UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Presence of Myxoma Virus and Rabbit Hemorrhagic Disease Virus in *Sylvilagus andinus* in the Antisana Hydrological Conservation Area

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

Autor:

Guzmán Arias Cristian

Tutor:

Dr. Tellkamp Tietz Markus, PhD

Co-tutor:

Dr. García Bereguiain Miguel, PhD

Urcuquí, julio de 2021

Urcuquí, 28 de junio de 2021

SECRETARÍA GENERAL
(Vicerrectorado Académico/Cancillería)
ESCUELA DE CIENCIAS BIOLÓGICAS E INGENIERÍA
CARRERA DE BIOLOGÍA
ACTA DE DEFENSA No. UITEY-BIO-2021-00016-AD

A los 28 días del mes de junio de 2021, a las 16:00 horas, de manera virtual mediante videoconferencia, y ante el Tribunal Calificador, integrado por los docentes:

Presidente Tribunal de Defensa	Dr. ALVAREZ BOTAS, FRANCISCO JAVIER , Ph.D.
Miembro No Tutor	Dr. GONZALES ZUBIATE, FERNANDO ALEXIS , Ph.D.
Tutor	Dr. TELLKAMP TIETZ, MARKUS PATRICIO , Ph.D.

El(la) señor(ita) estudiante **GUZMAN ARIAS, CRISTIAN AYMAR**, con cédula de identidad No. **1105923294**, de la **ESCUELA DE CIENCIAS BIOLÓGICAS E INGENIERÍA**, de la Carrera de **BIOLOGÍA**, aprobada por el Consejo de Educación Superior (CES), mediante Resolución **RPC-SO-37-No.438-2014**, realiza a través de videoconferencia, la sustentación de su trabajo de titulación denominado: **Presence of Myxoma Virus and Rabbit Hemorrhagic Disease Virus in Sylvilagus andinus in the Antisana Hydric Conservation Area.**, previa a la obtención del título de **BIÓLOGO/A**.

El citado trabajo de titulación, fue debidamente aprobado por el(los) docente(s):

Tutor	Dr. TELLKAMP TIETZ, MARKUS PATRICIO , Ph.D.

Y recibió las observaciones de los otros miembros del Tribunal Calificador, las mismas que han sido incorporadas por el(la) estudiante.

Previamente cumplidos los requisitos legales y reglamentarios, el trabajo de titulación fue sustentado por el(la) estudiante y examinado por los miembros del Tribunal Calificador. Escuchada la sustentación del trabajo de titulación a través de videoconferencia, que integró la exposición de el(la) estudiante sobre el contenido de la misma y las preguntas formuladas por los miembros del Tribunal, se califica la sustentación del trabajo de titulación con las siguientes calificaciones:

Tipo	Docente	Calificación
Miembro Tribunal De Defensa	Dr. GONZALES ZUBIATE, FERNANDO ALEXIS , Ph.D.	9,8
Presidente Tribunal De Defensa	Dr. ALVAREZ BOTAS, FRANCISCO JAVIER , Ph.D.	9,8
Tutor	Dr. TELLKAMP TIETZ, MARKUS PATRICIO , Ph.D.	10,0

Lo que da un promedio de: **9.9 (Nueve punto Nueve)**, sobre 10 (diez), equivalente a: **APROBADO**

Para constancia de lo actuado, firman los miembros del Tribunal Calificador, el/la estudiante y el/la secretario ad-hoc.

Certifico que *en cumplimiento del Decreto Ejecutivo 1017 de 16 de marzo de 2020, la defensa de trabajo de titulación (o examen de grado modalidad teórico práctica) se realizó vía virtual, por lo que las firmas de los miembros del Tribunal de Defensa de Grado, constan en forma digital.*

GUZMAN ARIAS, CRISTIAN AYMAR
Estudiante

FRANCISCO JAVIER ALVAREZ BOTAS
Digitally signed by FRANCISCO JAVIER ALVAREZ BOTAS
Date: 2021.07.02 14:12:56 -05'00'

Dr. ALVAREZ BOTAS, FRANCISCO JAVIER , Ph.D.
Presidente Tribunal de Defensa

Firmado electrónicamente por:
MARKUS PATRICIO
TELLKAMP TIETZ

Dr. TELLKAMP TIETZ, MARKUS PATRICIO , Ph.D.
Tutor

Firmado electrónicamente por:
FERNANDO ALEXIS
GONZALES ZUBIATE

Dr. GONZALES ZUBIATE, FERNANDO ALEXIS , Ph.D.
Miembro No Tutor

KARLA ESTEFANIA ALARCON FELIX
Firmado digitalmente por KARLA ESTEFANIA ALARCON FELIX
Fecha: 2021.06.28 17:36:44 -05'00'

ALARCON FELIX, KARLA ESTEFANIA
Secretario Ad-hoc

AUTORÍA

Yo, **CRISTIAN AYMAR GUZMÁN ARIAS**, con cédula de identidad 1105923294, declaro que las ideas, juicios, valoraciones, interpretaciones, consultas bibliográficas, definiciones y conceptualizaciones expuestas en el presente trabajo; así cómo, los procedimientos y herramientas utilizadas en la investigación, son de absoluta responsabilidad de el/la autora (a) del trabajo de integración curricular. Así mismo, me acojo a los reglamentos internos de la Universidad de Investigación de Tecnología Experimental Yachay.

Urcuquí, julio de 2021.

Cristian Aymar Guzmán Arias

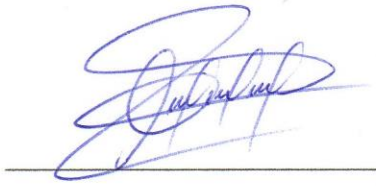
CI: 1105923294

AUTORIZACIÓN DE PUBLICACIÓN

Yo, **CRISTIAN AYMAR GUZMÁN ARIAS**, con cédula de identidad 1105923294, cedo a la Universidad de Investigación de Tecnología Experimental Yachay, los derechos de publicación de la presente obra, sin que deba haber un reconocimiento económico por este concepto. Declaro además que el texto del presente trabajo de titulación no podrá ser cedido a ninguna empresa editorial para su publicación u otros fines, sin contar previamente con la autorización escrita de la Universidad.

Asimismo, autorizo a la Universidad que realice la digitalización y publicación de este trabajo de integración curricular en el repositorio virtual, de conformidad a lo dispuesto en el Art. 144 de la Ley Orgánica de Educación Superior

Urcuquí, julio de 2021.



Cristian Aymar Guzmán Arias

CI: 1105923294

For the two most important women in my life Mia and Shary.

Acknowledgments

To my tutor, Dr. Markus Tellkamp, for his guidance and support during the development of this thesis. To my co-tutor, Dr. Miguel Ángel García, for his technical support and the provision of materials. To my external advisor, Dr. Jorge Mosquera, for his logistical support and resource management. To the professor in charge of the Molecular Biology laboratory at Yachay Tech University, Dr. Javier Álvarez, for his help with the elaboration of laboratory protocols. To EPMAPS, FONAG, and Estación Científica Agua y Páramos for financing this project. To Ing. Luna Delerue and Ing. Fabiola Guzmán for managing the necessary resources to carry out the investigation. To the Antisana Hydrological Conservation Area paramo guards, especially Mr. Manuel Simba, for his help in capturing specimens and taking samples. To Yachay Tech University, the school of biological sciences and engineering, and its teachers for all the knowledge and support provided during my training at this institution. Finally, to my family, especially my parents Magali and Santos, for their infinite support and for always believing in me.

Funding

This work was financed by the Empresa Pública Metropolitana de Agua Potable y Saneamiento de Quito (EPMAPS) and the Fondo Para la Protección del Agua (FONAG) through the Scholarship program of the Estación Científica Agua y Páramos (ECAP) [Programa de Becas de la Estación Científica Agua y Páramos 2019].

Resumen

Sylvilagus andinus (conejo andino) es una de las especies de mamíferos nativas más comunes en el área de amortiguamiento de la Reserva Ecológica Antisana al este de Quito, Ecuador, y su población influye en la estabilidad ecológica de los páramos andinos. Su importancia recae en ser un eslabón importante de la cadena alimentaria y en el mantenimiento de la cobertura vegetal y, por tanto, del ciclo hidrológico. Por ello, el estudio de los factores que pueden afectar a su población, como los virus, es importante para mantener el equilibrio ecológico en el Área de Conservación Hídrica Antisana, que se encuentra bajo programas de restauración ecológica. Dos de los virus más importantes que afectan a los conejos son el virus del Mixoma y el virus de la Enfermedad hemorrágica del conejo, que causan las enfermedades mortales Mixomatosis y Enfermedad hemorrágica del conejo, respectivamente, en conejos domésticos y silvestres del género *Oryctolagus*. Sin embargo, la presencia de estos virus en *Sylvilagus* spp. ha sido poco estudiada y nunca se han detectado en *Sylvilagus andinus*. En este estudio, se usó un ensayo de reacción en cadena de la polimerasa (PCR) convencional para detectar la presencia del virus del Mixoma a través del gen M000.5L / R, y un ensayo de reacción en cadena de la polimerasa con transcripción inversa (RT-PCR) para identificar la presencia del virus de la Enfermedad hemorrágica del conejo a través del gen VP60, en muestras obtenidas de conejos silvestres capturados en el Área de Conservación Hídrica Antisana. Los amplicones fueron secuenciados para confirmar la presencia del virus del Mixoma y del virus de la Enfermedad hemorrágica del conejo en *Sylvilagus andinus*, lo que implica la posibilidad de que la Mixomatosis y/o la Enfermedad hemorrágica del conejo afecten la dinámica poblacional del conejo andino, pero también la posibilidad de que los conejos domésticos cercanos a los páramos contraigan estas enfermedades.

Palabras clave: Virus del mixoma, Mixomatosis, Virus de la enfermedad hemorrágica del conejo, PCR, RT-PCR, *Sylvilagus andinus*, conejo andino, Área de Conservación Hídrica Antisana.

Abstract

Sylvilagus andinus (Andean rabbit) is one of the native mammal species most commonly seen in the Antisana Hydrological Conservation Area, east of Quito, Ecuador, and its population influences the ecological stability of the Andean moorlands. Its importance rests on being an important link in the food chain and its role in maintaining an intact vegetation and thus hydrological cycle. For this reason, the study of factors that can affect its population size, such as viruses, is important for keeping the ecological balance in the Antisana Hydrological Conservation Area, which is under management for ecological restoration. Two of the most important viruses that affect rabbits are the Myxoma virus (MYXV) and Rabbit hemorrhagic disease virus (RHDV), which causes the fatal diseases Myxomatosis and Rabbit hemorrhagic disease, respectively, in domestic and wild rabbits of the genus *Oryctolagus*. However, the presence of these viruses in *Sylvilagus* spp. has been poorly studied and they have not been documented in *Sylvilagus andinus*. In this study, we used a conventional polymerase chain reaction assay (PCR) to detect the presence of MYXV through the M000.5L/R gene and a reverse transcription polymerase chain reaction assay (RT-PCR) to identify the presence of RHDV through the VP60 gene, on samples obtained from wild rabbits captured in the Antisana Hydrological Conservation Area. The amplicons were sequenced to confirm the presence of Myxoma virus and Rabbit hemorrhagic disease virus in *Sylvilagus andinus*. The presence of Myxomatosis and Rabbit hemorrhagic disease suggests that these diseases might affect the population dynamics of the Andean rabbit and also reveals the possibility that domestic rabbits in and near the paramo highlands may harbor them.

Key words: Myxoma virus, Myxomatosis, Rabbit hemorrhagic disease virus, PCR, RT-PCR, *Sylvilagus andinus*, Andean rabbit, Antisana Hydrological Conservation Area.

INDEX

1. INTRODUCTION

Sylvilagus andinus (Thomas, 1897) is primarily found in paramo grassland, moorlands, and adjacent high Andean forests. It is a herbivorous, solitary, and terrestrial species, of blackish, mottled black and fawn fur, and with short rounded ears (Vallejo, 2018). *Sylvilagus andinus*, commonly named Andean rabbit or Andean cottontail, belongs to the Order Lagomorpha, family Leporidae, and it is distributed from Venezuela in the north to Peru in the south between 2,500 and 4,800 meters (Ruedas & Smith, 2019). It is hypothesized that *S. andinus* lineage split from the lineage leading to *S. audobonii* + *S. nuttallii* about 3.09 million years ago, and that clade joined to *Sylvilagus brasiliensis* separated 3.88 million years ago from *Sylvilagus floridanus*, which inhabits North America (Ruedas, et al., 2017).

Sylvilagus andinus builds its burrows under grass tussocks and in rock crevices, and feeds on the vegetation surrounding its burrows. It is more active early in the morning and at noon, though it keeps active during the day and night (Tirira, 2015). One of the areas where the Andean rabbit is most commonly seen is in the moor and grasslands of the Antisana Ecological Reserve (Reserva Ecológica Antisana or REA for short), in the provinces of Pichincha and Napo, near to the Antisana volcano (Ministerio del Ambiente, 2015). Usually, the Andean rabbit is not aggressive, but in some cases might use its incisors and hind claws to protect itself from predators.

Predator populations in the buffer zone of the Antisana Ecological Reserve have not been quantified, but include small numbers of Andean Fox (*Lycalopex culpaeus*), puma (*Puma concolor*), feral dogs (*Canis familiaris*), Variable Hawks (*Buteo polyosoma*), Short-eared Owls (*Asio flammeus*), among others (M. Tellkamp, personal communication; Zapata-Ríos & Branch, 2016). Nevertheless, the population of rabbits appears to go through great population cycles. In 2009 park guards claim not having seen a single rabbit (I. Bautista, personal communication), but by November 2018, rabbits were hyperabundant (M. Tellkamp, personal communication), diminishing progressively ever since. Several dead rabbits in the grassland appear nearly intact, and it appears that they suddenly die while feeding (M. Simba, personal communication). Given the apparent low numbers of predators, the death of rabbits might be caused by viral infections, which could diminish the population

rapidly, due to high contagiousness (Kerr, 2012). The remaining rabbits, which become immune, restore the population until the next epidemic (Bhopal, 2016).

Viral pathogens can be diagnosed by using molecular biology methods such as the Polymerase Chain Reaction assay (PCR). This method utilizes a pair of synthetic oligonucleotides called primers, and each primer hybridizes to one of the double-stranded DNA (dsDNA) targets covering the region that will be exponentially replicated by a DNA Polymerase. The most commonly used DNA Polymerase is derived from the thermophilic archaebacterium *Thermus aquaticus*, called *Taq* Polymerase. The process begins with dsDNA separation at temperatures higher than 90°C (denaturation), then, the temperature is reduced to 50°C for primer annealing on the denatured DNA strand, and finally, the DNA polymerizes during the elongation phase at approximately 72°C to create a complementary, antiparallel strand to the template DNA. The three steps are repeated cyclically by controlling the temperature, ramp rate, length of incubation at each temperature, and the number of cycles, in a Thermocycler. This way, after 30 to 40 cycles, the target DNA region has been amplified several billion-fold (NIH, 2020).

