# DOCTOR OF PHILOSOPHY

# Molecular Detection of Totiviruses in Medically Important Arthropods and Parasites

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*Award date:*
2021

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# Molecular Detection of Totiviruses in Medically Important Arthropods and Parasites


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A thesis submitted for the degree of
*Doctor of Philosophy*

July 2020

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## Abstract

The *Totiviridae* is a family of unsegmented, icosahedral, small dsRNA viruses in the realm of *Riboviria*, which has been historically characterised by host diversity, morphology and host impact on differences in strategies for transmission. Human hosts include parasites like *Leishmania,* the cause of leishmaniasis a widespread and sometimes fatal disease, *Trichomonas*, the cause of trichomoniasis*,* the most common non-viral sexually transmitted infection, and *Giardia,* which causes giardiasis - an acute or chronic gastrointestinal disease. *Eimeria* causes serious diseases of domestic animals, particularly chickens, cattle and rabbits. Hosts from which they have been isolated included plant parasitic oomycetes, many yeasts and fungi, red macroalgae (seaweeds), diatoms (single celled algae), woodlice (terrestrial crustaceans), many insects such as flies, mosquitoes, ants and wasps and shrimp (marine crustaceans). However, also fish, freshwater snails that are intermediate hosts to parasites, and plants like papaya, notoginseng, maize, and wild petunias. The totiviruses increase the virulence of the parasites in *Leishmania* and *Trichomonas* (hypervirulence) and sometimes decreases fungal virulence (hypovirulence) such as in oats. Totivirus is myocarditis and myonecrosis in salmon, smelt and shrimp, and is asymptomatic in golden shiners. It is not infectious and vertically transmitted in *Leishmania, Trichomonas,* and other fungi and plants, while in *Giardia* it is transmitted horizontally by fish, shrimps, papaya.

Totiviruses evolve so fast that there is currently no systematic method to search for them. The commercial antibody J2, specific for dsRNA, has been evaluated as such a tool to detect totivirus. While the sensitivity of the antibody was promising, the lack of specificity for dsRNA rendered it useless. To enable a systematic survey, conserved regions in the RNA-dependent RNA polymerase gene of individual virus species and lineages were identified and primers developed for *Leishmaniavirus1*, *Leishmaniavirus*2, *Leishmania aethiopica virus*, *Giardiavirus*, *Eimeriavirus*, and *Trichomonasvirus*. Outside of Leishmaniaviruses, PCR results were limited by the absence of available virus-positive host samples, and the reasons for failures to detect virus in test samples is discussed. The following viruses new to science were discovered: *Leishmania infantum virus*, *Leishmania major virus*, *Leishmania panamensis virus*, *Leishmania hertigi virus*, *Leishmania mexicana virus*, *Leishmania amazonensis virus*, *Leishmania venezuelensis virus*, *Leishmania chagasi virus*, *Leishmania donovani virus*, *Leishmania gerbilli virus,* and *Leishmania tarentolae virus.* Outside the current taxonomic grouping of parasites, *Totivirus*es new to science were discovered in the genus *Endotrypanum*, in the species *Herpetomonas megaseliae*, and in the species *Blastocrithidia culicis* of the *Trypanosomatidae*. In addition, the first totivirus was discovered in *Bodo caudatus* (*Bodonida*: *Kinetoplastida*), widely expanding the range of vertically transmitted totiviruses and the probable time when these viruses entered their host lineage.

Sandflies as most common vectors of *Leishmania* parasites were investigated with next generation whole genome sequencing for arthropod derived *Totiviridae* to resolve where the infection came from to *Leishmania*.

Using alignments of all available sequences, a new conserved motif of the RNA-dependent RNA polymerase of dsRNA viruses was discovered. Based on these alignments, a new phylogeny of the totiviruses was reconstructed and generic and whole-family delineations discussed.

## Acknowledgments

First and foremost, I would like to thank my God (Allah) for giving me the ability and opportunity to complete this, my PhD. It was a long and beautiful adventure, and full of challenges, without his support, help and guidance I would not have been able to complete it and receive my PhD certificate.

A special thanks and much gratefulness go to the person who was behind this accomplishment and my achievement of this enormous project, Dr. Henk R. Braig. Also, I want to thanks Dr. Nathalie Fenner, Chirman Prof. Simon Creer, Internal Prof James Mcdonald, Dr Alexander Papadopulos for their help and fully support to finish my thesis.

Also, special thanks to my family in my home country, Saudi Arabia, my mother Eishah and my father Ali, and my family here in UK, my wife Amal, and children Leen, Mohammad, Salman, and Ali.

Last not least, I express my acknowledgment and thanks to the Ministry of Health in Saudi Arabia, for funding my scholarship here at Bangor University and sponsoring my living expenses in the UK.

## List of Abbreviations

| | |
|---|---|
| ATL | American tegumentary leishmaniasis |
| CDC | Center for Disease Control |
| cDNA | complementary Deoxyribonucleic Acid |
| CEB | Centre of Environmental Biotechnology |
| CeRV1 | *Chalara elegans RNA Virus 1* |
| CL | Cutaneous leishmaniasis |
| CP | Capsid Protein |
| DCL | Dissemination Cutaneous leishmaniasis |
| DMVs | Double-membrane vesicles |
| dsDNA | Double-stranded Deoxyribonucleic Acid |
| dsRNA | Double-stranded Ribonucleic Acid |
| EAV | equine arteritis virus |
| ELISA | enzyme-linked immunosorbent assay |
| GLV | *Giardia lamblia virus* |
| HvV190S | *Helminthosporium victoriae virus 190S* |
| IBDV | Infectious Bursal Disease Virus |
| ILTV | Infectiously Laryngotracheitis Virus |
| IPNV | Infectious pancreatic necrosis virus |
| ICTV | Committee on Virus Taxonomy |
| LRV1 | *Leishmania RNA virus 1* |
| LRV2 | *Leishmania RNA virus 2* |
| mAb | Monoclonal antibody |
| MCL | Mucocutaneous leishmaniasis |
| NWL | New World Leishmania |
| ORF | Open Reading Frames |
| OWL | Old World Leishmania |
| PBV | Picobirnaviruses |
| PCR | Polymerase Chain Reaction |
| PKDL | Post-Kala-Azar Dermal Leishmaniasis |
| PnLV | *Poinsettia* latent virus cultured |
| PVDF | Polyvinylidene fluoride |
| RdRp | RNA-dependent RNA polymerase |
| RNA | Ribonucleic Acid |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| ScV-L-A | *Saccharomyces cerevisiae* virus L-A |
| ScV-L-BC | *Saccharomyces cerevisiae* virus L-BC |
| ssRNA | Single-stranded Ribonucleic Acid |
| SYNV | *Sonchus* yellow net virus cultured |
| T | Triangulated |
| TBE | Tris/Borate/EDTA |
| TL | Tegumentary leishmaniasis |
| TLR3 | Toll-like receptor 3 |
| TVV | *Trichomonas vaginalis* virus |
| UmV-H1 | *Ustilago maydis* virus H1 |
| VL | Visceral *Leishmania* |
| VLP | Virus-like particle |
| WHO | World Health Organisation |
| HPLC | High performance liquid chromatography |
| IFNs | interferons |

