. J., R. B. J. C. Thomson Jr, and A. I. Reviews. 2004. Review of rapid diagnostic tests for influenza. **4**:151-172.

Gottschalk, A. J. B. e. b. a. 1957. Neuraminidase: the specific enzyme of influenza virus and Vibrio cholerae. **23**:645-646.

Hatta, M., H. Goto, and Y. J. J. o. v. Kawaoka. 2004. Influenza B virus requires BM2 protein for replication. **78**:5576-5583.

Hause, B. M., M. Ducatez, E. A. Collin, Z. Ran, R. Liu, Z. Sheng, A. Armien, B. Kaplan, S. Chakravarty, and A. D. J. P. p. Hoppe. 2013. Isolation of a novel swine influenza virus

from Oklahoma in 2011 which is distantly related to human influenza C viruses. **9**:e1003176.

Heilek, G. 2016. Nucleic Acids—The Use of Nucleic Acid Testing in Molecular Diagnostics. Nucleic Acids-From Basic Aspects to Laboratory Tools. IntechOpen.

Hirst, G. K. 1941. The Agglutination of Red Cells by Allantoic Fluid of Chick Embryos Infected with Influenza Virus. **94**:22-23.

Huang, X., G. Tang, N. Ismail, and X. J. E. Wang. 2022. Developing RT-LAMP assays for rapid diagnosis of SARS-CoV-2 in saliva. **75**.

Imai, M., S. Watanabe, A. Ninomiya, M. Obuchi, and T. J. J. o. v. Odagiri. 2004. Influenza B virus BM2 protein is a crucial component for incorporation of viral ribonucleoprotein complex into virions during virus assembly. **78**:11007-11015.

Imai, M., S. Watanabe, and T. J. A. o. v. Odagiri. 2003. Influenza B virus NS2, a nuclear export protein, directly associates with the viral ribonucleoprotein complex. **148**:1873-1884.

Jang, W. S., D. H. Lim, J. Nam, D.-C. Mihn, H. W. Sung, C. S. Lim, and J. J. P. O. Kim. 2020. Development of a multiplex isothermal amplification molecular diagnosis method for on-site diagnosis of influenza. **15**:e0238615.

Kaczmarek, M., R. Owen, and I. G. Barr. 2010. Annual Report of the National Influenza Surveillance Scheme, 2008. Communicable Diseases Intelligence Quarterly Report **34**:8-22.

KATO, K., Y. HAMAGUCHI, S. OKAWA, E. ISHIKAWA, K. KOBAYASHI, and N. KATUNUMA. 1977. Use of Rabbit Antibody IgG Bound onto Plain and Aminoalkylsilyl Glass Surface for the Enzyme-Linked Sandwich Immunoassay. The Journal of Biochemistry **82**:261-266.

KAWANO, J., T. ONTA, H. KIDA, and R. J. J. J. o. V. R. YANAGAWA. 1978. Distribution of antibodies in animals against influenza B and C viruses. **26**:74-80.

Kordyukova, L. V., E. V. Shtykova, L. A. Baratova, D. I. Svergun, O. V. J. J. o. B. S. Batishchev, and Dynamics. 2019. Matrix proteins of enveloped viruses: a case study of Influenza A virus M1 protein. **37**:671-690.

Koutsakos, M., T. H. Nguyen, W. S. Barclay, and K. J. F. m. Kedzierska. 2016. Knowns and unknowns of influenza B viruses. **11**:119-135.

Koutsakos, M., A. K. Wheatley, K. Laurie, S. J. Kent, and S. J. N. R. M. Rockman. 2021. Influenza lineage extinction during the COVID-19 pandemic? **19**:741-742.

L'helgouach, N., P. Champigneux, F. S. Schneider, L. Molina, J. Espeut, M. Alali, J. Baptiste, L. Cardeur, B. Dubuc, and V. J. M. Foulongne. 2020. EasyCOV: LAMP based rapid detection of SARS-CoV-2 in saliva.2020.2005. 2030.20117291.