The DNA product, also called amplicons, can be visualized on agarose or polyacrylamide gels. As the net charge of the DNA molecules is negative, they move towards the positive electrode when the charge circulates through a salt-enriched medium such as TAE or TBE buffer. Because of the porosity of the gel, the DNA fragments are separated by size, the smaller fragments move faster towards the positive electrode and the longer fragments move more slowly. After that, the DNA fragments form separated bands, and a DNA intercalant such as Ethidium Bromide, Gel Red, or SYBR is added to make it visible under UV light (Hall, 2019). The PCR method has received modifications and it has been combined with other methods to be used in a wide range of procedures, such as the RT-PCR. It is highly reliable and time-efficient, having displaced some of the other methods used for viral detection such as cell culture, antigenemia, serological assays (Mackay, Arden, & Nitsche, 2002). For this reason, we used a conventional PCR and an RT-PCR for detecting viral particles of Myxoma virus and Rabbit hemorrhagic disease virus in wild rabbit samples.

2. THEORETICAL BACKGROUND

2.1 Andean Rabbits and their impact on the Antisana Hydrological Conservation Area

The Antisana Hydrological Conservation Area (ACHA) covers 8,200 ha of Andean grass and moorland west of the Antisana volcano between 3,300 and 4,800 meters above sea level (m.a.s.l.). This area is part of the watersheds that provide drinking water for the south of the metropolitan district of Quito (DMQ), the Ecuadorian capital (Quito Informa, 2020). The ACHA is part of the buffer zone of the Antisana Ecological Reserve (REA), one of the 59 protected areas conserved in Ecuador through the National System of Protected Areas (SNAP) (Ministerio del Ambiente, 2020). Since 2011, It is an area dedicated to the conservation and recovery of paramo vegetation under the care of the Fund for the Protection of Water (FONAG) to ensure the supply and quality of water for the DMQ (Coronel, 2019). Although most of the REA is constituted by Andean paramo-type ecosystems, the biodiversity of the reserve is remarkably high. In the REA, 418 species of birds, 73 species of mammals, and 61 species of amphibians and other reptiles have been recorded (Ministerio del Ambiente, 2015). Among these species, there are spectacled bears (*Tremarctos ornatus*), cervicabras (*Mazama rufina*), white-tailed deers (*Odocoileus virginianus ustus*), dwarf deers (*Pudu mephistopholes*), mountain tapirs (*Tapirus pinchaque*), pumas, Andean cats (*Leopardus colocola*), foxes, condors (*Vultur gryphus*), curiquingues (*Phalcoboenus carunculatus*), Andean gulls (*Chroicocephalus serranus*), lizards, guagsas (*Stenocercus* spp.), Andean rabbits (*Sylvilagus andinus*), and the Antisana osornosapo (*Osornophryne antisana*), a brown miniature frog that lives among the rocks of the moorland, very rare to find and in danger of extinction (Ministerio del Ambiente, 2015).

Apart from birds, the most commonly seen species in the ACHA are the white-tailed deer and Andean rabbits, the latter in large numbers. According to an assessment of the population of *S. brasiliensis andinus* made in the paramos surrounding the areas of the REA and the Cayambe-Coca National Park, the rabbit densities vary between 23 to 92 individuals/ha (García, Suárez, & Zapata-Ríos, 2016). In some areas, the population density is notably high, which could have an impact on the ecology of the ecosystem, mainly on the vegetation and the population of predators. Even though the Andean rabbit feeds on a large variety of plant species, up to 67 different species, there is a preference for five to nine species in the rabbit's

diet (Valero & Durant, 2001). Consequently, the impact on those species is concerning for the ecological balance and the conservation of the Andean paramo ecosystem, to the extent that rabbits are exclusive primary consumers and could act as seed dispersers (Gibbens, Havstad, Billheimer, & Herbel, 1993).

Nowadays, the population of *S. andinus* seems to be declining as the paramo guards reported. According to a paramo guard report, the population of *S. andinus* cycles up and down every nine to ten years, whereby an overpopulation of rabbits is followed by a complete absence of the species (I. Bautista, personal communication). Additionally, an assessment of the rabbit population from December of 2012 to July of 2013, shows an exponential increase in the population density (García, Suárez, & Zapata-Ríos, 2016). On the other hand, a population evaluation of the Andean rabbit at the Jerusalem protective forest, made from December of 2017 to April of 2018, shows a decrease in the number of individuals in the area (Zapata, 2018). Unfortunately, more information about the population dynamics of Andean rabbits in the Ecuadorian Andes is scant.

Species interaction in the buffer zone of the REA has been altered by human activities during the last five decades. Large extensions of the natural landscape were converted into agricultural lands and pastures for livestock, with the consequent loss of habitat for several native species, primarily by wetlands drainage (Coronel, 2019). As a consequence, the ecological disturbance has altered the number of individuals of populations of native animal and vegetal species (Aguirre, Torres, & Velasco-Linares, 2013). However, the dramatic decrease of the Andean rabbit population in the ACHA cannot be explained by other reasons than by the abundance of available food species (García, Suárez, & Zapata-Ríos, 2016; Zapata, 2018), which has been changing lately because of the ecosystem recovery programs implemented by FONAG (Coronel, 2019). Moreover, the scarcity of predators (M. Simba, personal communication), food availability, and weather conditions almost remained unaltered during the last ten years (Díaz, 2018), make it difficult to account for the large population cycles in the Andean rabbit population by ecological and environmental factors alone.

2.2 Diseases in wild rabbits

Another factor to consider is the presence of viral diseases, as the epidemiology of certain diseases can establish patterns of population dynamics under certain conditions (Bhopal, 2016). The diseases that have had the greatest impact on rabbit populations of the genera *Sylvilagus* and *Oryctolagus* are Myxomatosis, Rabbit hemorrhagic disease (RHD), and European brown hare syndrome (EBHS) (Tizzani, Lavazza, Capucci, & Meneguz, 2002).

Myxomatosis is caused by the Myxoma Virus (MYXV) which has one of the highest rates of fatality cases of species-specific viruses with 90% to 99% in European rabbits (Kerr, 2012). MYXV belongs to the genus Leporipoxvirus, subfamily Chordopoxvirinae, family Poxviridae (Kerr, et al., 2015). During the 1950s, the Myxoma Virus was introduced into the Australian wild rabbit population to control the increasing number of individuals that caused crop damages. Then, it was introduced in Britain and France causing the spread of the virus throughout Europe, generating a dramatic reduction of wild rabbit populations, and spreading serious infections among farm rabbits of the genus *Oryctolagus* (Kerr, 2012). Infected rabbits with Myxomatosis present conjunctival inflammation with elevated temperature about 4 days after infection. After 6 days, anogenital swelling is observed with cutaneous papular secondary lesions on the face and ears, accompanied with mucopurulent discharge from nostrils and conjunctivae (Fenner & Ratcliffe, 1965). Then, 8 to 10 days after infection, the acute stage of Myxomatosis can be diagnosed by a swollen head and face, swollen drooping ears, mucopurulent blepharoconjunctivitis with swollen closed eyes, and mucopurulent rhinitis. Multiple cutaneous tumors or myxomas appear over the body, head and neck became extended, and respiration is slow during the late stage of the disease. Death occurs 8 to 12 days after infection, and the virus persists and is transmissible through the secretions and skin lesions of dead animals for several days by direct contact or via biting arthropods (Kerr, 2012).

The genome of the MYXV contains 171 open reading frames (ORFs) in 161.8 kb of dsDNA. The genome also contains 11,577 bp of terminal inverted repeats (TIRs) composed of 12 genes with two copies each (Cameron, et al., 1999). The genome of the MYXV (Lausanne strain) is shown in Figure 1 with the TIRs indicated with a blue arrow, and the gene M000.5 colored in red.

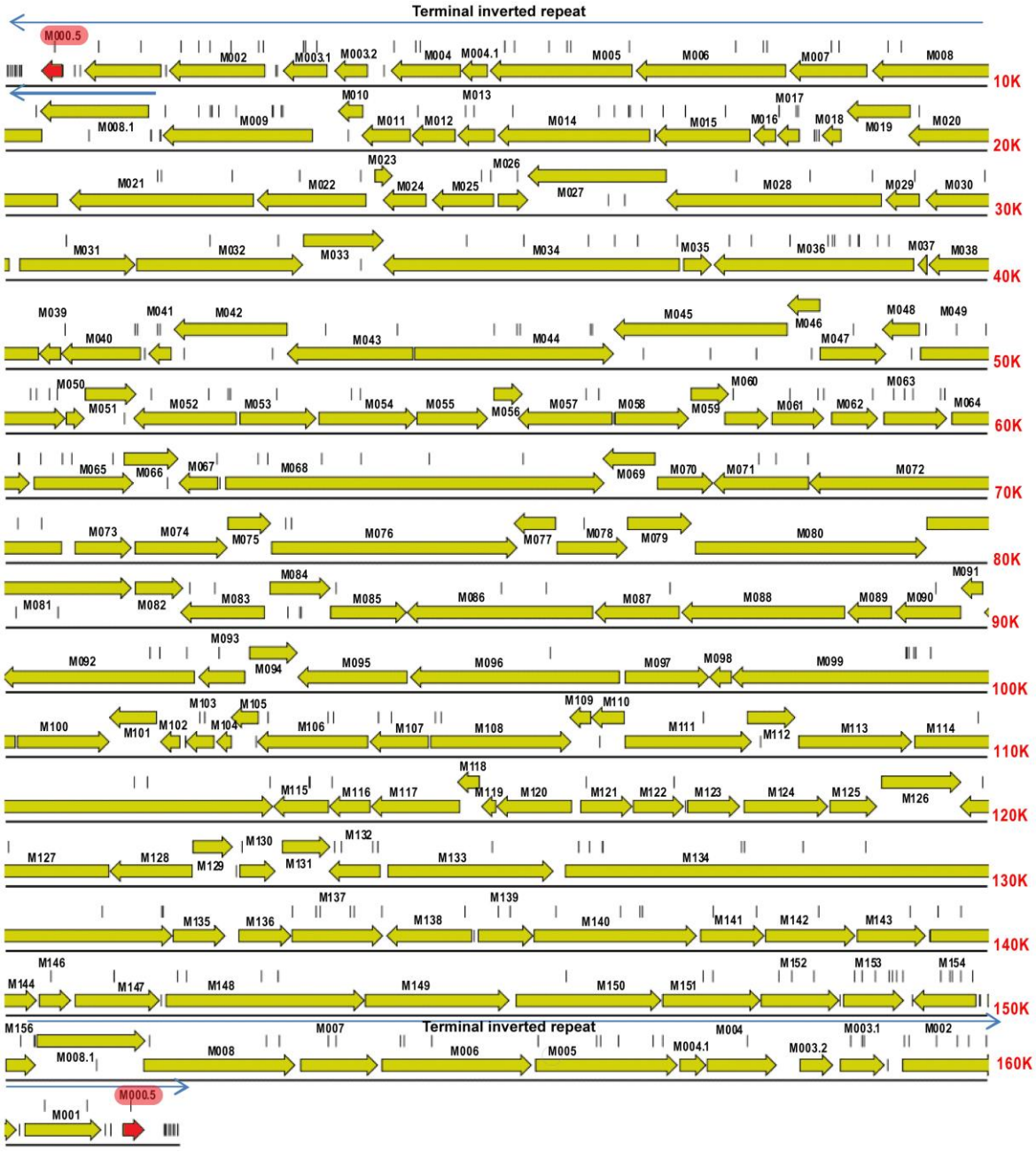


Figure 1. *Gene map of MYXV (Lu strain).* Yellow arrows show the open reading frames and their direction of transcription. The blue light arrow at each end of the genome shows the 11,577 bp terminal inverted repeats. The genes are named with the M letter followed of the numbers 0.5 to 156. The red arrows show the target duplicated gene (M000.5L/R) for the detection of MYXV and vertical lines show the location of mutations in Australian isolates. Taken from Kerr P. J., et al. (2012).

Another important disease affecting leporids is the Rabbit hemorrhagic disease caused by the Rabbit hemorrhagic disease virus (RHDV), which has a rate of more than 60% mortality in

Oryctolagus spp (Belz, 2004; Le-Gall-Reculé, Zwingelstein, Portejoie, & Le Gall, 2001), but also infects *Sylvilagus* spp. (Montana Public Radio, 2021). RHDV belongs to the genus Lagovirus, family Caliciviridae, and, as other caliciviruses, RHDVs are small non-enveloped virions. The RNA molecule is protected by a capsid protein, which forms cup-shaped depressions arranged in an icosahedral symmetry (Valicek, Smid, Rodak, & Kudrna, 1990). The virions contain the single-stranded, positive-sense genomic RNA (gRNA) of 7,437 nucleotides consisting of two overlapping open reading frames (ORF), and an additional subgenomic RNA (sgRNA) of 2.2 kb (Abrantes, van der Loo, Le Pendu, & Esteves, 2012). The ORF1 contains 7034 nucleotides, encodes a large polyprotein composed of non-structural proteins involved in viral replication and the major structural VP60 protein, which is duplicated in the sgRNA alongside with VP10 protein of the ORF2 (Figure 2).

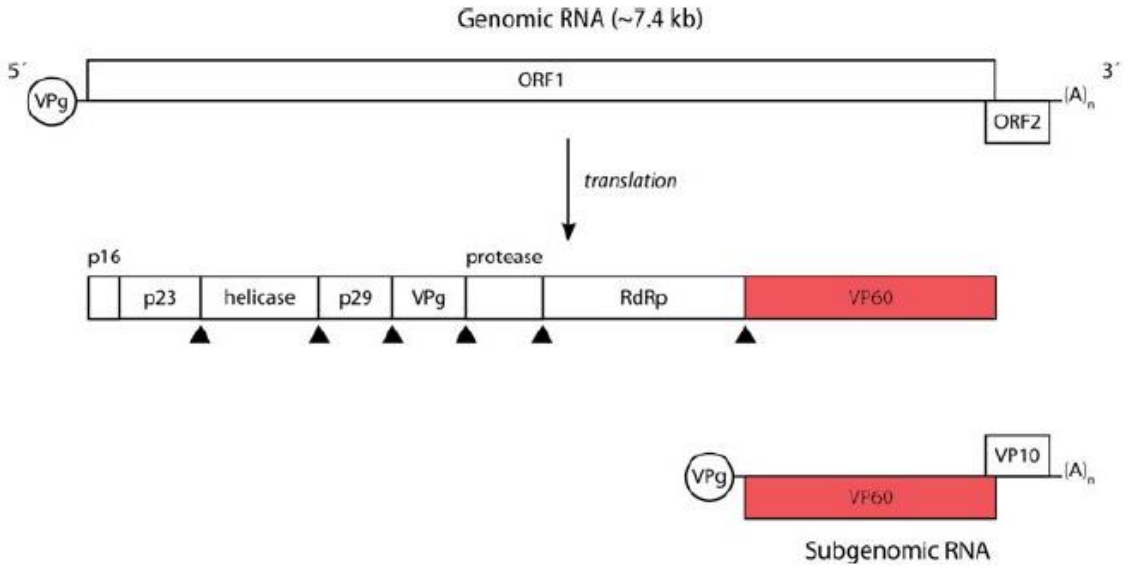


Figure 2. *Genomic organization of RHDV*. RHDV genome is composed of two ORFs, ORF1 encodes for a several non-structural proteins and the major structural protein for the capsid VP60 (in red). ORF2 encodes for a minor structural protein VP10. The sgRNA can be found in viral particles encoding the structural proteins VP60 and VP10. Taken from Abrantes, et al. (2012).