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# *Chapter 1:* INTRODUCTION

## *1.1* RNA Viruses

Viruses are one of the smallest parasitic or pathogenic entities or agents, but cannot replicate except in a host. When a virus infects a susceptible host cell, it uses the cell's reproductive apparatus to replicate itself, sometimes in massive numbers. Viruses have either RNA or DNA or both as genomic material; the nucleic acid can be single or double-stranded. Infectious viral particles known as virions are made up of nucleic acid, sometimes enzymes, protein matrices, capsids, and envelopes (Lodish *et al.*, 2000). Some RNA viruses do not form virions. These viruses are most common in the families *Narnaviridae*, *Hypoviridae*, and *Endornaviridae* but are also scattered among a divers list of other lineages; they often referred to as capsid-less viruses (Dolja & Koonin, 2012). They are mainly dsRNA viruses encoding an RNA-dependent RNA polymerase (RdRp). They likely evolved several times independently from positive-sense ssRNA viruses that have lost their ability to code for capsids. Viroids on the hand are composed of circular single-stranded RNA and do not code for an RdRp or any protein for that matter (Bengone-Abogourin *et al.*, 2020; Shrestha & Bujarski, 2020; Ramesh *et al.*, 2021).

Most RNA viruses use their own viral RdRp to replicate their RNA, Hepatitis Delta virus and viroids are an exception. There are three classes of RNA virus genomes:

- single stranded, either (+) or (-) sense (ssRNA);
- closely related ambisence ssRNA has regions that are (+) sense or (−) sens; and
- double-stranded RNA viruses (dsRNA).

In each of these groups, transcription is the first synthetic occurrence following infection. Below is a recent classification of RNA viruses published up to 2015 (Figure 1.1) (Koonin *et al*., 2015). RNA viruses infect a wide range of hosts (Payne, 2017).

RdRp is one of the most versatile enzymes of all RNA viruses, important for replicating and transcribing genomes. Although RdRp sequences evolve very fast, RdRp core structural features remain. The RdRp structure resembles a right-hand cup shape, with subdomains of fingertips, palm and thumb. Catalysis involves the presence of conserved aspartate and divalent metal ions. RdRps' complex structures with substrate, inhibitors, and metal ions may provide a full overview of their functional mechanism and also provide useful insights into the production of antivirals; it is the sequence of this enzyme that is the main focus of assays in this study.