Lanciotti, R. S. J. A. i. v. r. 2003. Molecular amplification assays for the detection of flaviviruses. **61**:67-99.

Leirs, K., P. Tewari Kumar, D. Decrop, E. Perez-Ruiz, P. Leblebici, B. Van Kelst, G. Compernolle, H. Meeuws, L. Van Wesenbeeck, and O. J. A. c. Lagatie. 2016. Bioassay development for ultrasensitive detection of influenza a nucleoprotein using digital ELISA. **88**:8450-8458.

Leyva-Grado, V. H., S. Mubareka, F. Krammer, W. B. Cárdenas, and P. J. E. i. d. Palese. 2012. Influenza virus infection in guinea pigs raised as livestock, Ecuador. **18**:1135.

Li, Z.-N., K. M. Weber, R. A. Limmer, B. J. Horne, J. Stevens, J. Schwerzmann, J. Wrammert, M. McCausland, A. J. Phipps, and K. J. J. o. v. m. Hancock. 2017. Evaluation of multiplex assay platforms for detection of influenza hemagglutinin subtype specific antibody responses. **243**:61-67.

Lin, M., Q.-C. Yao, J. Liu, M. Huo, Y. Zhou, M. Chen, Y. Li, Y. Gao, and Y. J. V. Ge. 2023. Evolution and reassortment of H6 subtype avian influenza viruses. **15**:1547.

Lindström, P., and O. J. S. j. o. i. Wager. 1978. IgG autoantibody to human serum albumin studied by the ELISA-technique. **7**:419-425.

Liu, R., Z. Sheng, C. Huang, D. Wang, and F. J. C. o. i. v. Li. 2020. Influenza D virus. **44**:154-161.

Ma, W. J. V. M. 2022. Orthomyxoviridae.573-588.

Mahony, J., S. Chong, D. Bulir, A. Ruyter, K. Mwawasi, and D. J. J. o. C. V. Waltho. 2013. Multiplex loop-mediated isothermal amplification (M-LAMP) assay for the detection of influenza A/H1, A/H3 and influenza B can provide a specimen-to-result diagnosis in 40 min with single genome copy sensitivity. **58**:127-131.

Mansour, S. M., H. Ali, C. C. Chase, and A. J. A. H. R. R. Cepica. 2015. Loop-mediated isothermal amplification for diagnosis of 18 World Organization for Animal Health (OIE) notifiable viral diseases of ruminants, swine and poultry. **16**:89-106.

Matias, G., R. Taylor, F. Haguinet, C. Schuck-Paim, R. Lustig, V. J. I. Shinde, and o. r. viruses. 2014. Estimates of mortality attributable to influenza and RSV in the U nited S tates during 1997–2009 by influenza type or subtype, age, cause of death, and risk status. **8**:507-515.

Matsuzaki, Y., N. Katsushima, Y. Nagai, M. Shoji, T. Itagaki, M. Sakamoto, S. Kitaoka, K. Mizuta, and H. Nishimura. 2006. Clinical Features of Influenza C Virus Infection in Children. The Journal of Infectious Diseases **193**:1229-1235.

McCLELLAND, L., and R. J. C. P. H. J. Hare. 1941. The adsorption of influenza virus by red cells and a new in vitro method of measuring antibodies for influenza virus. **32**:530-538.

Meagher, R. J., A. Priye, Y. K. Light, C. Huang, and E. J. A. Wang. 2018. Impact of primer dimers and self-amplifying hairpins on reverse transcription loop-mediated isothermal amplification detection of viral RNA. **143**:1924-1933.

Nair, H., W. A. Brooks, M. Katz, A. Roca, J. A. Berkley, S. A. Madhi, J. M. Simmerman, A. Gordon, M. Sato, and S. J. T. L. Howie. 2011. Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. **378**:1917-1930.

Negi, G., A. Sharma, M. Dey, G. Dhanawat, and N. J. B. R. Parveen. 2022. Membrane attachment and fusion of HIV-1, influenza A, and SARS-CoV-2: resolving the mechanisms with biophysical methods. **14**:1109-1140.