Rabbit hemorrhagic disease was first observed in Europe and China in the 1980s killing millions of domestic and wild rabbits (*Oryctolagus cuniculus*). The disease presents an aggressive peracute form killing rabbits suddenly 48 to 72 hours after infection with convulsions in opisthotonus position (Kerr & Donnelly, 2013). Also, acute infections can be identified by anorexia, apathy, congestion of the palpebral conjunctiva, and neurologic symptoms such as paralysis and ataxia. Respiratory signs, such as foamy and bloody nasal discharge, lacrimation, ocular hemorrhages, and epistaxis can occasionally occur. Additionally, a chronic form of the disease also exists with symptoms including jaundice, anorexia, lethargy, and death after 1 to 2 weeks (Patton, 1989). RHDV affects primarily the liver, lung, and spleen causing hemorrhages in the lungs, heart and, kidneys. Its transmission may occur by direct contact with infected animals, feces, secretions, and arthropods vectors (Abrantes, van der Loo, Le Pendu, & Esteves, 2012).

Also, an important disease is the EBHS caused by the European brown hare syndrome Virus (EBHSV) which has a rate of mortality of 60% to 90% (Le-Gall-Reculé, Zwingelstein, Portejoie, & Le Gall, 2001). For reasons of time and resources, only MYXV and RHDV were considered for this study. Both of them are highly infectious, with a short period of incubation, and fatal within few days, affecting multiple organs such as skin, lungs, and respiratory tract in the case of MYXV (Bertagnoli, Messud-Petit, & Marlier, 2006; Kerr, 2012), and liver, spleen, kidneys, and heart in the case of RHDV (Belz, 2004). These diseases are amply studied in European rabbits (*Oryctolagus*), but are poorly studied in American rabbits (*Sylvilagus*), and have never been tested for *Sylvilagus andinus*.

3. OBJECTIVES

3.1 General Objective: To determine the presence of Myxoma Virus and Rabbit hemorrhagic disease Virus in *Sylvilagus andinus* in the Antisana Hydrological Conservation Area.

3.2 Specific Objectives:

a) To capture Andean rabbits (*Sylvilagus andinus*) and take blood, and nasal swabs samples.
b) To take necrotic tissue samples from dead Andean rabbits.

c) To detect the presence of two viruses (Myxoma virus and Rabbit hemorrhagic disease virus) in blood, mucus, and tissue samples from Andean rabbits.

4. MATERIALS AND METHODS

4.1 Field Sampling

4.1.1 Site desccription

The area of study was located in the western buffer zone of the REA, within the area of the ACHA, next to the "Humboldt House" and the "Antisana House." Two transects of 500 meters were established to capture specimens, blood and mucus sampling, on both sides of the Micahuaycu creek. Another transect of 2000 meters was established to look for dead rabbits to collect necrotic tissue samples. The entire area of study was located within a diameter of 2000 meters around the GPS coordinates: 0°30'39.394''S, 78°12'56.494''W, between 4,060 and 4,200 m.a.s.l. This site was chosen because of easy access by road, and the proximity to the campsite at the Antisana House, thus facilitating the transport of the material required for the capture and sampling of specimens.

4.1.2 Sampling

To take the samples of blood and mucus, the specimens were captured using Tomahawk live traps with the plot-trap and the burrow-trap methods, following the procedures of Insuasty, et al. (2008). The bait was composed of peanut butter, oat, and fruits as described Tirira (2015). Fifty-two Tomahawk traps were located in the first transect during the course of 3 days (20 on the first and second days, and 12 on the third day) 50 meters apart for the plot-trap method (Insuasty, Ramírez, & Mejía, 2008) with a bait (Tirira, 2015). Additionally, seventy Tomahawk traps were located on the second transect distributed during 5 days (8 on the first day, 20 on the second day, and 14 on each of the next three days) at 30 meters apart for the burrow-trap method (Insuasty, Ramírez, & Mejía, 2008). All the traps were revised three times per day to reduce stress from capture and capture myopathy.

The captured individuals were carefully handled with rubber palm gloves and seized by the neck and hind legs to avoid bites and scratches. The blood sample was taken by professional

veterinarian from the central atrial artery and marginal ear vein with 3ml syringes, after disinfecting the puncture site with a 70% v/v alcohol swab (Penadés, et al., 2011). Then, the sample of blood was transferred to BD Vacutainer 4ml K2 EDTA tubes, 1ml RNA stabilization solution was added for RNA preservation, and samples were stored at 4°C. The mucus sample was taken from the nasal cavities, by gently introducing a cotton swab and turning to scrape epithelial cells off the walls of the nasal cavity (Penadés, et al., 2011). Then the cotton swab was introduced in a cryovial with 1ml of PBS-Glycerol transport medium and stored to 4°C. Both, the swabs and blood samples were stored in a -20°C freezer in the lab for longer preservation. For the tissue collection, we used dead specimens that were found in excellent conditions. The samples were taken using scissors, forceps, and scalpels following the recommendations of Parkinson, et al. (2011). The collected samples were submerged in 1ml of RNA stabilization solution and kept at 4 °C for transportation, and finally stored at -20°C until further use.

4.2 Laboratory Methods

4.2.1 RNA stabilization solution

This solution is used for preserving samples collected in field conditions for stabilization of rapidly degrading RNA for long-term storage. RNA stabilization solution rapidly permeates tissues to protect the RNA (Passow, et al., 2018). For its synthesis, we needed to prepare 0.5M EDTA and 1M sodium citrate. A total of 100ml to 0.5M EDTA was obtained by adding 18.61g of EDTA (Fisher Scientific) to 70ml ultra-pure water (Type I), and the pH was brought to 8.0 with 2.3g of NaOH pellets (Emsure) while stirring on hot plate at room temperature. The volume was brought to 100ml with ultra-pure water and autoclaved to ensure that it was sterile. In the same way, 1M sodium citrate was obtained by adding 29.4g of sodium citrate dihydrate (Fisher Scientific) to 70ml of ultra-pure water with stirring, and the volume was brought to 100ml with ultra-pure water. As before, the sodium citrate solution was autoclaved for 40 minutes at 120°C for ensuring sterility.

To prepare RNA stabilization solution, 6ml of 0.5M EDTA and 3.75ml of 1M sodium citrate were mixed in a 500ml flask while stirring. During this time, 105g of ammonium sulfate 99.0% (Sigma – Aldrich) was added in 20g amounts to ensure proper dissolution. Stirring

continued for around another 30 minutes on a stirring hot plate set to low until crystals of ammonium sulfate completely dissolved. The final solution was cooled for 1 hour to room temperature and stored at 4°C until use. The RNA stabilization solution has a final concentration of 25mM sodium citrate, 20mM EDTA, 70g ammonium sulfate for every 100ml of solution, and a pH of 6.97.

4.2.2 PBS-Glycerol transport medium

For the preservation of viral content in swab samples, we used a viral transport medium following the guidelines for collecting, preserving, and shipping animal specimens for the diagnosis of influenza (World Health Organization, 2006), since there is no information on handling samples for diagnosis of Leporipoxviruses nor Lagoviruses. The viral transport medium is composed of a 1:1 mix of PBS (Fisher Chemical) and 99.5% glycerol (Sigma – Aldrich). First, 25ml of PBS was autoclaved to 120°C for 40 minutes to sterilize it and mixed with 25ml of sterile glycerol to make a 50ml solution with a pH 7.13 (at 22°C). Then, 1ml of PBS-Glycerol transport medium was dispensed into sterile plastic screw-cap vials (cryovials). The cryovials were stored at –20°C and transported to 4°C for usage following the guide's recommendations.

4.2.3 TBE buffer 10X

Tris-borate-EDTA (TBE) buffer is one the most common running buffers used in nucleic acid electrophoresis. Its properties, such as constant pH and electric conductivity are necessary for separating nucleic acids by electric charge. In addition, some of its advantages are high buffer capacity, enzyme activity inhibition, and slow DNA migration, which results in a good resolution for small (less than 0.3 kb) DNA fragments (Merck, 2021). According to the formula, we need a 0.5M EDTA solution dissolving 7,445g of EDTA (Fisher Scientific) into 40ml of ultra-pure water on a stirring hot plate. To get EDTA completely dissolved, we adjusted pH at 8.0 by adding sodium hydroxide pellets (Emsure) while stirring and measuring pH with a pH meter (Thermo Scientific). Subsequently, 108g of Tris-base (Fisher Scientific) and 55g of boric acid (Sigma – Aldrich) were dissolved in 800ml of ultra-

pure water while stirring, then 40ml of 0.5M EDTA were added to the solution, and the final volume was adjusted to 1 liter by adding more water (Merck, 2021).

4.2.4 Nucleic Acid Extraction

DNA/RNA extraction was performed using the CommaXP® Virus DNA/RNA Extraction Kit (Biocomma), which uses the silica membrane adsorption technology. The collected samples were kept at –20°C for better nucleic acid preservation. For nucleic acid extraction from blood, the samples were thawed at 4°C and centrifuged in a refrigerated centrifuge (ThermoFisher Scientific) at 1,000 to 2,000 x g (G-force) for 10 minutes set at 4°C, then the supernatant was transferred into a 1.5ml Eppendorf tube (Thermo Fisher Scientific, 2007). For nucleic acid extraction from nasal swabs stored in PBS-Glycerol viral transport medium, the swab was carefully pressed against the tube several times while rotating it, then the homogenate was transferred to a 1.5ml Eppendorf tube. For nucleic acid extraction from tissue samples, we first refrigerated the mortars, pistils, PBS, tweezers, and scalpels to –20°C to keep the sample temperature low while handling. The samples were thawed at 4°C, and approximately 0.1g of tissue was carefully cut with the help of tweezers and the scalpel. The 0.1g sample was placed in a mortar, adding 1ml of PBS, and ground until homogenous. It is important to use a sterile set of mortar, pistil, tweezers, and scalpel for each sample to avoid cross-contamination. This process was carried out in a biosafety cabinet. The homogenized tissue was transferred to a 2ml tube and centrifuged in a refrigerated centrifuge at 7,000 rpm for 5 minutes set at 4°C. The supernatant was transferred to a 1.5 ml Eppendorf tube as per kit manufacturer's instruction (BIOCOMMA LIMITED, 2020).

Continuing in a biosafety cabinet, 300µl of each processed sample was added to a new 1.5ml Eppendorf tube, along with 500µl of Buffer GLX and 20µl of Proteinase K (20mg/ml), mixed by Vortex mixer for 1 minute, and placed at room temperature for 5 minutes in order to lyse the cells. Afterward, the supernatant was transferred into the spin column RC2®, which was placed inside a 2ml collection tube, centrifuged at 12,000 rpm for 1 minute, and the flow-through was discarded. Before starting the wash step, it was necessary to add 15ml of isopropanol to the buffer PD and 60ml of absolute ethanol (≥ 99%) to the buffer PW. For the wash step, we added 500µl of buffer PD to the spin column, centrifuged it at 12,000 rpm

for 1 minute, and discarded the flow-through. Then, 700µl of buffer PW were added to the spin column, which was centrifuged at 12,000 rpm for 1 minute, and the flow-through was discarded. This step was repeated twice, with the final centrifugation set at 12,000 rpm for 2 minutes to eliminate all traces of the buffers. The spin column RC2® was kept with the lid open for around 10 minutes at room temperature to remove the remaining ethanol. Finally, the DNA and RNA were eluted with 50µl of RNase-free double distilled water preheated in a water bath at 65°C to increase elution efficiency and centrifuged at 12,000 rpm for 2 minutes to collect the DNA/RNA as recommended by the manufacturer (BIOCOMMA LIMITED, 2020). Eluted DNA/RNA was collected into 1.5ml Eppendorf tubes and quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific), following the spectrophotometer manual. Finally, samples were store at –80°C.

4.2.5 Conventional PCR for MYXV detection

To detect the presence of the MYXV, we used a conventional PCR based on the presence or absence of amplicons. The conditions to perform the PCR was established by following the methodology of development and validation of a PCR protocol for the detection of the MYXV based on the diploid gene M000.5L/R (Duarte, et al., 2014), which has a high specificity, high sensitivity, and a detection limit of 2.6 copies of DNA. The primers 000.5R/L-F and 000.5R/L-R (Eurofins Genomics) amplify a 125 bp segment of the M000.5R/L gene (Table 1). Additionally, a positive control (Synbio Technologies) was designed with an 835 bp oligonucleotide composed of a target segment of the M000.5R/L gene of the MYXV, and a segment of the VP60 gene of RHDV. The same positive control was used for the detection of both MYXV and RHDV.

Table 1. Primers for MYXV and RHDV detection. The sequences for M000.5L/R gene detection were taken from Duarte, et al. (2014) and the sequences for VP60 gene detection were taken from Yang, et al. (2008).

Primer	Sequence 5'-3'	bp	Position	Gene	Ref.number
000.5R/L-F	CGACGTAGATTTATCGTATACC	125	558–537 & 161216–161237	M000.5L/R	AF170726
000.5R/L-R	GTCTGTCTATGTATTCTATCTCC		434–456 &		

			161340–161318		
RHDV-F	CAGCCGTACTGAGCCAGATGTA	269	5612–5880	VP60	AY269825
RHDV-R	AAGGACTAGTGGGAACAAGG				

The PCR reaction was prepared using the LightMix® Lyophilized 1-step RT-PCR Polymerase Mix (TIB Molbiol), which ensures high specificity and sensitivity for extremely low-copy-number targets in pathogen detection. The 1-step RT-PCR Polymerase Mix contains a mixture of a reverse transcription (RT) enzyme and a thermostable *Taq* DNA polymerase at 2x concentration. The lyophilized polymerase mix was first prepared by adding 990µl of ice-cold qRT-PCR Reconstitution Buffer and mixed gently without vortexing, then aliquoted and stored at –20°C to minimize freeze-thaw cycles, as the manual recommends (TIB MOLBIOL, 2020). The 1µg lyophilized positive control was reconstituted in 100µl TE Buffer to obtain a 10ng/µl concentration, aliquoted in several dilutions to obtain 1ng/µl to 1pg/µl concentrations. The lyophilized primers were first reconstituted by centrifuging for 1 minute at 8,000 rpm to collect all possible free DNA particles in the tube, then resuspended in TE Buffer for better DNA preservation. The 32nmol lyophilized forward primer 000.5R/L-F was reconstituted in 320µl of TE Buffer for a stock solution at 100µM. In the same way, the 36.6nmol lyophilized reverse primer 000.5R/L-R was reconstituted in 366µl of TE Buffer for a stock solution at 100µM. Then an aliquote of 50µl was prepared with 10µl of the stock solution of each primer and 40µl of ice-cold double distilled RNase-free water for a final concentration of 20µM each.