## *1.2* Double Stranded RNA Viruses

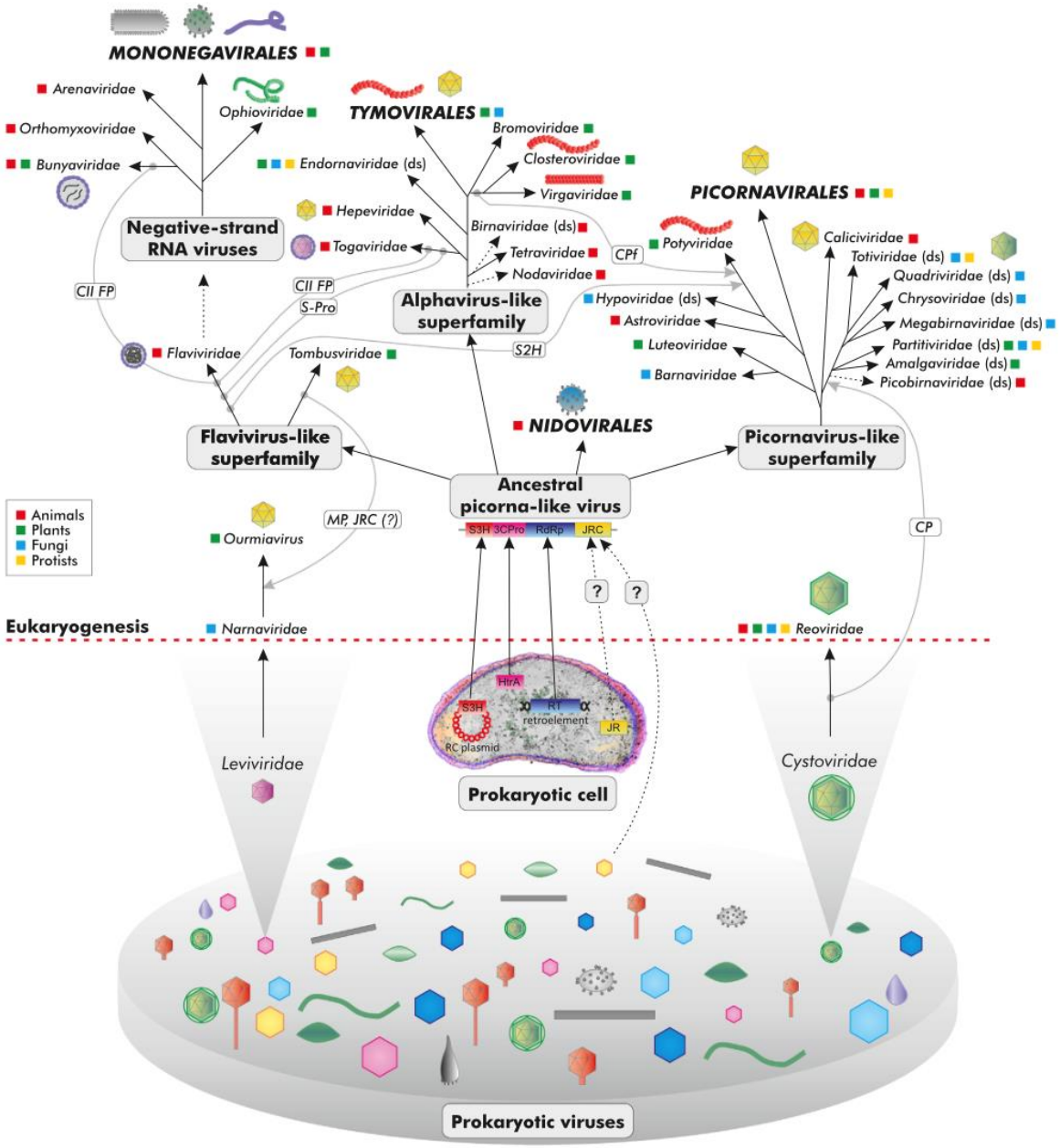


Figure 1.1: Classification of RNA viruses
After Koonin *et al*., 2015.

The dsRNA viruses are classified into five major groups; they differ considerably in host ranges, which include humans, plants, fungi, animals, and bacteria and rarely Archaea. With the exception of members of the *Totiviridae* and a few other virus families like the *Endornaviridae*, *Hypoviridae*, *Amalgaviridae*, most dsRNA viruses have multiple segments of dsRNA in their genome. A large family of dsRNA viruses, the *Reoviridae,* which infect a wide variety of hosts, including plants, animals, and humans, and cause mild- to life-threatening illnesses, have 10 – 12 unique dsRNA segments in their genomes. Generally, the dsRNA segments in the *Reoviridae* are monocistronic, which means open reading frames are transcribed and translated one at a time, whereas polycistronic segments are more common in other families of dsRNA viruses where on mRNA codes for several proteins. All the well-characterised dsRNA viruses have icosahedral capsids, and,

except for φ6, a prototypical bacterial virus in the family *Cystoviridae*, they are nonenveloped. Perhaps necessitated by the general requirement for cell entry and a specialised requirement for endogenous transcription, the capsids of these viruses, with few exceptions, consist of multiple layers (Prasad & Prevelige Jr, 2003).

Structures of several dsRNA viruses have been studied, these include:

- L-A virus (*Totiviridae*) with a single segment;
- Infectious bursal disease virus (IBDV, *Birnaviridae*) with two segments;
- φ6 (*Cystoviridae*) with three segments;
- several members of the *Reoviridae* representing various genera, including rotaviruses
- Bluetongue virus (BTV), *Orthoreovirus* , *Aquareovirus*
- Rice dwarf virus and *Cypovirus*.

Their structures have been analysed using cryo-EM techniques. X-ray structures of L-A virus, and transcriptionally competent cores of bluetongue virus, Orthoreovirus and Rice dwarf virus have been determined to near 3 Å resolutions. Despite noticeable differences, with the exception of *Cypovirus* and L-A virus, the outer capsid layer is generally based on triangulated (T) = 13 icosahedral symmetry.

Several virus families are known to infect fungi, three families are dominant: *Chrysoviridae, Partitiviridae* and *Totiviridae,* their genomes are quadripartite, bipartite, and monopartite, respectively. They are naturally isometric particles with diameters ranging from 25 - 50 nanometres. *Partitiviruses* are thought to likely derive from a *Totivirus* ancestor, due to the sequence similarity of their RNA dependent RNA polymerase. A fourth family also infects fungi, the *Alternaviridae* was also identified recently and has a quadripartite genome (Payne, 2017).

A list of dsRNA families, as currently recognised, is in Table 1.1; they are described below.

Table 1.1: Families of dsRNA Viruses

Data from the International Committee for Taxonomy of Viruses (ICTV) (King *et al.*, 2011).

| Family | Size (kb) | Host | Comments |
|---|---|---|---|
| *Amalgaviridae* | ~3.5 | Plants | Capsid not detected |
| *Alternaviridae* | 1 - 4 | Filamentous fungi | Genomes consist of 4 segments |
| *Birnaviridae* | 6 - 7 | Vertebrates and invertebrates | Bipartite genomes |
| *Chrysoviridae* | 12 - 13 | Fungi | Genomes consist of 4 segments |
| *Endornaviridae* | 14 - 17.6 | Fungi, oomycetes and plants | Capsid-less viruses |
| *Megabirnaviridae* | 16 | Fungi | Bipartite genomes |
| *Partitiviridae* | 3 - 5 | Fungi, plants and protists | Bipartite genomes |
| *Picobirnaviridae* | 4 - 4.5 | Vertebrates | Bipartite genomes |
| *Quadriviridae* | 16.8 - 17.1 | Fungi | Genomes consist of 4 segments |
| *Reoviridae* | 18.5 - 29 | Vertebrates, arthropods, molluscs, plants, green alga and fungi | Largest family of dsRNA viruses; genomes contain from 9 to 12 segments |
| *Totiviridae* | 4.6 - 7 | Fungi, parasitic protists, arthropods, plants, fruits, fish and bats | Unsegmented, Currently has five genus; many related viruses remain unclassified |

### *1.2.1 Amalgaviridae*

Recently, a new group of dsRNA viruses has been described and classified into a new family: *Amalgaviridae,* ratified by the Committee on Virus Taxonomy (ICTV) membership in 2014. They have been isolated from numerous plant species as dsRNA molecules of ~3.5 kb. They have a bicistronic genome organisation, typical of dsRNA viruses of the family *Totiviridae*, which mainly infect eukaryotic organisms such as fungi and protists (Krupovic *et al.*, 2015) and also share similarities with members of the *Partitiviridae,* yet have significant differences in genome organisation (Nibert *et al.*, 2014). As yet, no virions have been isolated from this group, but the genomes are non-segmented and organised similarly to those of *Totiviridae*. They have an upstream Open Reading Frame (ORF), a sequence of nucleotide triplets that are read as codons specifying amino acids without a stop codon, but of unknown function, a downstream an RdRp ORF, and a ribosomal frameshifting signal between these ORFs. Of note, their RdRp sequence is recovered in a different phylogenetic clade, which is more similar to *Partitivirus* RdRps than *Totivirus* RdRps. They are referred to as amalga viruses due to their apparent “amalgamation” of *Totivirus* and *Partitivirus* properties (Koloniuk *et al.*, 2015).