Newton, D. W., J. J. Treanor, and M. A. J. A. J. M. C. Menegus. 2000. Clinical and laboratory diagnosis of influenza virus infections. **6**:S265-S275.

Ni, F., I. N. Mbawuike, E. Kondrashkina, and Q. J. V. Wang. 2014. The roles of hemagglutinin Phe-95 in receptor binding and pathogenicity of influenza B virus. **450**:71-83.

Notomi, T., K. Nagamine, Y. Mori, H. J. D. a. C. t. Kanda, and applications. 2004. Loop mediated isothermal amplification (LAMP) of DNA analytes.199-212.

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. J. N. a. r. Hase. 2000. Loop-mediated isothermal amplification of DNA. **28**:e63-e63.

Ohtsuka, K., K. Yanagawa, K. Takatori, Y. J. A. Hara-Kudo, and e. microbiology. 2005. Detection of Salmonella enterica in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of Salmonella isolates. **71**:6730-6735.

Osterhaus, A., G. Rimmelzwaan, B. Martina, T. Bestebroer, and R. J. S. Fouchier. 2000. Influenza B virus in seals. **288**:1051-1053.

Paget, J., S. Caini, M. Del Riccio, W. van Waarden, and A. J. E. Meijer. 2022. Has influenza B/Yamagata become extinct and what implications might this have for quadrivalent influenza vaccines? **27**:2200753.

Parida, M., S. Sannarangaiah, P. K. Dash, P. Rao, and K. J. R. i. m. v. Morita. 2008. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. **18**:407-421.

Peltzer, D., K. Tobler, C. Fraefel, M. Maley, and C. J. J. o. V. M. Bachofen. 2021. Rapid and simple colorimetric loop-mediated isothermal amplification (LAMP) assay for the detection of Bovine alphaherpesvirus 1. **289**:114041.

Pica, N., Y.-Y. Chou, N. M. Bouvier, and P. J. J. o. v. Palese. 2012. Transmission of influenza B viruses in the guinea pig. **86**:4279-4287.

Poon, L. L., C. S. Leung, K. H. Chan, J. H. Lee, K. Y. Yuen, Y. Guan, and J. S. J. J. o. c. m. Peiris. 2005. Detection of human influenza A viruses by loop-mediated isothermal amplification. **43**:427-430.

RA, L. J. F. v. 1995. Orthomyxoviridae: the viruses and their replication.

Ran, Z., H. Shen, Y. Lang, E. A. Kolb, N. Turan, L. Zhu, J. Ma, B. Bawa, Q. Liu, and H. J. J. o. v. Liu. 2015. Domestic pigs are susceptible to infection with influenza B viruses. **89**:4818-4826.

Reich, S., D. Guilligay, A. Pflug, H. Malet, I. Berger, T. Crépin, D. Hart, T. Lunardi, M. Nanao, and R. W. J. N. Ruigrok. 2014. Structural insight into cap-snatching and RNA synthesis by influenza polymerase. **516**:361-366.

Romvary, J., J. Meszaros, and K. J. A. M. A. S. H. Barb. 1980. Susceptibility of birds to type-B influenza virus. **27**:279-287.

Rota, P. A., T. R. Wallis, M. W. Harmon, J. S. Rota, A. P. Kendal, and K. J. V. Nerome. 1990. Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. **175**:59-68.

Schneider, J., B. Dauber, K. Melén, I. Julkunen, and T. J. J. o. v. Wolff. 2009. Analysis of influenza B Virus NS1 protein trafficking reveals a novel interaction with nuclear speckle domains. **83**:701-711.

Sederdahl, B. K., and J. V. J. V. Williams. 2020. Epidemiology and clinical characteristics of influenza C virus. **12**:89.

Soldevila, N., L. Basile, A. Martínez, N. Torner, M. Á. Marcos, M. D. M. Mosquera, A. Antón, C. Andrés, C. Rius, and T. J. J. o. M. V. Pumarola. 2022. Surveillance of influenza B severe hospitalized cases during 10 seasons in Catalonia: Does the lineage make a difference? **94**:4417-4424.