For preparing the reactions, all the reagents were thawed on a cooling block (Selleckchem) previously frozen to –20°C. The PCR tubes were labeled and placed in the cooling block, as well. Before preparing the PCR reactions, the primers, samples, and positive control were briefly vortexed to ensure homogenization of the DNA, and the Polymerase Mix was mixed gently without vortexing. While handling, the reagents were kept at 0°C. The PCR reactions were prepared using the reagents and quantities listed in Table 2 by pipetting water first, then the primers and DNA, and finally the Polymerase Mix. The amount of DNA for the positive control was 1pg, and the negative control was prepared with all of the reagents except for the DNA template. However, the amount of sample DNA was variable and depended on the amount of extracted DNA in every sample as can be seen in Appendix 1. The prepared

reactions were immediately transferred to the thermocycler (Applied Biosystems) and preheated to 95°C to avoid any enzymatic activity (New England BioLabs, 2021). Even though Duarte, et al. (2014) developed a highly sensitive PCR protocol, better results were obtained with a touchdown PCR, which increases the specificity and yield of the reaction. The touchdown PCR was performed with the parameters shown in Table 3, which were based on the thermal cycling parameters of a touchdown PCR protocol (Korbie & Mattick, 2008).

Table 2. PCR Reactions Mixture. Volume of each component for the 20 µl PCR reaction.

Component	Volume for 20 µl reaction	Notes
1-step RT-PCR Polymerase Mix (2x)	10 µl	Final concentration 1x
Primer forward (20 µM)	1 µl	Final concentration 1µM
Primer reverse (20 µM)	1 µl	Final concentration 1 µM
Sample	Variable	Used as much as needed
PCR grade water	Variable	Filled up to 20 µl

Table 3. Touchdown PCR parameters used for performing a touchdown PCR with a 1st amplification step in which temperature* decreases 1 °C each cycle during 13 cycles for increasing specificity. The 2nd amplification step uses the same annealing temperature for 22 more cycles for increasing the yield of the reaction. A total of 35 cycles of amplification were run in this PCR.

Step	Stage	Phase	Cycles	Temperature	Time
Initial denaturation	1		1	95 °C	2 min
1st Amplification	2	Denaturation	13	95 °C	30 sec
		Annealing		62 – 50 °C *	45 sec
		Elongation		72 °C	30 sec
2nd Amplification	3	Denaturation	22	95 °C	30 sec
		Annealing		51 °C	30 sec

		Elongation		72 °C	30 sec
Final Extension	4		1	72 °C	1 min

4.2.6 RT – PCR for RHDV detection

A RT-PCR was performed to detect the presence of RHDV. The parameters for the RT-PCR were obtained from Yang, et al. (2008) using the VP60 gene of the RHDV which encodes for a highly conservative capsid protein. The primers Rhdv – F and Rhdv – R (Eurofins Genomics) amplify a region of 269 bp of the VP60 gene of RHDV (Table 1). The same positive control used in the PCR for the detection of MYXV was used for this protocol, as it has a target segment of the VP60 gene of the RHDV. Before starting, the lyophilized primers were centrifuged for 1 minute at 8,000 rpm and reconstituted in TE Buffer. The 31.2nmol forward primer Rhdv – F was diluted in 312µl of TE Buffer for a stock solution of 100µM. The 29.5nmol reverse primer Rhdv – R was diluted in 295µl of TE Buffer for a stock solution of 100µM. An aliquote of both primers was prepared by diluting 10µl of stock solution in 40µl of double-distilled RNase-free water for a 20µM concentration.

All the reagents were prepared in the same way as for the conventional PCR by thawing them on a cooling block alongside PCR tubes. The reagents were briefly vortexed except for the Polymerase Mix. Keeping the reagents at 0°C, the reactions were prepared by adding water, primers, DNA, and the Polymerase Mix at the quantities detailed in Table 2. The positive control was prepared with 1µl of 1pg/µl of the Positive Control. The negative control was prepared by adding all the reagents except for DNA, which was substituted by water. The specific amount of sample added to the mix depended on the concentration of the extracted RNA, as can be seen in Appendix 1. Once the reactions were prepared, they were placed immediately into the Thermocycler (Applied Biosystems) preheated at 50°C, which is the temperature for reverse transcription (RT) enzyme activation. After reverse transcription, the initial denaturation at 95°C inactivates the RT enzyme; the remaining PCR protocol used the parameters show in Table 4. Those parameters were used by Yang, et al. (2008) with certain modifications for a better performance.

Table 4. Reverse Transcription PCR parameters used for performing an RT-PCR with 30 cycles of amplification.

Step	Stage	Phase	Cycles	Temperature	Time
Reverse Transcription	1		1	50 °C	5 min
RT inactivation/Initial denaturation	2		1	95 °C	2 min
Amplification	3	Denaturation	30	94 °C	60 sec
		Annealing		56 °C	45 sec
		Elongation		72 °C	30 sec
Final Extension	4		1	72 °C	1 min

4.2.7 Electrophoresis

PCR products were visualized on 1.8% agarose gels stained with ethidium bromide. The agarose gel was prepared in an Erlenmeyer flask by dissolving 1.5g of InstantAgarose™ (HydraGene) in 83ml of TBE Buffer 1x. To get agarose completely dissolved, the solution was microwaved until ebullition began. Then, 10µl of ethidium bromide (EtBr) was added. A 10mg/ml EtBr solution was prepared by adding 10mg of EtBr powder in an Eppendorf tube of 1.5ml and filling it with ultra-pure water to a total volume of 1ml. The solution was mixed by pipetting for several minutes until the EtBr powder dissolved completely. This solution was prepared under a gas extraction cabin to avoid inhalation of vapors, as the EtBr is potentially carcinogenic (Hong & Piette, 1976). The tube was covered by aluminum foil to protect it from light and stored at -20°C until use. Finally, the agarose solution with EtBr was poured onto the tray and allowed to cool until solid.

Once the gel solidified, it was placed inside a DNA electrophoresis chamber of a Wide Mini-Sub Cell GT Cell (BIO-RAD) filled to the mark with TBE Buffer 1x. Then, 5µl of each PCR product DNA was mixed with 1µl of TriTrack DNA Loading Dye 6x (Thermo Scientific), previously thawed from –20°C, and put inside each well alongside 2µl of GeneRuler™ 50 bp DNA Ladder (Thermo Scientific), also previously thawed from –20°C. TriTrack DNA Loading Dye contains three different dyes for visual tracking of DNA migration during electrophoresis: blue (Xylene cyanol FF 3030 bp), purple (bromophenol blue 220 bp), and

orange (Orange G <50 bp). It also helps to keep DNA in the well due to the glycerol and inhibits metal-dependent nucleases by the EDTA (Thermo Fisher Scientific, 2015). GeneRuler™ 50 bp DNA Ladder contains DNA fragments for sizing and approximate quantification of dsDNA on agarose gels with two reference bands at 500 and 250 bp for easy orientation (Thermo Fisher Scientific, 2018). The DNA electrophoresis chamber was connected to a power supply PowerPac™ Basic Power Supply (BIO-RAD) and set at 100V for 50 minutes. The DNA bands on gels were visualized with a UV Transilluminator (Enduro GDS Touch).

4.2.8 Sequencing

Four confirmed PCR products were sequenced by Macrogen (Seoul, South Korea) through the laboratory IDgen (Quito, Ecuador) to corroborate the results. The PCR products were run in agarose gel 2% at 80V for 2 hours for a better band separation. Because of the presence of several bands, the DNA of interest was extracted from agarose gel with a PureLink™ Quick PCR purification kit (Invitrogen). Finally, the DNA was amplified to obtain more product using the same primer sets (Table 1) and PCR parameters (Table 3 and Table 4) and sent to Macrogen for a Sanger Sequencing. The reads of two amplicons positive for RHDV were assembled with Geneious Prime® 2021.1.1 software using the De Novo Assemble algorithm. The consensus sequences obtained were aligned using the Basic Local Alignment Search Tool for nucleotide sequences (BLASTn) (Altschul, Gish, Miller, Myers, & Lipman, 1990) with the sequence databases of the National Center for Biotechnology Information (NCBI). Two sequences positive for MYXV were assembled with MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) software with a ClustalW alignment algorithm (Thompson, Higgins, & Gibson, 1994) using as reference the NCBI genomic databases.

5. RESULTS

5.1 Field sampling

To capture individuals for sample collection, 122 Tomahawk live-traps were used following the plot-trap and burrow-trap methods (Insuasty, Ramírez, & Mejía, 2008). No individual

could be captured with the plot-trap method with baited traps. In contrast, 23 individuals were captured with the burrow-trap method, which represents a 33% trapping rate. From those, 1 rabbit escaped and 2 were too small to get samples from, so samples were obtained from 20 rabbits. Regarding the trap sites, 14 individuals were captured on the Antisana house transect, which represents 61% of the captured specimens, whereas 9 individuals were captured on the Humboldt house transect or 39% of the total captured rabbits (Table 5). In terms of sex, 8 rabbits were female and 15 male rabbits (Appendix 2).

Table 5. Capture successful rate of two methods used on three transects.

Method	Transect	Deployed traps	Captured rabbits	Successful rate
Plot-Trap	Micahuaycu creek	52	0	0%
Burrow-Trap	Antisana house	38	14	36.84%
	Humboldt house	32	9	28.13%

In addition, we found three dead rabbits from which 3 tissue samples each could be obtained. In total, 20 samples of blood, 14 nasal swabs, and 9 tissue samples were taken (Appendix 2). The type of tissue required corresponds to the lung, liver, and spleen (Belz, 2004; Yang, et al., 2008; Kerr, 2012; Duarte, et al., 2014). One of the specimens was found partially eaten by a *Phalcoboenus carunculatus* "Curiquingue" and no longer had lungs nor liver; in this case, penis and testicle samples were taken (Kerr, 2012; Duarte, et al., 2014).

5.2 Laboratory

5.2.1 Nucleic Acid Extraction

The quantity of DNA and RNA from the 43 samples in 50µl solution varies widely between sample types (Table 6). Some of the samples had barely the amount necessary for PCR. Other samples had too much DNA and they had to be diluted for the PCR. Some contamination is present in several samples and quantified with the 260/280 and 260/230 ratio (Table 6 and Appendix 3).

Table 6. Summary of spectrophotometric measurement of nucleic acid extractions. The values for Min, Max, Median, and Mean calculations for DNA and RNA (units included) were taken from the total DNA/RNA

extracted from the samples, and the rest of the values were calculated with the DNA/RNA concentration in 50 µl solution.

	DNA	260/280	260/230	RNA	260/280	260/230
Minimum	40 ng	0.88	0	25 ng	0.88	0
Maximum	33.61 µg	27.23	7.2	27.15 µg	10.85	4.49
Median	420 ng	2.11	0.25	335 ng	2.09	0.25
Mean	3.674 µg	3.06	0.83	2.683 µg	2.69	0.74
Standard Deviation	139.15	3.95	1.377	107.79	1.779	0.972
Variance	19361.78	15.6	1.895	11619.54	3.164	0.944

5.2.2 PCR for MYXV detection

The PCR product from the 43 samples could only be measured qualitatively by electrophoresis in agarose gel. The resulting bands vary in intensity in several samples showing a clear band at 125 bp. The samples 8B (Figure 3), 5S (Figure 4), and 14S, 1T, and 4T samples (Figure 5) contain a visible band at 125 bp. Other samples have a very faint band but still visible at 125 bp such as 20B, 21B, 4S, 6S, 7S, 8S (Figure 4) and 13S, and 6T (Figure 5). In total, 13 positive samples for MYXV belonging to 9 individuals represent 39% of the sampled rabbits (Appendix 4). The rest of the samples show no bands between 100 and 150 bp.

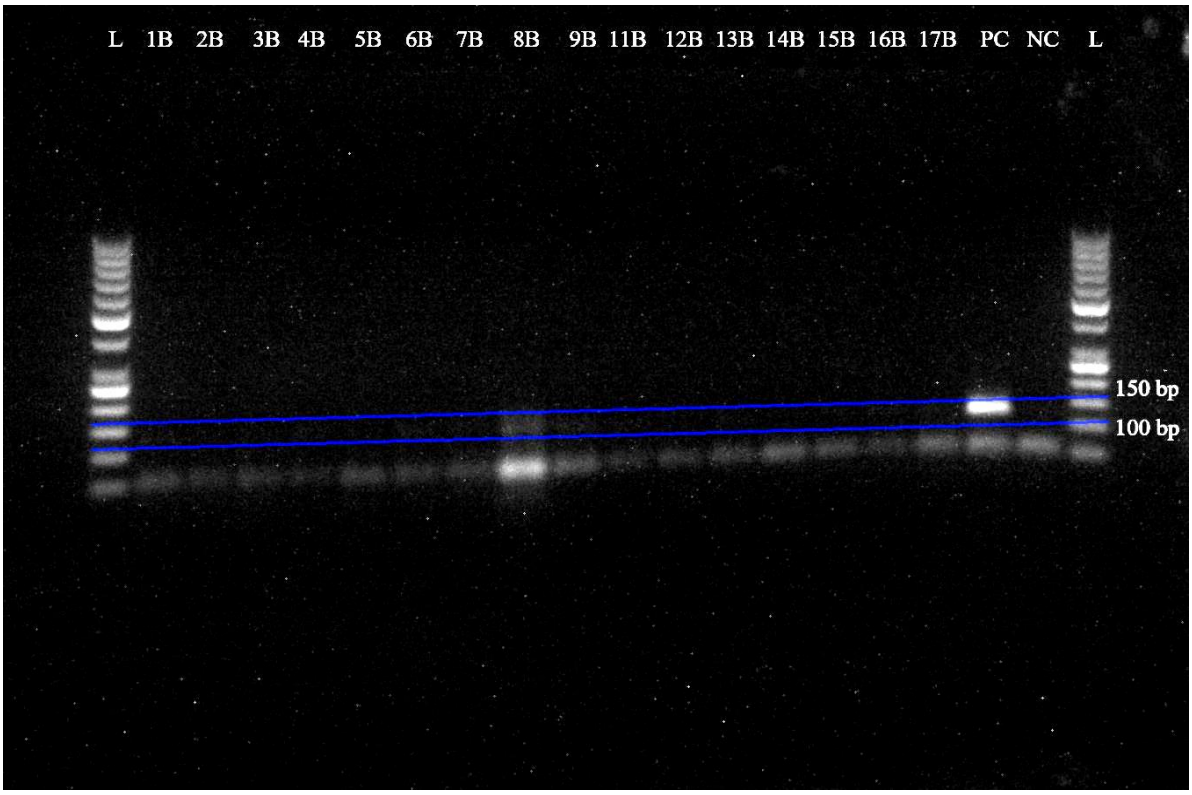


Figure 3. *Agarose gel (EtBr) with PCR products 1B-17B under UV-light.* L: 50 bp DNA ladder with 250 bp and 500 bp reference (more intense bands); 1B – 17B: samples; PC: Positive control; NC: Negative control. PC has a band at 125 bp as well as 8B sample. The other samples seem to be negative.