### *1.2.2 Alternaviridae*

The *Alternaviridae* have also been proposed only recently and comprise three viruses infecting filamentous fungi (Kozlakidis *et al.*, 2013). The first of these viruses has been known for over forty years (Ratti & Buck, 1972). The family name is derived from one of the fungal hosts, *Alternaria*

*alternata,* a fungus reported in more than 380 host plant species that causes leaf spot and other diseases. *A. alternata* is an opportunistic pathogen in many hosts that causes leaf spots, rots and is a scourge of many plant tissues (Mehrabi *et al.*, 2011; Tsuge *et al.*, 2013; Palou *et al.*, 2013). In humans, this fungus has been implicated in persistent and severe cases of asthma (Knutsen *et al.,* 2012), fungal melanonychia (nails) (Finch *et al.*, 2012), mycotic keratitis (cornea) (Thomas *et al.*, 2013), cerebral phaeohyphomycosis (Silveira *et al.*, 2013), and many conditions arising in transplant and HIV-infected patients (Revankar & Sutton, 2010). *Alternaviridae* viruses have so far been found in three fungal host species, *Alternaria alternata, AaV-1* (Aoki *et al.*, 2009), *Asperigillus foetidus, AfV-F* (Kozlakidis *et al.*, 2013) and *Aspergillus niger, ASV341* (Hammond *et al.*, 2008).

The dsRNA of the virus comes in four segments of 1 - 4 kb in length, the plus strands of which are polyadenylated. Each of the segments is a single ORF. The segments are probably packaged in separate virion particles (Kozlakidis *et al.*, 2013). The proposed type virus, *Alternaria alternatavirus-1* (AaV-1), impairs mycelial growth, leading to aerial mycelial collapse, unregulated pigmentation and cytolysis. Virus detection can be achieved through extracting total nucleic acids then digesting ssRNA with S1 nuclease and DNA with DNAse, separating the dsRNA on an agarose gel, and visualisation the banding pattern with ethidium bromide. No Polymerase Chain Reaction (PCR) essay has yet been described for this family. The virus load in fungal isolates tested this way varied by a factor of ten. Inside the fungal cell, most of the viral RNA is present as genomic dsRNA and little as the single-stranded replicative form. Consequently, for developing a PCR assay, there seems to be no benefit from specifically targeting the polyadenylated strand. Of the four open reading frames, only one has so far been identified as an RNA-dependent RNA polymerase (Aoki *et al.,* 2009; Kozlakidis *et al.*, 2013).

### *1.2.3 Birnaviridae*

*Birnaviridae* have a non-enveloped single-shell T = 13 icosahedral capsid with a diameter of approximately 70 nm and a composition of 260 virus protein 2 (VP2) trimers which form radially projected spikes from the capsid. Peptides produced from cleavages from pre-VP2 C-terminals remain in the virus. virus protein 3 (VP3) constitutes a genomic RNA ribonucleoprotein complex. The virion also contains tiny quantities of VP1. The segmented linear dsRNA genome has 2 segments (A, B), encoding 5 proteins. VP1 is located in free form, with 5' genomic RNA (VPg) attached covalently, segment size is ~2.3 kb, the average size of the genome is ~6 kb. Segment A codes for the structural polyprotein matured in cis by VP4 and an alternate ORF that can be converted by leaky scanning (VP5). Segment B encodes VP1.

*Birnaviridae* consists of four genera, including *Avibirnavirus*. *Avibirnavirus* has been detected in human faeces following ingestion of its hosts, which include chickens and other fowl. There is only one species in this genus, which causes Contagious Bursal Disease. *Aquabirnavirus* causes

infectious pancreas necrosis virus (IPNV) in salmonid fishes, characterised by a severely inflamed bursa of Fabricius, leading to significant acute morbidity and mortality. Extreme immune suppression of the bursa of Fabricius arises when immature B-lymphocytes are killed (Hon *et al.*, 2008).

### *1.2.4 Chrysoviridae*

*Chrysoviridae* is a recently classified dsRNA virus family, which infects fungi only, containing the single genus *Chrysovirus* (Coutts *et al.,* 2004), which was formerly part of the *Partitiviridae*. It was isolated from *Aspergillus niger*, as four polyadenylated *Chrysovirus* dsRNAs in mixed or individual infections (Jamal *et al.*, 2010). *Chrysovirus* has four species, including: *Helminthosporium victoriae virus 145S*, hosted by Victoria blight in oats, *Penicillium brevicompactum virus*, *Penicillium chrysogenum virus*, hosted by the dietary mould *Penicillium chrysogenum,* and *Penicillium cyaneo-fulvum virus*, hosted by *Penicillium cyaneo-fulvum.* Both PCR and northern blot with a hybridisation probe have found *Penicillium chrysogenum* virus in *Aspergillus fumigatus* (Jiang & Ghabrial, 2004; Urayama *et al.*, 2012). Other hosts have been identified using Reverse Transcription PCR (RT-PCR), ligase-mediated Rapid Amplification of cDNA Ends ((RLM)-RACE) PCR methodologies, and northern hybridisation analysis, which identifies single segment sequences (Coutts *et al.,* 2004). In addition, sequence analysis, was used to identify *Cryphonectria nitschkei chrysovirus 1*, *Fusarium oxysporum chrysovirus 1*, and *Verticillium chrysogenum* virus, along with the unclassified *Agaricus bisporus Virus 1* and *Fusarium oxysporum chrysovirus* (Jamal *et al.*, 2010). High-throughput sequencing has also recently successfully identified chrysoviruses and other mycoviruses in grapevine (Al Rwahnih *et al.*, 2011).