Song, D. S., Y. J. Lee, O. M. Jeong, Y. J. Kim, C. H. Park, J. E. Yoo, W. J. Jeon, J. H. Kwon, G. W. Ha, and B. K. J. J. o. v. s. Kang. 2009a. Evaluation of a competitive ELISA for antibody detection against avian influenza virus. **10**:323.

Song, D. S., Y. J. Lee, O. M. Jeong, Y. J. Kim, C. H. Park, J. E. Yoo, W. J. Jeon, J. H. Kwon, G. W. Ha, and B. K. J. J. o. v. s. Kang. 2009b. Evaluation of a competitive ELISA for antibody detection against avian influenza virus. **10**:323-329.

Spackman, E. 2014. Animal influenza virus. Springer.

Steinhauer, D. A. J. V. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. **258**:1-20.

Stevens, M. P., and W. S. Barclay. 1998a. The N-Terminal Extension of the Influenza B Virus Nucleoprotein Is Not Required for Nuclear Accumulation or the Expression and Replication of a Model RNA. **72**:5307-5312.

Stevens, M. P., and W. S. J. J. o. v. Barclay. 1998b. The N-terminal extension of the influenza B virus nucleoprotein is not required for nuclear accumulation or the expression and replication of a model RNA. **72**:5307-5312.

Strachan, S., M. Chakraborty, M. Sallam, S. A. Bhuiyan, R. Ford, and N.-T. J. M. Nguyen. 2023. Maximising Affordability of Real-Time Colorimetric LAMP Assays. **14**:2101.

Suarez, D. L. J. A. i. 2016. Influenza A virus.1-30.

Sun, Y., L. Zhao, M. Zhao, R. Zhu, J. Deng, F. Wang, F. Li, Y. Ding, R. Tian, and Y. J. J. o. v. m. Qian. 2014. Four DNA extraction methods used in loop-mediated isothermal amplification for rapid adenovirus detection. **204**:49-52.

Tanner, N. J. I., MA: New England Biolabs. 2019. Optimized integration of New England Biolabs loop-mediated isothermal amplification (LAMP) reagents with Axxin ISO instruments.

Taylor, R. J. A. J. o. P. H., and t. N. Health. 1949. Studies on survival of influenza virus between epidemics and antigenic variants of the virus. **39**:171-178.

Thompson, W. W., M. R. Moore, E. Weintraub, P.-Y. Cheng, X. Jin, C. B. Bridges, J. S. Bresee, and D. K. J. A. j. o. p. h. Shay. 2009. Estimating influenza-associated deaths in the United States. **99**:S225-S230.

Trilla, A., G. Trilla, and C. Daer. 2008. The 1918 “Spanish Flu” in Spain. Clinical Infectious Diseases **47**:668-673.

Tsai, C. P., H. J. J. I. Tsai, and o. r. viruses. 2019. Influenza B viruses in pigs, Taiwan. **13**:91-105.

Van De Sandt, C. E., R. Bodewes, G. F. Rimmelzwaan, and R. D. J. F. m. De Vries. 2015. Influenza B viruses: not to be discounted. **10**:1447-1465.

ViralZone, 2020. Betainfluenzavirus. https://viralzone.expasy.org/80?outline=all_by_species

Wacker, M. J., and M. P. J. J. o. b. t. J. Godard. 2005. Analysis of one-step and two-step real-time RT-PCR using SuperScript III. **16**:266.

Wagner, R., M. Matrosovich, and H. D. J. R. i. m. v. Klenk. 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. **12**:159-166.

Wang, J., R. M. Pielak, M. A. McClintock, J. J. J. N. s. Chou, and m. biology. 2009. Solution structure and functional analysis of the influenza B proton channel. **16**:1267-1271.

Wang, Q., F. Cheng, M. Lu, X. Tian, and J. J. J. o. v. Ma. 2008. Crystal structure of unliganded influenza B virus hemagglutinin. **82**:3011-3020.