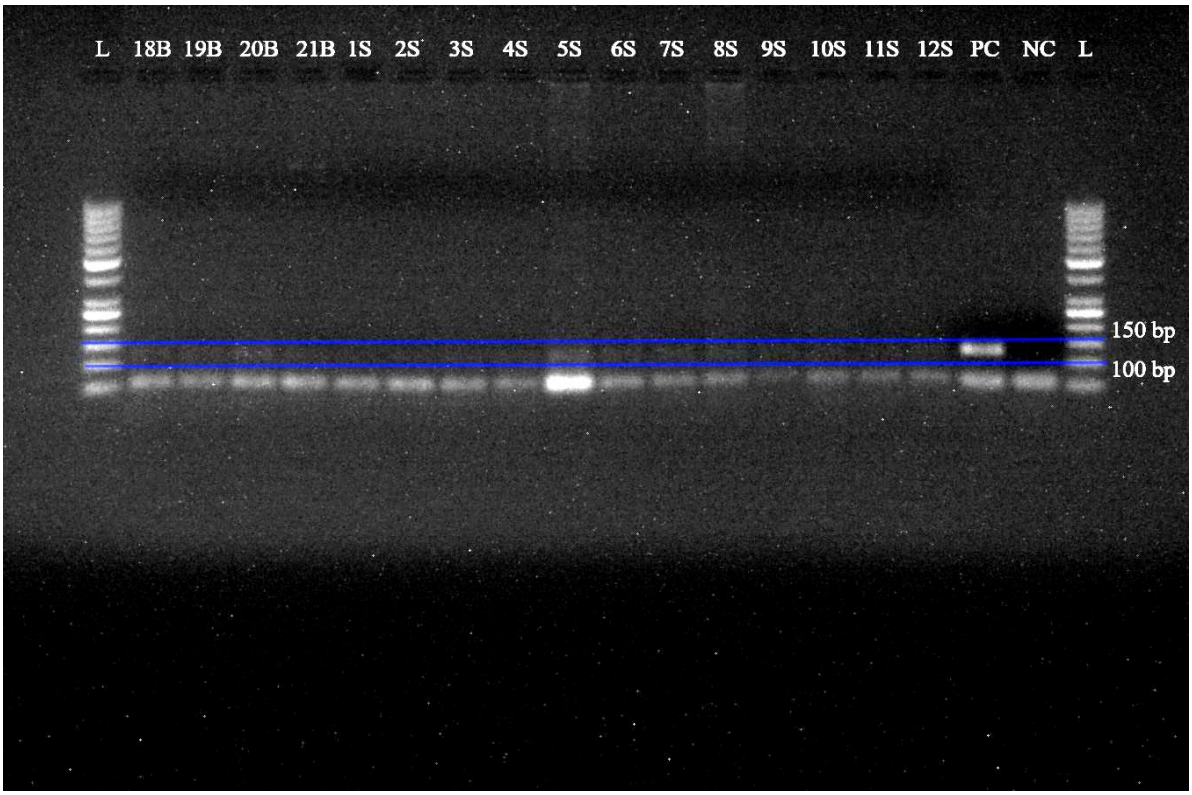


Figure 4. *Agarose gel (EtBr) with PCR products 18B-12S under UV-light.* L: 50 bp DNA ladder with 250 bp and 500 bp reference (more intense bands); 18B – 12S: samples; PC: Positive control; NC: Negative control. PC has a band at 125 bp as well as the 5S sample. 20B, 21B, 4S, 6S, 7S, and 8S show a very faint band. The other samples seem to have a faint band but we considered negative to avoid false positives.

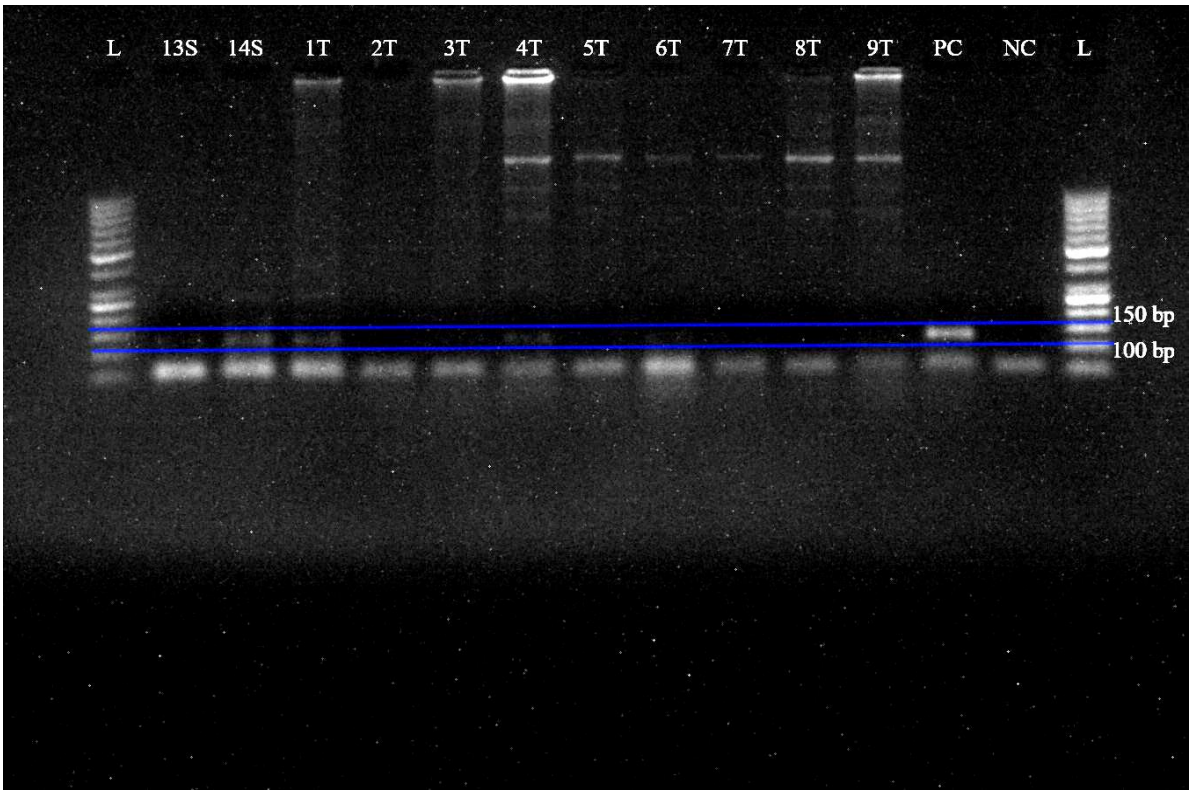


Figure 5. *Agarose gel (EtBr) with PCR products 13S-9T under UV-light.* L: 50 bp DNA ladder with 250 bp and 500 bp reference (more intense bands); 13S – 9T: samples; PC: Positive control; NC: Negative control. PC has a band at 125 bp as well as 14S, 1T, and 4T samples. 13S, and 6T show a very faint band. The other samples seem to be negative.

5.2.3 RT-PCR for RHDV detection

The RT-PCR products of the 43 samples could only be visualized in agarose gel 1.8% qualitatively. For sample 14B, we observed a clear band at the 269 bp lane (Figure 6). However, a less visible band could be observed for the 4S, 12S, and 5T samples (Figure 7 and Figure 8). Finally, a barely visible band appears for the sample 6T at 269 bp (Figure 8). In total, 5 positive samples for RHDV belonging to 3 individuals represent 13% of the sampled rabbits (Appendix 4). The rest of the samples showed no bands between 250 and 300 bp lines.

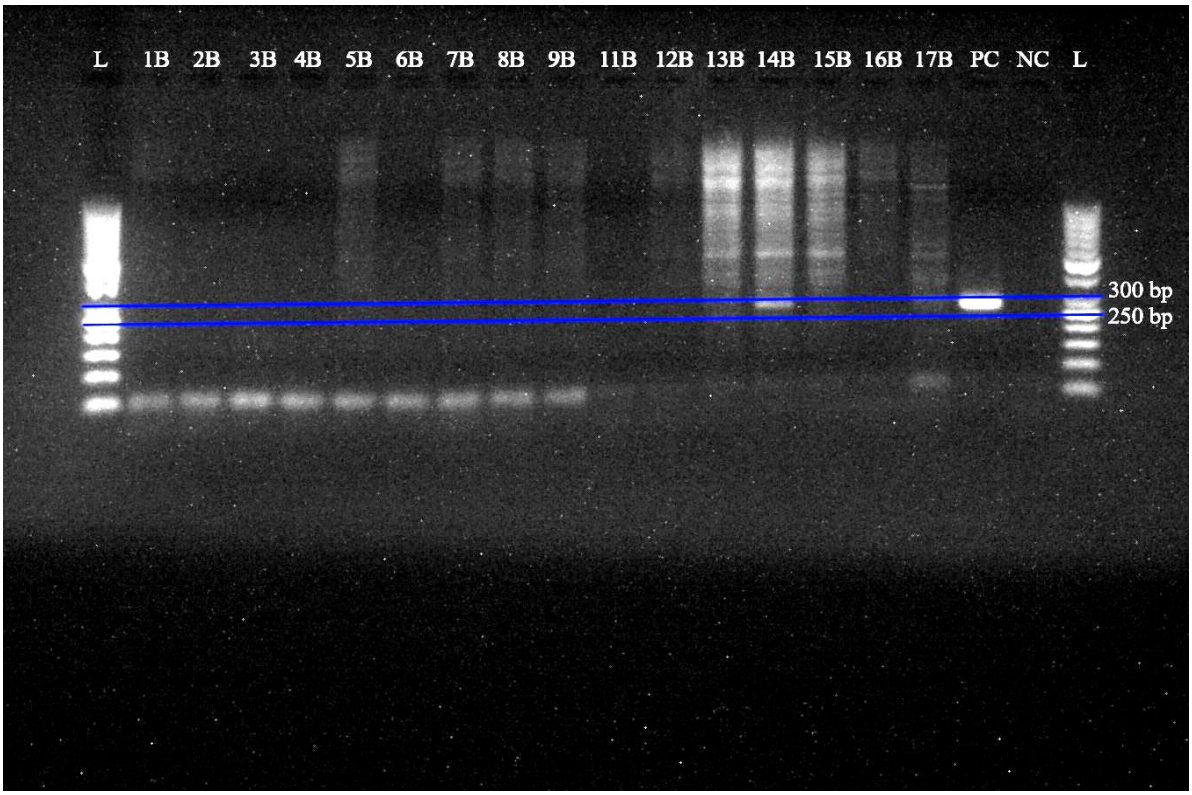


Figure 6. *Agarose gel (EtBr) with RT-PCR products 1B-17B under UV-light.* L: 50 bp DNA ladder with 250 bp and 500 bp reference (more intense bands); 1B – 17B: samples; PC: Positive control; NC: Negative control. PC has a band at 269 bp as well as 14B sample. The other samples seem to be negative.

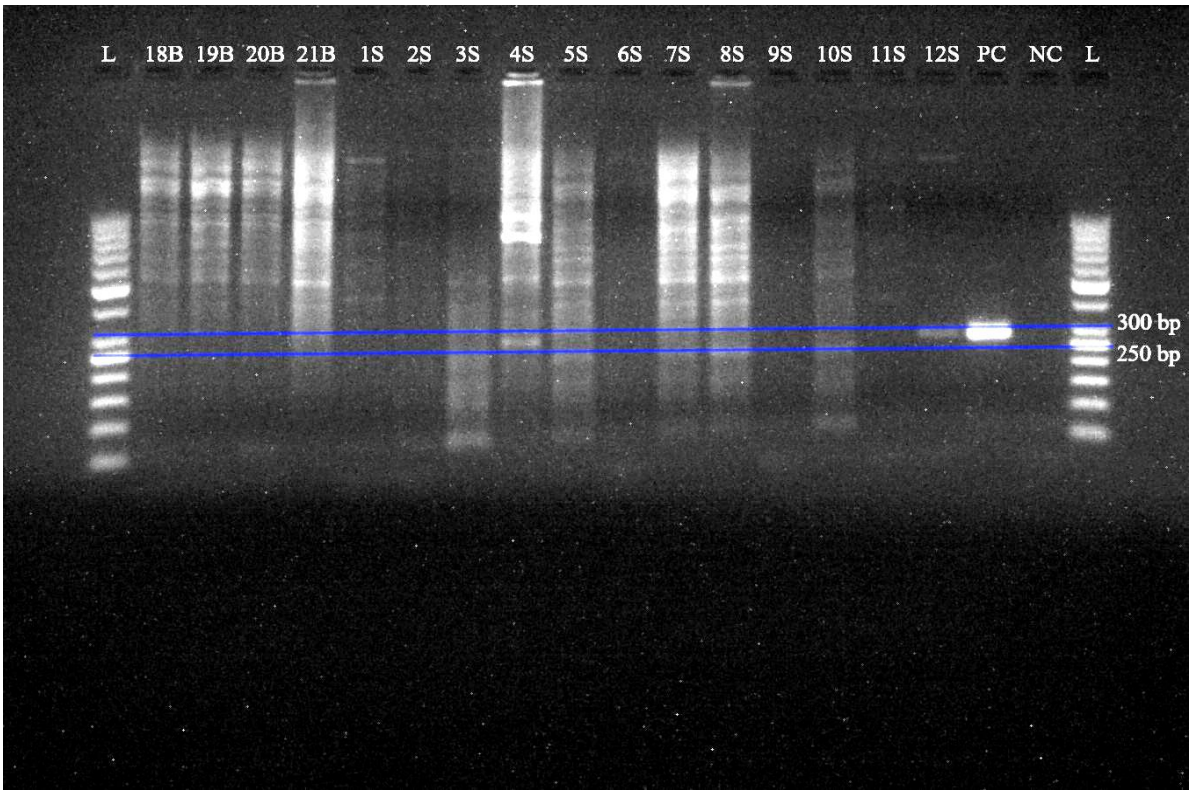


Figure 7. *Agarose gel (EtBr) with RT-PCR products 18B-12S under UV-light.* L: 50 bp DNA ladder with 250 bp and 500 bp reference (more intense bands); 18B – 12S: samples; PC: Positive control; NC: Negative control. PC has a band at 269 bp as well as 4S, and 12S samples. The other samples seem to be negative.