*Chrysovirus* has 60 monomers in a subunit of proteins in a T = 1 icosahedral symmetry in isometric virions. Its four genome segments are monocistronic. The second segment, dsRNA-2, encodes the capsid protein (CP), and is the longest, with dsRNA-1 it encodes the RdRp. The dsRNA-3 and dsRNA-4 segments have unknown functions (King *et al.*, 2011; Castón *et al.*, 2013).

### *1.2.5 Endornaviridae*

*Endornaviridae* viruses are found naturally in several plants, and fungi including oomycetes, it is transmitted both horizontally through asexual reproduction (generally spores) within the same generation or vertically from mother to daughter cells, i.e. between generations. Replication occurs in cytoplasmic vesicles, its (+) RNA is copied through its anti-genomic RNA into new genomes as dsRNA. There are currently 8 species in one genus, *Endornavirus*, in which characteristically the viral replicase enzyme is encapsulated with no real capsid. The genome has one ORF which codes for multiple polypeptides and transcription makes viral RdRp and other proteins possible (Koonin & Dolja, 2012).

### *1.2.6 Megabirnaviridae*

*Megabirnaviridae* has one genus, *Megabirnavirus*, fungi are the natural hosts, including, for example *Rosellinia necatrix, which hosts Rosellinia necatrix* Megabirnavirus. This family is linked to reduction in host virulence. The icosahedral capsid is not enveloped and has a diameter of ~ 50 nm, with, it is presumed, icosahedral symmetry of T = 1. The segmented genome has linear dsRNA, thought to comprise two segments of ~ 7 kb and ~ 9 kb encoding four proteins. The RNA-1 and RNA-2 coding strands have two tandem ORFs which do not overlap. RNA-1 is encoded with a single large 135 kDa capsid protein (Wu *et al.*, 2012).

### *1.2.7 Partitiviridae*

There are four genera in this family, one infects only fungi and is known as *Partitivirus*. *Partitivirus* has 19 species: *Agaricus bisporus virus 4, Aspergillus ochraceous virus 1, Atkinsonella hypoxylon virus, Ceratocystis resinifera virus 1, Discula destructiva virus 1, Discula destructiva virus 2, Fusarium poae virus 1, Fusarium solani virus 1, Gaeumannomyces graminis virus 019/6-A, Gaeumannomyces graminis virus T1-A, Gremmeniella abietina RNA virus MS1, Helicobasidium mompa virus, Heterobasidion annosum virus, Ophiostoma partitivirus 1, Penicillium stoloniferum virus F, Penicillium stoloniferum virus S, Pleurotus ostreatus virus 1, Rhizoctonia solani virus 717,* and *Rosellinia necatrix virus 1*.

*Atkinsonella hypoxylon virus* that infects *Atkinsonella hypoxylon*, has been detected by RT-PCR and northern blot analysis (Osaki *et al.,* 2002; Urayama *et al.*, 2012). *Gaeumannomyces graminis virus 019/6-A*, *Gaeumannomyces graminis virus T1-A and Gremmeniella abietina RNA virus MS1*, which infect *Gaeumannomyces graminis*, were detected by PCR and confirmed by RNA blot hybridisation (Batten *et al.,* 2000). *Rosellinia necatrix virus 1* that infects *Rosellinia necatrix* fungi was detected by RT-PCR (Chiba *et al.,* 2013). Some other species were detected by RT- PCR and RNA ligase-mediated (RLM)-RACE PCR procedures (Coutts *et al.,* 2004), with the remainder by electron microscopy (King *et al.*, 2011). *Partitivirus* has recently been detected using high-throughput sequencing (Al Rwahnih *et al.*, 2011).

The viruses of this family are associated with latent infection in fungal, plant and protozoan hosts. Intracellular transmission occurs during host cell division, sporogenesis, and hyphal anastomosis. Fungal purified partitiviruses have been identified in fungal protoplasts as has infection of the pollen and embryos of plant ovules by cryptoviruses of the genera *Alphacryptovirus and Betacryptovirus* (Ghabrial, 1998). No transmission by grafting or from intracellular transportation without cell division has been observed in this family (King *et al.*, 2011).

The virions of this family are morphologically isometric, 30 - 43 nm in diameter, with a capsid built of 120 copies of the capsid protein and have no envelope. Virions are composed of two non-related and similarly sized, linear dsRNA segments in all virus species (Jamal *et al.*, 2010). The small and larger linear segments code for the capsid protein (Park *et al.*, 2005), as well as the virion-related RNA polymerase. Such dsRNA segments have a different particle encapsulation (King *et al.*, 2011). This structure contributes to their success as immunogens; no serological relationships between fungal and plant viruses were found in this family (King *et al.*, 2011).

### *1.2.8 Picobirnaviridae*

*Picobirnavirus* is the only genus in the *Picobirnaviridae*. Amniotes are widely recognised natural hosts, especially mammals, but studies have reported *Picobirnavirus* (PBV) in a wide variety of other organisms. At present there are only two known species, including a human PBV. Diseases caused by PBV include animal and human gastroenteritis, although the relationship of the virus species in these hosts is uncertain. It is a dsRNA virus and has small, unenveloped, bi-segmented genome (Mondal & Majee, 2014).

### *1.2.9 Quadriviridae*

*Quadriviridae* is a family with one genus and one species, *Rosellinia necatrix quadrivirus 1,* which infects the fungus of that name. It has capsid with a diameter of ~48 nm, the four segments of the genome may be independently encapsulated. It has a genome of ~16.8 kb linear dsRNA, comprising four single-protein encoding segments. Segments 2 and 4 produce structural proteins for virion assembly (Kondo *et al.*, 2013; Lin *et al.*, 2013).