Wang, Q., X. Tian, X. Chen, and J. J. P. o. t. N. A. o. S. Ma. 2007. Structural basis for receptor specificity of influenza B virus hemagglutinin. **104**:16874-16879.

Wang, X., Y. Li, K. L. O'brien, S. A. Madhi, M.-A. Widdowson, P. Byass, S. B. Omer, Q. Abbas, A. Ali, and A. J. T. L. G. H. Amu. 2020. Global burden of respiratory infections associated with seasonal influenza in children under 5 years in 2018: a systematic review and modelling study. **8**:e497-e510.

Wang, X., J.-p. Zhu, Q. Zhang, Z.-g. Xu, F. Zhang, Z.-h. Zhao, W.-z. Zheng, and L.-s. J. J. o. v. m. Zheng. 2012. Detection of enterovirus 71 using reverse transcription loop-mediated isothermal amplification (RT-LAMP). **179**:330-334.

WHO, W. r. J. B. 1979. Reconsideration of influenza A virus nomenclature: a WHO Memorandum. **57**:1-7.

Wille, M., and E. C. J. C. S. H. p. i. m. Holmes. 2020. The ecology and evolution of influenza viruses. **10**.

Wolff, T., and M. J. E. o. V. Veit. 2021. Influenza B, C and D viruses (orthomyxoviridae).561.

Xie, S., Y. Chai, Y. Yuan, L. Bai, R. J. B. Yuan, and Bioelectronics. 2014. Development of an electrochemical method for Ochratoxin A detection based on aptamer and loop-mediated isothermal amplification. **55**:324-329.

Yorde, D. E., E. A. Sasse, T. Y. Wang, R. O. Hussa, and J. C. Garancis. 1976. Competitive enzyme-liked immunoassay with use of soluble enzyme/antibody immune complexes for labeling. I. Measurement of human choriogonadotropin. Clinical Chemistry **22**:1372-1377.

Zaraket, H., A. C. Hurt, B. Clinch, I. Barr, and N. J. A. R. Lee. 2021. Burden of influenza B virus infection and considerations for clinical management. **185**:104970.

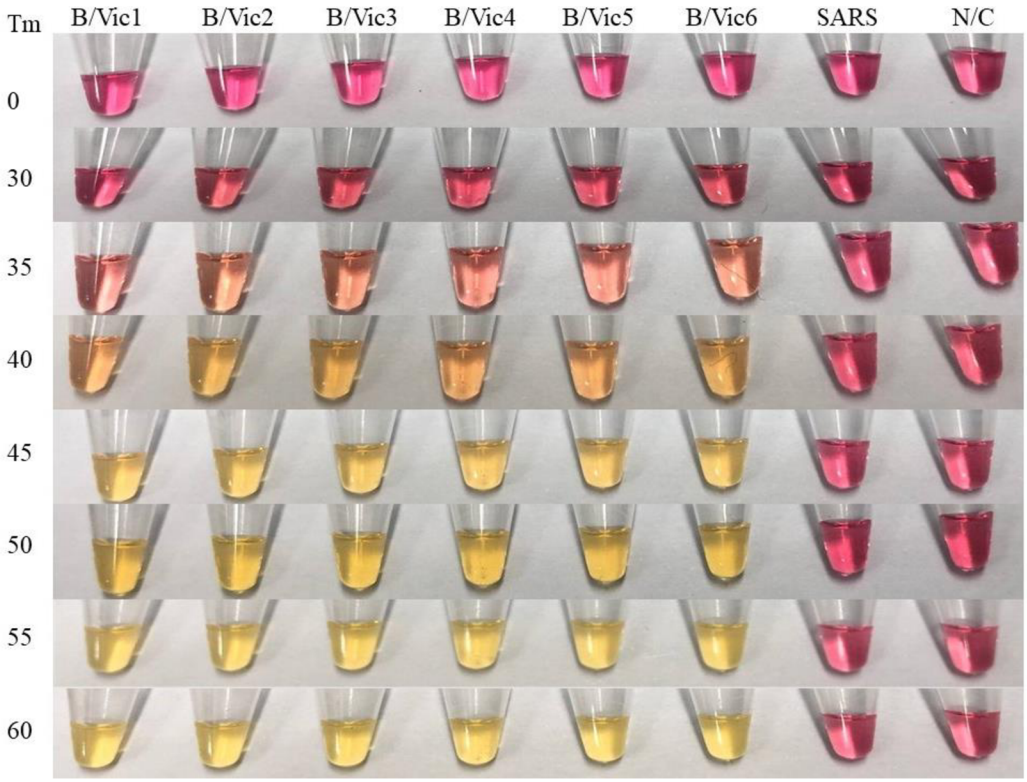
Zhang, W., and D. H. J. J. o. v. m. Evans. 1991. Detection and identification of human influenza viruses by the polymerase chain reaction. **33**:165-189.