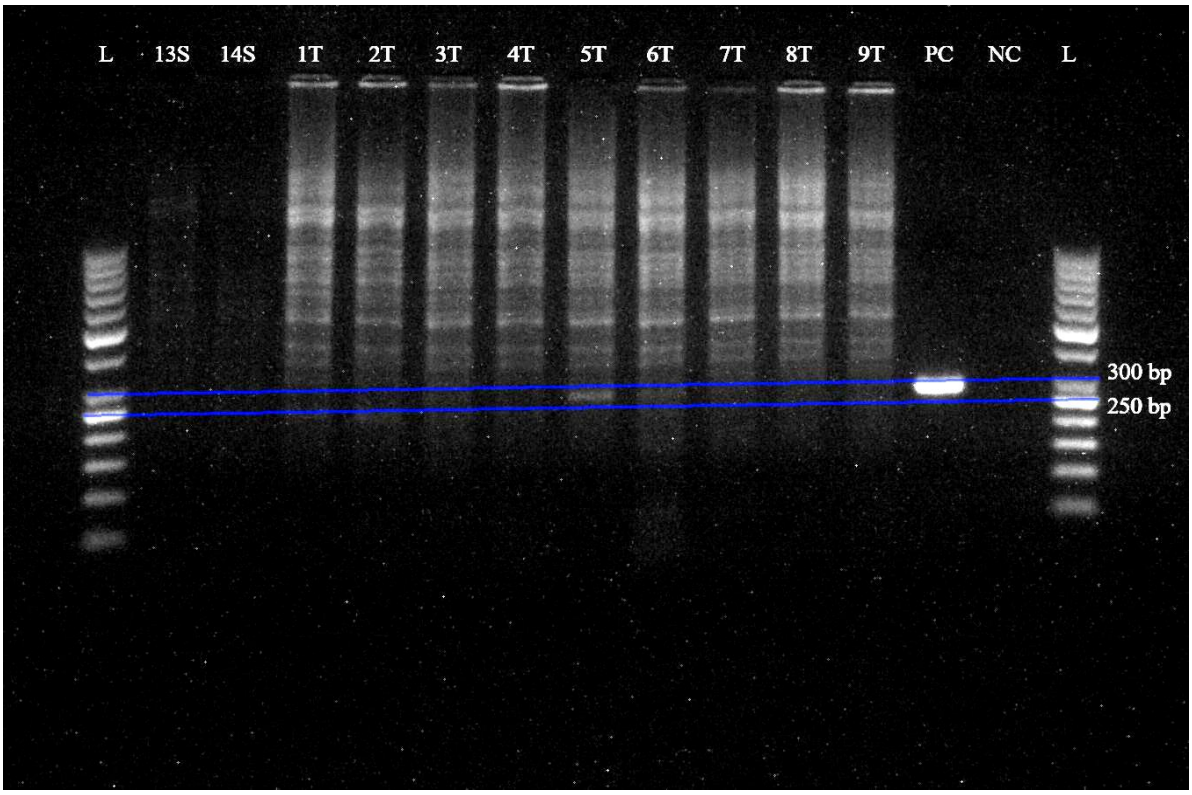


Figure 8. *Agarose gel (EtBr) with RT-PCR products 13S-9T under UV-light.* L: 50 bp DNA ladder with 250 bp and 500 bp reference (more intense bands); 13S – 9T: samples; PC: Positive control; NC: Negative control. PC has a band at 269 bp as well as 5t sample. A very faint band is seen in sample 6T. The other samples seem to be negative.

5.2.4 Sequencing

Four samples could be sequenced and assembled into a consensus sequence (Table 7). The 14B and 5T samples were successfully aligned with a Rabbit hemorrhagic disease virus genome, and the 1T and 4T samples were aligned with a Myxoma virus genome. From the 14B sample, an assembled fragment of 161 bp with 99.4% of quality was obtained, and it was successfully aligned with 100% identity with the Rabbit hemorrhagic disease virus (Table 8) (IDgen, 2021). The sequence matched a fragment of the polyprotein gene of RHDV that codes for the VP60 protein (Figure 9). From the 5T sample, an assembled fragment of 218 bp with 92.2% of quality was obtained, and it was aligned with 99.54% similarity with the Rabbit hemorrhagic disease virus (Table 8) (IDgen, 2021). The sequence matched with the same fragment of the VP60 gene of RHDV (Figure 10). The results of the BLASTn for

14B and 5T sequences are in Appendix 5. From 1T sample, a consensus sequence of 76 bp was obtained, and it was aligned with 88.16% similarity with the Myxoma virus (Table 8). The sequence matched with a fragment of the diploid M000.5L/R gene on both sides of the TIRs (Figure 11). From the 4T sample, a consensus sequence of 129 bp was obtained, and it was aligned with 84.5% similarity with the Myxoma virus (Table 8). The consensus sequence was aligned with the same fragment of the diploid gene M000.5L/R, also on both sides of the TIRs (Figure 12).

Table 7. Consensus sequences of 14B and 5T sequences assembled by Geneious Prime assembling algorithm. 1T and 4T sequences were assembled by MEGAX.

Sample	Nucleotide sequence 5' – 3'
14B	TAGTTGCTGGATCAGGCGTGTTTGGTGGGCGACTGGTCGCGGCTGT GATACCACCAGGCATCGAGATTGGACCAGGGTTAGAGGTCAGGCA ATTTCCTCATGTTGTTATCGACGCCCGTTCACTCGAACCTGTTACCA TCACCATGCCAGACTTGCGCCC
1T	CTAGGTCTGCCCGGAGGATTTCCCGAGTGTTCGTTTTTCTACGTCTA CCGGAAGGAGATCGAATACATACACAGAC
4T	CGACGTAGATTTATCGTATACCCGTCTGCCCAGATTAGGAGGCGGA CGAGGTCTAGTCTGCCCGAGGATAGACCCGAGTATTTCGTTTTTCTA CTGCTACAGGGAGGATATAGTACACCTACACCGAC
5T	TGGGCTGGTGGCATGCAGTTCCGCTTYATAGTTGCTGGATCAGGCG TGTTTGGTGGGCGACTGGTCGCGGCTGTGATACCACCAGGCATCGA GATTGGACCAGGGTTAGAGGTCAGGCAATTTCCTCATGTTGTTATC GACGCCCGTTCACTCGAACCTGTTACCATCACCATGCCAGACTTGC GCCCCAACATGTACCATCCAACTGGTGACCCTGG

Table 8. BLASTn results of positive samples for RHDV and MYXV aligned using BLASTn with the NCBI database.

Sample	Length	Quality (%)	Organism	Identity (%)	GenBank Acc. N°
14B	161	99.4	*Rabbit hemorrhagic disease virus*	100	MK895974.1

5T	218	92.2	*Rabbit hemorrhagic disease virus*	99.54	MK895974.1
1T	76	-	*Myxoma Virus*	88.16	MK388144.1
4T	129	-	*Myxoma Virus*	84.5	MK388144.1

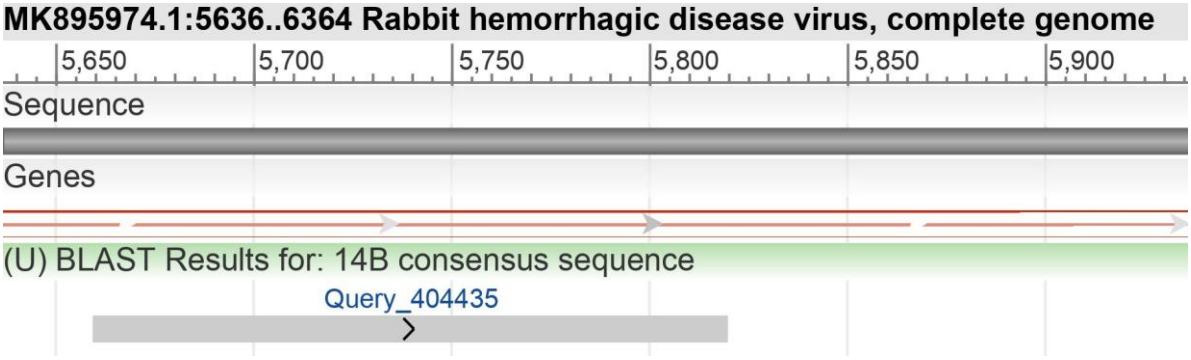


Figure 9. Blast results for 14B sequence (Query) aligned to RHDV complete genome (5660-5819). GenBank Accession number MK895974.1

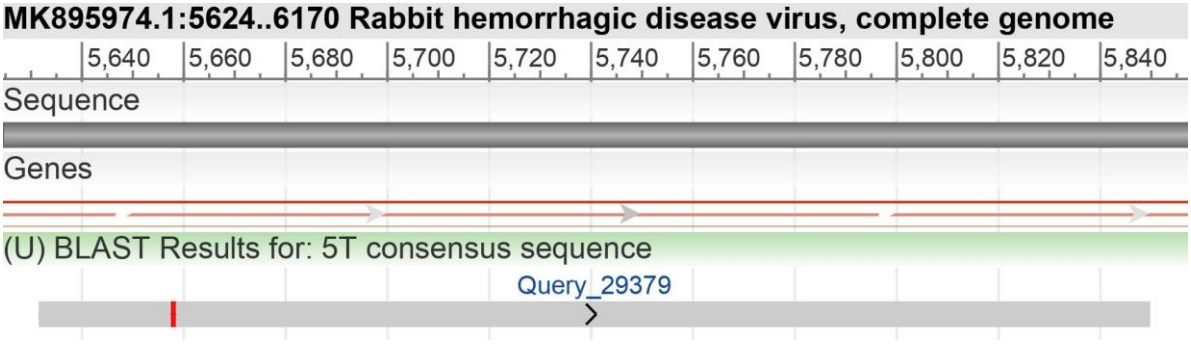


Figure 10. Blast results for 5T sequence (Query) aligned to RHDV complete genome (5632-5849). GenBank Accession number MK895974.1

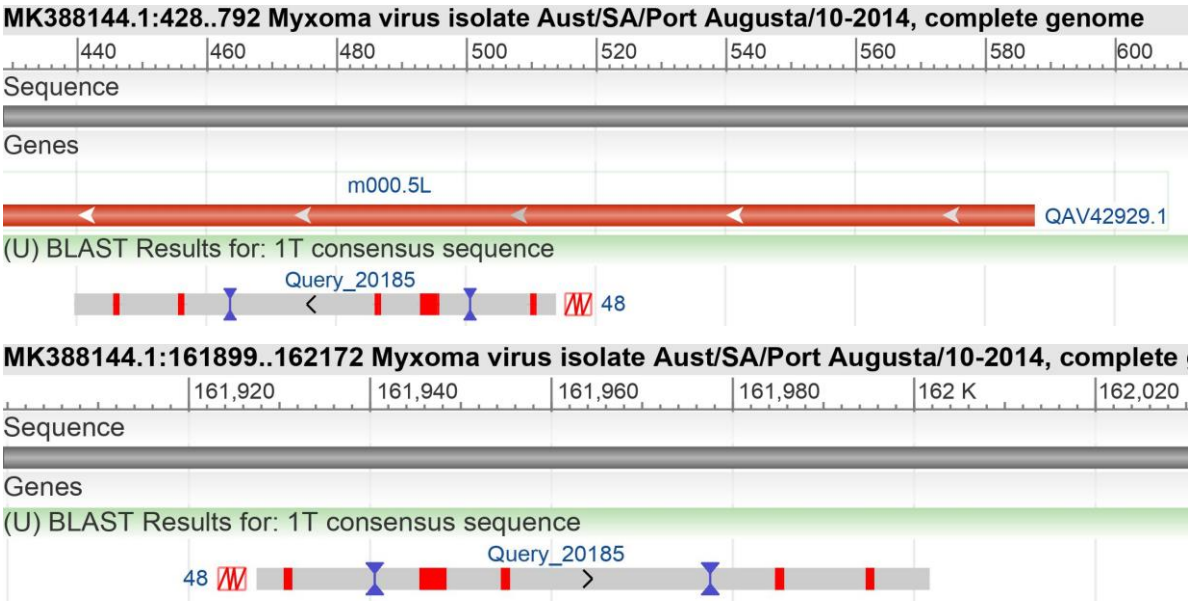


Figure 11. Blast results for 1T sequence (Query) aligned to MYXV complete genome (440-513) at the top. Second alignment to MYXV complete genome (161928-162001) at the bottom. The two sites of the union are because the target gene is the diploid gene m000.5L/R. GenBank Accession number MK895974.1

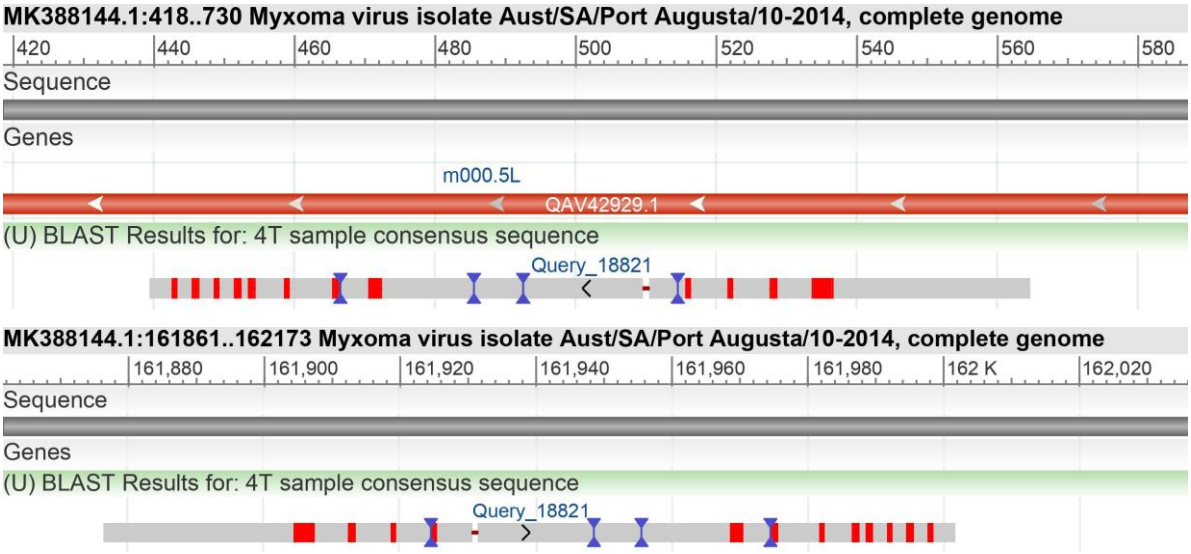


Figure 12. Blast results for 4T sequence (Query) aligned to MYXV complete genome (440-564) at the top. Second alignment to MYXV complete genome (161877-162001) at the bottom. The two sites of union are because the target gene is the diploid gene m000.5L/R. GenBank Accession number MK895974.1

6. DISCUSSION

In this study we proved for the first time the presence of MYXV and RHDV in Andean rabbits from the Ecuadorian paramo. Four amplicons of the PCR and RT-PCR product of the positive samples obtained in the electrophoresis (Figure 3 to 8) were sequenced to confirm they are fragments of the MYXV or the RHDV. Two sequences (14B and 5T samples) could be assembled using Geneious prime® software, and two sequences (1T and 4T samples) were assembled using a pairwise alignment in MEGAX software. 5T was a lung sample belonged to a juvenile male rabbit (individual 13, Appendix 2) found dead in the Antisana house transect, near to the Antisana House, with a pathological analysis without abnormalities. Besides the lung sample, the nasal swab and liver sample of the same individual also gave positive for RHDV in the RT-PCR, but blood and spleen samples gave negative (Appendix 4). 14B blood sample was taken from an adult female in the Humboldt house transect (individual 15, Appendix 2), but the nasal swab of the same rabbit did not show positive results in the RT-PCR (Appendix 4). The rabbit had a bleeding nose but it was not clear if the hemorrhage was caused by injuries caused by trying to escape from the trap or by a disease. 1T was a penis sample obtained from a dead juvenile rabbit with most of its organs absent (individual 10, Appendix 2). The rabbit was found near the Micahuaycu creek to 1500 meters from Antisana House, approximately. Also, testicle and spleen samples were taken which gave negative for MYXV (Appendix 4). Although the rabbit could not be pathologically analyzed for the lack of important organs, it had a serious case of taeniasis and cysticercosis.