### *1.2.10 Reoviridae*

*Reoviridae* is a group of dsRNA viruses hosted by a wide range of eukaryotic organisms. They lack envelopes and are exceptional in packaging their genomes in poorly understood multi-layered, capsids. They are icosahedral, with the same internal capsid equivalent, with the exception of *Cypovirus* and *Dinovernavirus*. The outer capsid has T = 13 icosahedra symmetry, the inner with T = 2 icosahedra symmetry. The linear, dsRNA genome contains between 10 and 12 protein coding segments of sizes ~0.2 – ~3.0 kb, total genome size is ~18.2 – ~30.5 kb.

In this family, the dsRNA genomes are never completely uncoated, to avoid antiviral activation in the host cell. Every section of dsRNA is assisted by viral polymerase. Such mRNAs are passed to the cell cytoplasm for translation. Leaky protein processing produce additional proteins (Calisher *et al.*, 1988).

### *1.2.11 Totiviridae*

*Totiviridae* is a family of dsRNA viruses that infect fungal parasites and yeasts. Virions of this family are usually mono-segmented and isometric, with a diameter of 30 - 40 nm; the capsid is made with a single protein and with no envelope, and has T = 2 icosahedral symmetry (Ghabrial, 2008). The genome resides on a single RNA containing two large, overlapping ORFs which encode CP and RdRp. The RdRp manifests as a fusion protein CP / RdRp as a result of ribosomal frame shifting or as a direct fusion with CP in some genera (e.g., *Giardiavirus*, *Leishmaniavirus*, and *Totivirus*) (Kang *et al.,* 2001). It is expressed as a distinct, non-fused protein in others (e.g. *Victorivirus*).

Viruses belonging to the *Totiviridae* family have been identified in numerous protozoan parasites, including, *Trichomonas vaginalis, Giardia lamblia, Leishmania braziliensis* and *Eimeria stiedae* (Wang & Wang 1986; Aldritt & Wang, 1986; Tarr *et al.,* 1988; Revets *et al.,* 1989; Roditi *et al.,* 1994; Del Cacho *et al.,* 2001; Han *et al.,* 2011; Fraga *et al.,* 2006). *Leishmania RNA virus 1 (LRV-1)* has been demonstrated to increase the inflammatory response by triggering Toll like receptor 3 (TLR3) signalling (Ives *et al.,* 2011). In addition, several reports suggested that the virus might increase the pathogenicity of the parasites (Wang *et al.,* 1987, Ogg *et al.,* 2003, Jenkins *et al.,* 2008, Fichorova *et al.,* 2012). A number of protozoan parasites, particularly *Giardia lamblia*, have been investigated using viral RNA-transfection studies (Yu *et al.,* 1996; Yu & Wang, 1996; Dan *et al.,* 2000; Davis-Hayman SR 2002).

The mostly persistent and non-cytopathic infectious hosts are known to carry more than 25 species of *Totivirus*es currently recognised by the ICTV. In all, 4 genera are recognised: *Giardiavirus*, *Leishmaniavirus*, *Totivirus*, and *Victorivirus (*King *et al.*, 2011). The two former include those viruses that infect protozoa, and the latter two infect fungi (Huang & Ghabrial,1996).

*a) Totivirus*

*Totivirus*, from "totus" means "undivided". *Totivirus* has three species (King *et al.*, 2011) and infects yeasts, smut fungi and protozoa (Cheng *et al.*, 2003). It was first isolated from *Aspergillus niger*, as both mixed and individual infections of four polyadenylated dsRNAs (Jamal *et al.*, 2010). The three species are:

- *Saccharomyces cerevisiae virus L-A* (ScV-L-A) that infects the yeast *Saccharomyces cerevisiae;*
- *Saccharomyces cerevisiae virus L-BC* (ScV-L-BC) in the same host (Ribas & Wickner, 1996); and
- *Ustilago maydis virus H1* (UmV-H1) that infects the fungus *Ustilago maydis* (Kang *et al.*, 2001).

The virions of *Saccharomyces cerevisiae virus L-A* (ScV-L-A) are icosahedral particles with 39 nm in diameter, each containing a single dsRNA molecule. ScV-L-A has a single, major coat protein

called GAG, and a minor fusion protein, GAG-POL. Most ScV-L-A strains carry a satellite dsRNA known as M dsRNA, which encodes a secreted, toxic protein (the "killer" toxin). There are many types of M dsRNA, which has been exploited for phenotypic differentiation in analyses of the ScV-L-A virus group (Ribas & Wickner, 1996; Wickner, 1996; Wickner *et al.*, 2008;). ScV-L-A was detected by electron microscopy (Fried & Fink, 1978; King *et al.*, 2011) and by X-ray crystallography (Naitow *et al.,* 2002).

*Saccharomyces cerevisiae L-BC* (ScV-L-BC) is a species of *Totivirus* found in the same host as ScV-L-A and is closely related to ScV-L-A (Ribas & Wickner, 1996). It was recently described following detection by high-throughput sequencing (Al Rwahnih *et al.*, 2011).

*Ustilago maydis virus* (UmV) comprises three obvious size groups of dsRNA segments, H (heavy), M (medium), and L (light). Segments of H have been suggested to encode most of the main viral proteins, but without any molecular evidence. M and L segments are associated with satellites and defective dsRNAs. The toxic protein KP6 has been found to be encoded by UmV (Steinlauf *et al.*, 1988).

The *Ustilago maydis virus H1* (UmV-H1) was detected using gel analysis and revealed by sequence analysis as a single, long ORF. Northern blot analysis was used to identify the H1 segment from H2 (Kang *et al.*, 2001). UmV-H1 was also detected by electron microscopy (King *et al.*, 2011) and recently, it has been detected in *Xanthophyllomyces dendrorhous* strains by RT-PCR (Baeza *et al.*, 2012).