Zhang, X., S. B. Lowe, J. J. J. B. Gooding, and Bioelectronics. 2014. Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP). **61**:491-499.

Appendices

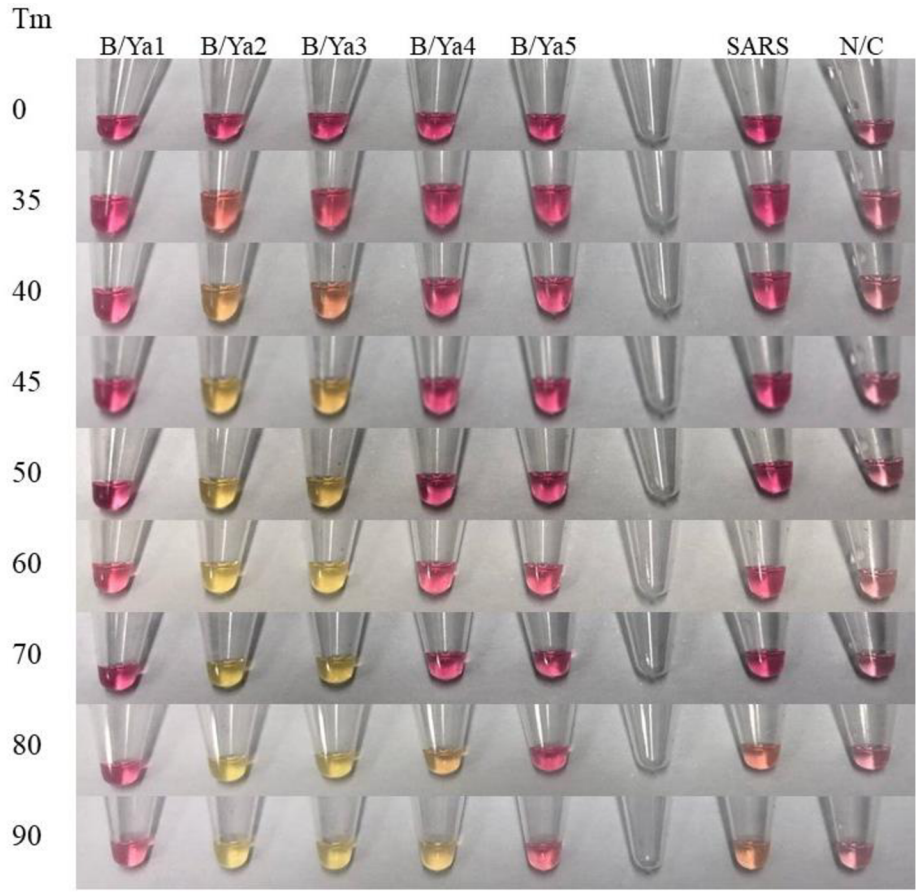
List of the Appendices:

Appendix 1: Result of sensitivity determine of the primers by colorimetric RT-LAMP on B/Victoria, and species-specificity cross-reaction on SARS-CoV-2.