Finally, the 4T was a spleen sample of the same 13th individual (Appendix 2) that had RHDV. Besides the spleen, a liver and nasal sample was also positive for MYXV, but blood and lung samples were negative (Appendix 4). Individual 13, has had a co-infection of MYXV and RHDV, however, its pathological analysis did not show abnormalities. Besides that, individual 20 (Appendix 2) was found dead near the Humboldt house transect, and it had hemorrhagic lungs, the apex of the heart with possible clots, dark liver, and necrotic gallbladder with dense bile. From this rabbit, it was taken blood, mucus, spleen, liver, and lung samples, that were negative for both RHDV and MYXV (Appendix 4). Individual 23 (Appendix 2) had red, swollen eyes, and it was positive for MYXV in the nasal swab and blood samples. Other than that, there were not seen more signs of Rabbit hemorrhagic disease nor Myxomatosis in captured rabbits.

39% of the sampled rabbits were positive for MYXV, which is a high prevalence without apparent signs of Myxomatosis disease. Except for one rabbit with red, swollen eyes, neither alive nor dead rabbits with signs of Myxomatosis were found. A reasonable explanation for the high prevalence of MYXV in *Sylvilagus andinus* is the coevolution of the virus with *Sylvilagus* spp., which reduces the disease's mortality rate (Kerr, et al., 2015). Also, the hypothesis that MYXV is naturally found in *Sylvilagus* spp. causing innocuous cutaneous fibromas which disappear in several weeks and rarely causing deaths, but in European rabbits, it causes Myxomatosis, a highly lethal systemic disease (Fenner & Ratcliffe, 1965) could explain a high prevalence of MYXV in *Sylvilagus andinus*.

For the RHDV, 13% of the sampled rabbits were positive. The prevalence of the RHDV in *Sylvilagus andinus* is low and the signs of the Rabbit hemorrhagic disease were absent, except for bleeding nose in one rabbit. The positive samples were obtained from one rabbit alive with a bleeding nose and two dead rabbits without pathological abnormalities. However, a low prevalence of the RHDV can be caused by a high mortality rate. A study of the incidence of the Rabbit hemorrhagic disease over the prevalence of the RHDV could clarify the rol of the RHDV in the rabbit populations (Pita-Fernández, Pértegas-Díaz, & Valdés-Cañedo, 2004). Also, the dead rabbits in opisthotonus position found near to the transects and the communication of the paramo guards that the rabbits suddenly die while feeding could explain the rabbit population decrease as a consequence of the high mortality of the Rabbit hemorrhagic disease. More samples and a follow-up of the sampled rabbits could confirm this assumption. Nevertheless, an RHDV fragment and an MYXV fragment were detected and successfully confirmed in two samples each, which implies the presence of both RHDV and MYXV in *Sylvilagus andinus* populations from the ACHA.

Regarding the methodology, the Tomahawk live trap is one the best techniques for capturing small mammals such as rabbits without harming the animal (Schemnitz, Batcheller, Lovallo, White, & Fall, 2009). However, some rabbits hurt their nose and mouth against the cage trying to escape, which also confused the pathological diagnosis of bleeding nose for Rabbit hemorrhagic disease. Other than that, the technique resulted convincing and combined with the burrow-trap method of capture was overwhelming with 32.85% capture success. It is

doubtless better than the plot-trap technique which had a null capture success. For future work, we recommend the use of the burrow-trap method and avoid the plot-trap method.

The nucleic acid extraction was challenging considering the scarce amount of blood obtained from such small rodents. Similarly, the nasal swabs got poor material due to difficulty in inserting the swab into the rabbits' noses. On the other hand, the tissue samples were well preserved and had a higher quantity and quality of DNA/RNA. Overall, the collected samples had a low quality which gave poor quality results in PCR. However, the sensitivity of the technique should be high enough to detect a low copy number of viral particles. For the PCR and RT-PCR optimization, the primer concentration, and sample amount were changed, and denaturation, annealing, and extension temperatures and times were also changed (Lorenz, 2012). The optimized protocols (Table 3 and Table 4) still showed smearing bands but with lower interference in the target lane which is enough to see the amplicons.

7. CONCLUSIONS

From this study, it was found that there is a presence of MYXV and RHDV in *Sylvilagus andinus* captured in the ACHA. This implies the possibility of finding infected wild and farm rabbits with Myxomatosis or Rabbit hemorrhagic disease in the surrounding areas to the ACHA, or even in other areas of the Ecuadorian Andes. Although Rabbit hemorrhagic disease seems to be killing the Andean rabbits from the ACHA, it is possible that Myxomatosis was also decreasing the population density. Consequently, more samples are needed for molecular analysis, and a population assessment has to be done in the *Sylvilagus andinus* population to find if the presence of these pathogens affects the number of individuals in populations of the ACHA.

Two positive samples for MYXV, 1T, and 4T were confirmed as fragments of the M000.5L/R gene of the MYXV (GenBank Accession number MK388144.1), and, two positive samples for RHDV, 14B, and 5T were confirmed as fragments of the VP60 gene of the RHDV (GenBank Accession number DQ205345.1). Assuming that the sequencing is correct, nine out of twenty-three sampled individuals would have MYXV, and the described protocol for conventional PCR (Table 3) may detect the presence of MYXV in blood, nasal swab, spleen, and penis samples. Additionally, three of the twenty-three sampled individuals

would have RHDV, and the described protocol for RT-PCR (Table 4) could successfully detect the presence of RHDV in blood, lung, and nasal swab samples from rabbits.

To continue this work, some considerations must be taken into accounts such as the sample type and the preservation of nucleic acids. The storage and transport of samples are very important to avoid degradation of the genetic material for the PCR protocol. The temperature of samples has to be kept below 4°C and immediately freeze to -20°C and repeating thaw-freeze cycles must be avoided. Once DNA/RNA has been extracted it must be frozen to -70°C and defrost on ice. The elution of the DNA/RNA was made on nuclease-free water, but an elution buffer would be better for long-term preservation. Taking these considerations into account, the fidelity of the results can considerably increase.

BIBLIOGRAPHY

Abrantes, J., van der Loo, W., Le Pendu, J., & Esteves, P. J. (2012). Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. *Veterinary Research, 43*(12), 1-19. doi:10.1186/1297-9716-43-12

Aguirre, N., Torres, J., & Velasco-Linares, P. (2013). *Guía para la restauración ecológica en los páramos del Antisana.* Ministerio del Ambiente, Quito. Retrieved from https://www.ambiente.gob.ec/wp-content/uploads/downloads/2014/07/Gu%C3%ADa-Metodol%C3%B3gica-restauracion-p%C3%A1ramos.pdf

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local aligment search tool. (S. Brenner, Ed.) *Journal of Molecular Biology, 215*(3), 403-410. doi:10.1016/S0022-2836(05)80360-2

Belz, K. (2004). Rabbit Hemorragic Disease. *Seminars in Avian and Exotic Pet Medicine, 13*(2), 100-104. doi:10.1053/j.saep.2004.01.006

Bertagnoli, S., Messud-Petit, F., & Marlier, D. (2006). Myxomatosis. *Recent Advances in Rabbit Sciences*, 139-145.

Bhopal, R. S. (2016). *Conceps of Epidemiology: Integrating the ideas, theories, principles, and methods of epidemiology* (Third ed.). Oxford University Press.

BIOCOMMA LIMITED. (2020). *CommaXP Virus DNA/RNA Extraction kit (Spin Column) Quick guide Version: BP111-1EN*. Guide, ShenZhen, China.

Cameron, C., Hota-Mitchell, S., Chen, L., Barrett, J., Cao, J.-X., Macaulay, C., . . . McFadden, G. (1999). The Complete DNA Sequence of Myxoma Virus. *Virology, 264*(2), 298-318. doi:10.1006/viro.1999.0001

Coronel, L. (2019). *Los caminos del agua FONAG: trabajos y aprendizajes.* Fondo para la Protección del Agua, Quito. Retrieved from http://www.fonag.org.ec/web/wp-content/uploads/2019/11/Sistematizaci%C3%B3n-FONAG-Espa%C3%B1ol-Web.pdf

Díaz, C. (2018). *Análisis del comportamiento de las frecuencias de valores extremos de temperatura y precipitación en zonas de alta montaña: caso de estudio volcán Antisana.* Bachelor's thesis, Quito. Retrieved from https://bibdigital.epn.edu.ec/bitstream/15000/19488/1/CD-8886.pdf

Duarte, M. D., Barros, S. C., Enriques, A. M., Fagulha, M. T., Ramos, F., Luís, T., & Fevereiro, M. (2014). Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. *Journal of Virological Methods, 196,* 219-224. doi:10.1016/j.jviromet.2013.11.014

Fenner, F., & Ratcliffe, F. N. (1965). Myxomatosis. *Cambridge University Press*.

García, J., Suárez, E., & Zapata-Ríos, G. (2016, May 24). An assessment of the populations of Sylvilagus brasiliensis andinus in Páramos with different vegetation structures in the northeastern Andes of Ecuador. *Neotropical Biodiversity, 2*(1), 72-80. doi:10.1080/23766808.2016.1179846

Gibbens, R., Havstad, K., Billheimer, D., & Herbel, C. (1993). Creosotebush vegetation after 50 years of lagomorph exclusion. *Oecologia, 94*, 210-217. doi:10.1007/BF00341319

Hall, A. C. (2019). A comparision of DNA stains and staining methods for Agarose Gel Electrophoresis. *bioRxiv*. doi:10.1101/568253

Hong, S.-J., & Piette, L. H. (1976). Electron Spin Resonance Spim-Label Studies of Intercalation of Ethidium Bromide and Aromatic Amine Carcinogens in DNA. *Cancer Research, 36*(3), 1159-1171.

IDgen. (2021). *Results Report N. A-209.* Report, Quito, Ecuador.

Insuasty, J., Ramírez, G., & Mejía, L. (2008). EPIDEMIOLOGIC STUDY OF THE WILD RABBIT Sylvilagus brasiliensis, IN “LOS NEVADOS” NATURAL NATIONAL PARK. *Boletín Científico. Centro de Museos. Museo de Historia Natural, 12*(1), 90-110.

Kerr, P. J. (2012). Myxomatosis in Australia and Europe: A model for emerging infectious diseases. *Antiviral Research, 93*(3), 387-415. doi:10.1016/j.antiviral.2012.01.009

Kerr, P. J., & Donnelly, T. M. (2013). Viral infections of rabbits. *Veterinary Clinics of North America: Exotic Animal Practice, 16*(2), 437-468. doi:10.1016/j.cvex.2013.02.002

Kerr, P. J., Ghedin, E., DePasse, J. V., Fitch, A., Cattadori, I. M., Hudson, P. J., . . . Holmes, E. C. (2012). Evolutionary History and Attenuation of Myxoma Virus on Two Continents. *PLOS Pathogens, 8*(10), e1002950. doi:10.1371/journal.ppat.1002950

Kerr, P. J., Liu, J., Cattadori, I., Ghedin, E., Read, A. F., & Holmes, E. C. (2015). Myxoma Virus and the Leporipoxviruses: An Evolutionary Paradigm. (E. J. Lefkowitz, & C. Upton, Eds.) *Viruses, 7*(3), 1020-1061. doi:10.3390/v7031020

Korbie, D. J., & Mattick, J. S. (2008). Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nature Protocols, 3*(9), 1452-1456. doi:10.1038/nprot.2008.133

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution, 35*(6), 1547-1549. doi:10.1093/molbev/msy096

Le-Gall-Reculé, G., Zwingelstein, F., Portejoie, Y., & Le Gall, G. (2001). Immunocapture-RT-PCR assay for detection and molecular epidemiology studies of Rabbit Haemorrhagic Disease and European Brown Hare Syndrome viruses. *Journal of Virological Methods, 97*(1-2), 49-57. doi:10.1016/S0166-0934(01)00336-6

Lorenz, T. C. (2012). Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal of Visualized Experiments, 63*(3998). doi:10.3791/3998

Mackay, I. M., Arden, K. E., & Nitsche, A. (2002). Real-time PCR in virology. *Nucleic Acids Research, 30*(6), 1292-1305. doi:10.1093/nar/30.6.1292

Merck. (2021). *TAE and TBE running buffers recipe*. Retrieved December 1, 2020, from https://www.sigmaaldrich.com/technical-documents/articles/biology/tae-and-tbe-running-buffers-

recipe.html#:~:text=TBE%20buffer%20is%20commonly%20prepared,at%20a%20pH%20of%208.0.

Ministerio del Ambiente. (2015). *Áreas protegidas del Ecuador*. Retrieved from http://areasprotegidas.ambiente.gob.ec/es/areas-protegidas/reserva-ecol%C3%B3gica-antisana

Ministerio del Ambiente. (2020, April). Retrieved from https://www.ambiente.gob.ec/wp-content/uploads/downloads/2020/04/2020_03_30-BOLETIN-FINAL.pdf

Montana Public Radio. (2021). *Highly Contagious, Fatal Rabbit Disease Detected in Montana*. (R. Cramer, Editor) Retrieved February 18, 2021, from https://www.mtpr.org/post/highly-contagious-fatal-rabbit-disease-detected-montana

New England BioLabs. (2021). *New England BioLabs Inc.* Retrieved December 10, 2020, from https://international.neb.com/tools-and-resources/usage-guidelines/guidelines-for-pcr-optimization-with-taq-dna-polymerase

NIH. (2020). *Polymerase Chain Reaction (PCR) Factt Sheet. National Human Genome Research Institute*. Retrieved September 1, 2020, from https://www.genome.gov/about-genomics/fact-sheets/Polymerase-Chain-Reaction-Fact-Sheet

Parkinson, C. M., O'Brien, A., Albers, T. M., Simon, M. A., Clifford, C. B., & Pritchett-Corning, K. (2011). Diagnostic Necropsy and Selected Tissue and Sample Collection in Rat and Mice. *Journal of Visualized Experiments, 54*(2966). doi:10.3791/2966

Passow, C. N., Kono, T. J., Stahl, B. A., Jaggar, J. B., Keene, A. C., & McGaugh, S. E. (2018). RNAlater and flash freezing storage methods nonrandomly influence observed gene expression in RNAseq experiments. *bioRxiv*, 379834. doi:10.1101/379834

Patton, N. M. (1989). Viral hemorrhagic disease. A major new disease problem of rabbits. *Journal of Applied Rabbit Research, 12*, 64-67.