*b) Leishmaniavirus*

The genus name *Leishmaniavirus* is derived from the trypanosomatid host name. *Leishmaniavirus* is one of the most dangerous genera to human health in the *Totiviridae* family, as it increases the virulence of infecting parasites (de Carvalho *et al.*, 2019; Kariyawasam *et al.*, 2019; Olivier & Zamboni, 2020; de Carvalho *et al.*, 2021). It is only known to infect *Leishmania*, responsible for the disease Leishmaniasis (Ives *et al.*, 2011). The *Leishmaniavirus* genus has 13 strains: *Leishmania RNA virus 1 – 1 (LVR),* found in the New World parasitic protozoa *Leishmania braziliensis, Leishmania RNA viruses 1 – 2 to 1 – 12 a*nd the distinct *Leishmania RNA virus 2 – 1,* which infects *Leishmania major* (King *et al.*, 2011). Leishmaniaviruses (types 1 and 2) infect only protozoa (Khoshnan & Aldetete, 1993).

The *Leishmaniavirus* is a non-envelope and non-segmented dsRNA genome of ~ 5,200 bp in length that has a single capsid protein. Virions particles are isometric, diameter 33 nm (King *et al.*, 2011). *Leishmania RNA virus 1* has three ORFs; ORF3 was identified as RNA-dependent RNA polymerase and ORF2 as capsid protein. All ORFs are detected by PCR and gel electrophoresis (Stuart *et al.*, 1992).

*Leishmania RNA virus* (LVR) has been detected by quantitative RT-PCR (qRT-PCR) and gel electrophoresis (Urayama *et al.*, 2012) and by a capsid-specific antibody, ELISA, and electron microscopy (Zangger *et al.*, 2013). The remaining species were detected by electron microscopy (King *et al.*, 2011).

*c) Giardiavirus*

*Giardiavirus* has one species which infects the flagellated protozoan *Giardia lamblia* after which the genus is named. The host is usually used to replicate *Giardia lamblia virus* (GLV). The virions are isometric, with a diameter of 36 nm and contain a single dsRNA molecule that holds the RNA polymerase and capsidic protein genes. In virions, the RNA polymerase is enclosed. Some *Giardia lamblia* strains are immune to GLV infection. GLV is not associated with effects on the virulence of its parasite host (King *et al.*, 2011). A RT-PCR (Miska *et al.*, 2009) with gel electrophoresis (Wang & Wang, 1986) was used detect *Giardia lamblia* virus and electron microscopy has also revealed, as yet poorly understood, GLV-like particles (Wang & Wang, 1986; King *et al.*, 2011).

*d) Victorivirus*

*Victorivirus* is a newly described genus in the family *Totiviridae* that infects fungi, the genus name is derived from the fungal host name. *Victorivirus* virions are isometric with a diameter of 35 – 45 nm and have non-segmented dsRNA. The *Victorivirus* genus has nine species that infect different fungal hosts (King *et al.*, 2011; Ghabrial & Nibert 2009). The *Helminthosporium victoriae virus 190S* (HvV190S) is found in *Helminthosporium victoriae* (Dunn *et al.*, 2013; Park *et al.*, 2005). Recently, the two sequenced species of *Eimeriavirus*, both of which infect protozoa, *Eimeria stiedae RNA virus* 1 and *Eimeria tenella* RNA virus 1, were both found to be included in a strongly supported Victorivirus clade, in an analysis of the entire *Totiviridae* (de Lima *et al.*, 2019).

Negative staining with uranyl acetate in electron microscopy is one recognised method of detection for this family. Northern blot and sequence analyses have been used to detect some species, such as *Chalara elegans RNA Virus 1* (CeRV1) and *Chalara elegans* RNA Virus 2 (CeRV2) (Park *et al.*, 2005). Transmission electron cryomicroscopy was used to detect *Helminthosporium victoriae virus 190S* (HvV190S), *Chalara elegans RNA Virus 1, Coniothyrium minitans RNA virus, Epichloe festucae virus 1, Gremmeniella abietina RNA virus L1, Helicobasidium mompa Totivirus 1-17, Magnaporthe oryzae virus 1, Sphaeropsis sapinea RNA virus 1* and *Sphaeropsis sapinea RNA virus 2* (Ghabrial & Nibert, 2009; King *et al.*, 2011). *Helminthosporium victoriae virus 190S* (HvV190S) was also detected using RT-PCR (Park *et al.*, 2005).

An example illustrating the promise of studying totiviruses is a recent publication on the dsRNA viriome of sponge, Figure 1.2 (Urayama *et al.*, 2020).

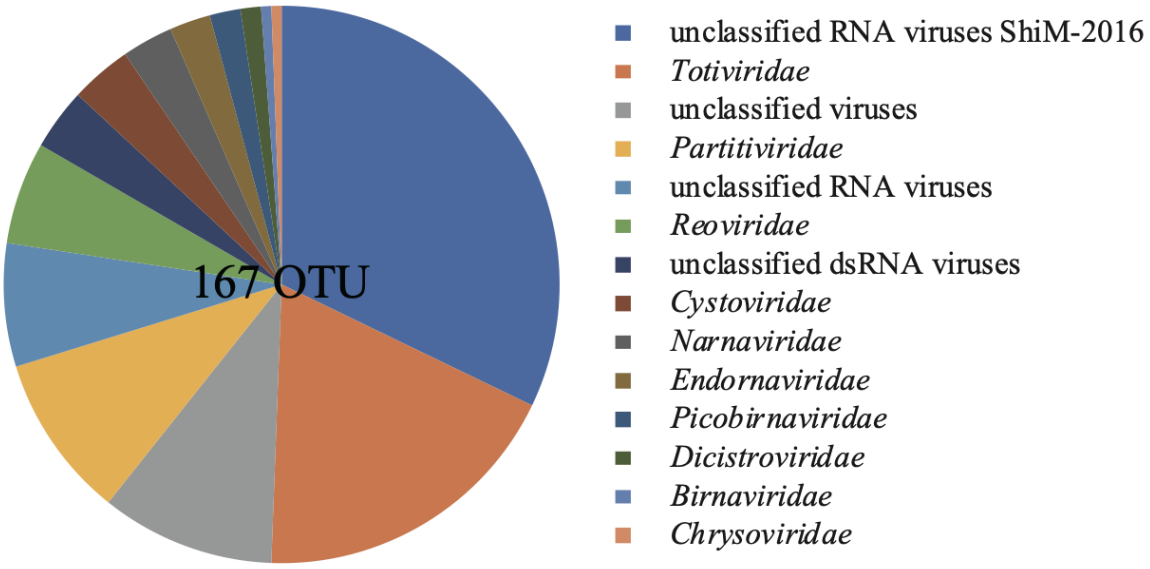


Figure 1.2: Totiviruses in orange are the dominant group aftyer unclassified RNA viruses in dsRNA viriome of a sponge (Urayama et al., 2020).