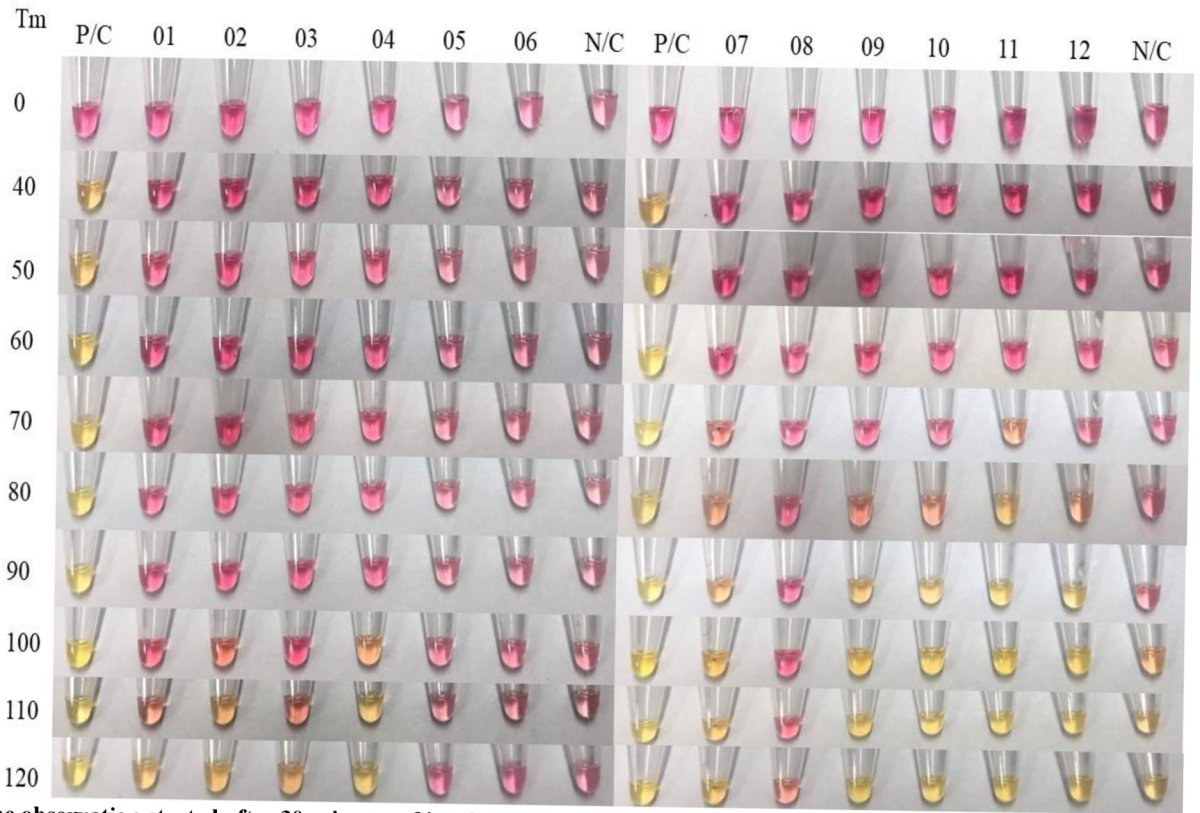
The observation was started after 30-minutes of incubation and 5 minutes interval. Color change represent the presence of RNA amplification (pink to yellow). The color change should be a yellow clear visible in 30-40 minutes of incubation (Biolabs 2020).

Appendix 2: Result of sensitivity determine of the primers by colorimetric RT-LAMP on B/Yamagata, and species-specificity cross-reaction on SARS-CoV-2.



The observation was started after 30-minutes of incubation and 5-10 minutes interval. Color change represent the presence of RNA amplification (pink to yellow) (Biolabs 2020).

Appendix 3: Result of specificity determine of the primers by colorimetric RT-LAMP on asymptomatic volunteer staff and students of FTZ.



The observation started after 30-minutes of incubation and 10 minutes interval. Color change represent the presence of RNA amplification (pink to yellow). Color change after 50 minutes would considered as a false-positive (Biolabs 2020).