Penadés, M., Guerrero, I., Ferrián, S., Selva, L., Corpa, J. M., & Viana, D. (2011). TOMA de MUESTRAS en explotaciones cunículas para enviar al laboratorio. *Cunicultura, 35*(209), 7-13. Retrieved from https://cunicultura.com/pdf-files/2011/2/5709-toma-de-muestras-en-explotaciones-cunicolas-para-enviar-al-laboratorio.pdf

Pita-Fernández, S., Pértegas-Díaz, S., & Valdés-Cañedo, F. (2004). Medidas de frecuencia de enfermedad: incidencia y prevalencia. *Cuadernos de atención primaria, 11*, 101-105.

Quito Informa. (2020, September 25). *Quito Informa*. Retrieved from http://www.quitoinforma.gob.ec/2020/09/25/trabajo-para-la-proteccion-de-fuentes-hidricas-en-el-antisana/

Ruedas, A. R., & Smith, A. T. (2019). *Sylvilagus andinus. The IUCN Red List of Threatened Species 2019: e.T142541491A142541563*. Retrieved May 30, 2019, from https://www.iucnredlist.org/species/142541491/142541563

Ruedas, L. A., Silva, S. M., French, J. H., Platt II, R. N., Salazar-Bravo, J., Mora, J. M., & Thompson, C. W. (2017). A Prolegomenon to the Systematics of South American Cottontail Rabbits (Mammalia, Lagomorpha, Leporidae: Sylvilagus): Designation of a Neotype for S. brasiliensis (Linnaeus, 1758), and Restoration of S. andinus (Thomas, 1897) and S. tapetillus Thomas 1913. Retrieved from https://deepblue.lib.umich.edu/handle/2027.42/136089

Schemnitz, S. D., Batcheller, G. R., Lovallo, M. J., White, H. B., & Fall, M. W. (2009). *The wildlife techniques manual: Capturing and Handling Wild Animals.* (N. J. Silvy, Ed.) Retrieved August 10, 2019, from https://digitalcommons.unl.edu/icwdm_usdanwrc/1191/

Thermo Fisher Scientific. (2007, January 1). *Plasma and Serum Preparation*. Retrieved from https://www.thermofisher.com/ec/en/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/elisa-sample-preparation-protocols/plasma-and-serum-preparation.html

Thermo Fisher Scientific. (2015). *6X TriTrack DNA Loading Dye.* Product Information. Retrieved from https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013168_6X_TriTrack_DNA_Loading_Dye_UG.pdf

Thermo Fisher Scientific. (2018). *GeneRuler 50 bp DNA Ladder.* Product Information. Retrieved from https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013016_GeneRuler_50bp_DNALadder_50ug_UG.pdf

Thomas, O. (1897). Description of new bats and rodents from South America. *Journal of Natural History, 20*(120), 544-553.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*(22), 4673-4680. doi:10.1093/nar/22.22.4673

TIB MOLBIOL. (2020). *Instructions For Use of Lyophilized 1-step RT-PCR Ploymerase Mix Version: V200415.* Guide, Berlin.

Tirira, D. G. (2015). *Caracterización de la fauna en el ecosistema páramo en la provincia de Tungurahua.* Quito: Geoinformática y Sistemas Cia. Ltda. Retrieved from http://rrnn.tungurahua.gob.ec/documentos/download/5583371a83ba88da0a70b933

Tizzani, P., Lavazza, A., Capucci, L., & Meneguz, P. (2002). Presence of infectious agents and parasites in wild population of cottontail (Sylvilagus floridanus) and consideration of its role in the diffusion of pathogens infected hares. *European Association of Zoo- and Wildlife Veterinarians (EAZWV) 4th scientific meeting, joint with the annual meeting of the European Wildlife Disease Association (EWDA)*, 245-248.

Valero, L., & Durant, P. (2001). DIET ANALYSIS OF THE PÁRAMO RABBIT, Sylvilagus brasiliensis meridensis Thomas, 1904 (Lagomorpha: Leporidae) IN MUCUBAJI, MÉRIDA, VENEZUELA. *Rev. Ecol. Lat. Am., 8*(2), 01-13.

Valicek, L., Smid, B., Rodak, L., & Kudrna, J. (1990). Electron and immunoelectron microscopy of rabbit haemorrhagic disease virus (RHDV). *Archives of Virology, 112*(3-4), 271-275. doi:10.1007/BF01323171

Vallejo, A. (2018). *Mamíferos del Ecuador*, 2018.0. (J. Brito, M. A. Camacho, V. Romero, A. F. Vallejo, Editors, & Museo de Zoología, Pontificia Universidad Católica del Ecuador) Retrieved March 10, 2019, from https://bioweb.bio/faunaweb/mammaliaweb/FichaEspecie/Sylvilagus%20andinus

World Health Organization. (2006). *Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1) virus infection. Guide for field operations.* Retrieved from https://www.who.int/csr/resources/publications/surveillance/WHO_CDS_EPR_ARO_2006_1/en/

Yang, L., Wang, F., Hu, B., Xue, J., Hu, Y., Zhou, B., . . . Xu, W. (2008). Development of an RT-PCR for rabbit hemorrhagic disease virus (RHDV) and the epidemiology of RHDV in three eastern provinces of China. *Journal of Virological Methods, 151*(1), 24-29. doi:10.1016/j.jviromet.2008.04.003

Zapata, A. (2018). *Evaluación poblacional del conejo andino Sylvilagus andinus: Leporidae en el bosque protector Jerusalem (Pichincha, Ecuador) a partir del conteo de fecas.* Bachelor's thesis, UCE, Facultad de Ciencias Biológicas, Quito. Retrieved from http://www.dspace.uce.edu.ec/bitstream/25000/17907/1/T-UCE-0016-CBI-016.pdf

Zapata-Ríos, G., & Branch, L. C. (2016). Altered activity patterns and reduced abundance of native mammals in sites with feral dogs in the high Andes. *Biological Conservation, 193*, 9-16. doi:10.1016/j.biocon.2015.10.016

APPENDICES

Appendix 1

Amount of sample and water for each 20 µl PCR and RT-PCR reaction

	PCR		RT-PCR	
Sample	**DNA (µl)**	**H_2O (µl)**	**RNA (µl)**	**H_2O (µl)**
1B	3	5	3	5
2B	1	7	2	6
3B	8	0	2	6
4B	1	7	3	5
5B	2	6	2	6
6B	4	4	3	5
7B	5	3	7	1
8B	2	6	3	5
9B	6	2	8	0
11B	8	0	8	0
12B	8	0	8	0
13B	8	0	8	0
14B	8	0	8	0
15B	8	0	8	0
16B	1	7	2	6
17B	1	7	1	7
18B	8	0	8	0
19B	8	0	8	0
20B	1	7	1	7
21B	8	0	8	0
1S	8	0	8	0
2S	8	0	8	0
3S	8	0	8	0
4S	8	0	8	0

5S	8	0	8	0
6S	8	0	8	0
7S	8	0	8	0
8S	6	2	8	0
9S	8	0	8	0
10S	8	0	8	0
11S	4	4	5	3
12S	8	0	8	0
13S	8	0	8	0
14S	8	0	8	0
1T	1	7	1	7
2T	3	5	3	5
3T	1	7	1	7
4T	1	7	1	7
5T	1	7	1	7
6T	1	7	1	7
7T	1	7	1	7
8T	1	7	1	7
9T	1	7	1	7

Appendix 2

Sampling data

N°	Collection date	Collection Site	Gender	Samples						
				Blood	Nasal swab	Tissue				
						Spleen	Liver	Lung	Penis	Testicle
1	12/6/2019	Antisana house	F	1B						
2	12/6/2019	Antisana house	F	2B						
3	12/6/2019	Antisana house	M	3B						
4	12/7/2019	Antisana house	M	4B						
5	12/7/2019	Antisana house	M	5B						
6	12/7/2019	Antisana house	M	6B						
7	12/7/2019	Antisana house	M	7B						
8	12/7/2019	Antisana house	F	8B						
9	12/7/2019	Antisana house	F	9B	3S					
10	12/8/2019	Micahuaycu creek	M			3T			1T	2T
11	1/17/2020	Antisana house	F		1S					
12	1/18/2020	Antisana house	M	11B	2S					
13	1/18/2020	Antisana house	M	12B	4S	4T	6T	5T		
14	1/18/2020	Antisana house	M	13B	5S					
15	1/18/2020	Crag	F	14B	6S					
16	1/18/2020	Humboldt house	M	15B	7S					
17	1/18/2020	Humboldt house	M		8S					
18	1/18/2020	Crag	F	16B	9S					
19	1/18/2020	Humboldt house	M	17B	10S					
20	1/18/2020	Humboldt house	F	19B	11S	9T	7T	8T		
21	1/18/2020	Humboldt house	M	18B	12S					
22	1/19/2020	Antisana house	M	20B	13S					
23	1/19/2020	Humboldt house	M	21B	14S					

Appendix 3

Spectrophotometry of the nucleic acid extracted

Sample	Date	DNA (ng/ul)	260/280	260/230	RNA (ng/ul)	260/280	260/230
1B	12/23/2020	20.5	2.12	4.96	23.6	2.16	1.58
2B	12/23/2020	313.4	1.5	0.64	34.9	4.89	1.95
3B	12/23/2020	4.2	1.8	0.45	39.5	2.61	0.53
4B	12/23/2020	115.1	1.8	0.91	17.8	2.17	2.31
5B	12/23/2020	42.6	2.18	1.09	33.7	2.25	1.07
6B	12/23/2020	13.1	2.1	7.2	17.6	2.04	1.29
7B	12/23/2020	10.6	2.37	0.79	8.3	2.47	0.76
8B	12/23/2020	30.2	2.11	1.1	23.4	2.09	1.08
9B	1/7/2021	9.3	2.37	0.19	6.4	2.5	0.17
11B	1/7/2021	1.2	4.76	0	1.1	5.47	0
12B	12/24/2020	3.6	3.37	0.01	2.6	3.15	0.01
13B	12/24/2020	2	27.23	0.01	2.4	6.78	0.01
14B	12/24/2020	0.9	6.4	0.02	1.1	10.85	0.03
15B	12/24/2020	2.1	1.61	0.04	1.4	1.1	0.03
16B	12/24/2020	52.8	0.88	0.08	48	0.88	0.09
17B	12/24/2020	94.9	2.09	1.61	75.4	2.07	4.49
18B	12/24/2020	2	5.22	0.08	1.4	2.42	0.07
19B	12/24/2020	6	1.84	0.04	4.7	2.08	0.04
20B	12/24/2020	109.1	0.94	0.26	102	0.95	0.33
21B	12/24/2020	3	3.26	0.19	2.8	1.93	0.13
1S	12/24/2020	4.7	1.99	0.25	5.3	2.02	0.29
2S	12/24/2020	0.8	7.97	0.04	0.5	1.53	0.02
3S	12/24/2020	5.5	2.38	0.09	4.3	2.4	0.09
4S	12/24/2020	3.9	2.13	0.04	3.2	2.01	0.04
5S	12/24/2020	5.8	1.81	0.11	4.3	1.71	0.1
6S	12/24/2020	1.9	2.7	0.12	0.7	6.26	0.06

7S	12/24/2020	2	3.15	0.14	1.4	4.26	0.12
8S	12/24/2020	8.4	2.15	0.03	6.7	2.45	0.02
9S	12/24/2020	4	2.1	0.07	3.3	1.95	0.06
10S	12/24/2020	4.6	2.42	0.26	3.8	1.92	0.25
11S	12/24/2020	12.8	1.74	0.39	10.2	1.79	0.38
12S	12/24/2020	1.5	2.42	0.03	1.6	4.3	0.04
13S	12/24/2020	4.9	2.11	0.09	4.3	2.2	0.09
14S	12/24/2020	2.3	2.55	0.13	2.1	2.28	0.14
1T	1/4/2021	180.6	1.86	0.72	139.7	1.86	0.68
2T	1/5/2021	23.7	2.07	2.54	18.7	2.1	2.56
3T	1/5/2021	129.1	1.88	1.85	102	1.89	1.85
4T	1/5/2021	433.4	1.97	2.11	350.5	1.97	2.12
5T	1/5/2021	74	1.95	0.59	55.4	1.93	0.56
6T	1/6/2021	672.2	2.07	1.74	543	2.05	1.75
7T	1/6/2021	207.1	2.11	0.46	165.3	2.11	0.48
8T	1/7/2021	125.2	1.95	2.18	100.7	1.94	2.16
9T	1/7/2021	414.7	1.98	1.92	332.4	1.97	1.91

Appendix 4

MYXV positive rabbits (blue), RHDV positive rabbits (green), MYXV and RHDV positive rabbits (red)

N°	Sample	MYXV	RHDV	Notes
1	1B	-	-	
2	2B	-	-	
3	3B	-	-	
4	4B	-	-	
5	5B	-	-	
6	6B	-	-	
7	7B	-	-	
8	8B	+	-	
9	9B	-	-	
	3S	-	-	
10	1T	+	-	Taeniasis and cysticercosis.
	2T	-	-	
	3T	-	-	
11	1S	-	-	
12	11B	-	-	
	2S	-	-	
13	12B	-	-	Without rigor mortis. Pathological examination without abnormalities.
	4S	+	+	
	4T	+	-	
	5T	-	+	
	6T	+	+	
14	13B	-	-	
	5S	+	-	
15	14B	-	+	Rash and alopecia in legs.
	6S	+	-	

16	15B	-	-	
	7S	+	-	
17	8S	+	-	
18	16B	-	-	
	9S	-	-	
19	17B	-	-	
	10S	-	-	
20	19B	-	-	Opisthotonous rigor mortis, hemorrhagic lungs, clot in heart, dark liver, and necrotic gallbladder.
	11S	-	-	
	7T	-	-	
	8T	-	-	
	9T	-	-	
21	18B	-	-	
	12S	-	+	
22	20B	+	-	
	13S	+	-	
23	21B	+	-	Red, and swollen eyes.
	14S	+	-	

Appendix 5

Results report of the sequencing, assembly, and alignment of two positive samples for RHDV from IDgen

Av. De los Granados E14-285 y Eloy Alfaro
Teléfono: 0998982450
e-mail: idgen.ecuador@gmail.com
R.U.C. 1713443479001

Informe de Resultados

Nombre del Proyecto: Purificación e identificación molecular a partir de muestras de ADN

Informe No.: A-209

Técnico Responsable: Francisco Garrido

Fecha: 19/03/2021

Resultados

Código IDgen	Muestra	Longitud	% de Calidad	Organismo	Fragmento	% de identidad	NºAccesión
5T	5T	218	92,2	*Rabbit hemorrhagic disease virus*	Rhd	99,54	MK895974.1
14B	14B	161	99,4	*Rabbit hemorrhagic disease virus*	Rhd	100	MK895974.1

Firma autorizada

Francisco Javier Flores Flor, PhD.

Propietario IDgen