Interestingly, the largest group in the dsRNA viriome study in Figure 1.2 are as of yet unclassified dsRNA viruses, showing that the full diversity of dsRNA has still to be discovered.

## *1.3* Study Outline (Framework)

### *1.3.1 Study Outline*

Chapter 1: Introduction to the diversity of dsRNA viruses. The *Totiviridae* family is compared to the other important dsRNA virus families, their hosts, genome structures and, importantly, the methods that have been successfully used to detect them.

Chapter 2: This is a series of dot blot analyses to determine the sensitivity and accuracy of the method for detecting new *Totiviridae*. Results from assays of a range of viruses and positive and negative controls are discussed. This method was found to be neither sensitive nor accurate, since both positive and negative controls gave positive signal, hence it was necessary to use PCRs to achieve improved results in Chapters 3 **–** 5.

Chapter 3: A general introduction to the *Leishmania* parasite and *Leishmaniavirus*, causes of serious disease in humans. Primers are designed, for both *Leishmania RNA Virus 1* previously known from New World host species, and *Leishmania RNA* Virus 2 from Old World hosts, based on identified protein motifs. A series of reverse transcriptase PCR and nested PCRs is then used to detect these viruses in amplified DNA products of a range of Old and New World *Leishmania* and related parasites.

Chapter 4: Reverse transcriptase PCR and newly designed primers are used with samples of *Giardia* from a variety of human and animal hosts to detect *Giardiavirus*, a recently recognised genus of

*Totiviridae*. Virus signal was not detected, but the potential causes of its absence, which is not uncommon, are discussed. In addition, *Eimeriavirus*, recently recognised as belonging to the *Victorivirus* genus of the *Totiviridae* was sought in clinical samples without success.

Chapter 5: This is a short, general introduction to the applications of phylogenetic trees, followed by an up-to-date Bayesian analysis of the whole of the *Totiviridae,* including some new sequences*.* The resulting phylogeny is presented in a phylogeny and discussed in the context of previous phylogenies and current thinking on the delineation of genera and of the family as a whole.

Chapter 6: Samples of sandflies were collected and for comparison samples from termites and birds obtained, in order to extract their RNA and process it to build a next generation sequence library, prior to Illumina MiSeq sequencing and appropriate analysis of the differences between the host families.

Chapter 7: Final conclusions, summarising the major findings from the experimental Chapters and highlighting the novelty and value of this research study.

### *1.3.2 Aims and Objectives*

This study focusses on double-stranded RNA viruses. Aims and objectives are as follows.

a) Determine how old, in evolutionary terms, host infections are.

b) Assess the time span for divergence in these infections, has it occurred in a few or millions of years? It is well known that divergence between Old and New World *Leishmania* happened some thirty-seven million years ago (Croan & Ellis,. 1997) and, consequently, host infection could have happened during that time. In Chapter 5, a phylogenetic tree is developed to contribute to our understanding of this issue.

c) To establish whether *Totivirus* and viruses from other genera in the *Totiviridae* comprise sister species or a group, and which are horizontally or vertically transmitted.

d) Consider if totiviruses infect human parasites as well as those of other animals.

e) Most published research work on *Totiviridae* pertains to human infections, Chapters 3 and 4 investigate the relationship between *Totiviridae* and the animal parasites they infect.

f) Evaluate the effects of *Totivirus* and other parasites such as *Giardiavirus* on their host's virulence.

g)

### *1.3.3 Ethical Approvals*

The Ethics Review Committee of The Vector Borne and Zoonotic Diseases Department of the Saudi Arabia Ministry of Health authorised and approved this study to collect blood, swab and similar

samples directly from patients for laboratory culture. The health and safety department of Bangor University also approved these experiments, ethical approval number: CNS2018AG01.

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# *Chapter 2:* SEARCHING FOR DSRNA VIRUSES WITH AN ANTIBODY

## *2.1* Introduction

Discovering new viruses is a challenge unless the brute force of shot-gun metagenomics is applied where everything is sequenced, virus, host, host microbiome, and environmental nucleic acids associated with the host (Massart *et al.*, 2014; Zhang *et al.*, 2019). Viruses are then discovered through the identification of viral hallmark genes like the RNA-dependent RNA polymerase (Plyusnin *et al.*, 2020; Tisza *et al.*, 2021) or with the subtraction of a virus-free reference genome of the host and subsequent mapping of the remaining sequence reads to a viral database. If only a few viral reads are available, the assembly of viral hallmark genes might become a challenge (Kruppa *et al.*, 2018). Shot-gun metagenomics might encompass both, the viral nucleic acid and any intermediates that are produced during replication. Yet, some viral sequences might be too divergent to be recognized. These sequences will belong to so-called dark viruses or the dark matter of the virome or viroshere (Rinke *et al.*, 2013; Webster *et al.*, 2015; Krishnamurthy & Wang, 2017; Obbard *et al.*, 2020). Although shot-gun metagenomics is hailed in some publications as an impartial method or a method without a bias as it starts out as a sequence-independent method, it eventually is sequence dependent but to a much lesser degree than metagenomic approaches using consensus PCR with primers for the very same viral hallmark genes. Sequence dependent methods suffer from the fact that viruses, and especially RNA viruses, mutate much faster than all other genomes. Figure 2.1 shows viral mutation rates compared with prokaryotic and eukaryotic genomes. This higher mutation rate of RNA viruses can be explained by the fact that the most viral RNA-dependent RNA polymerases do not have a proof-reading capability. A notable exception to this rule are the *Nidovirales* among which the coronaviruses are.

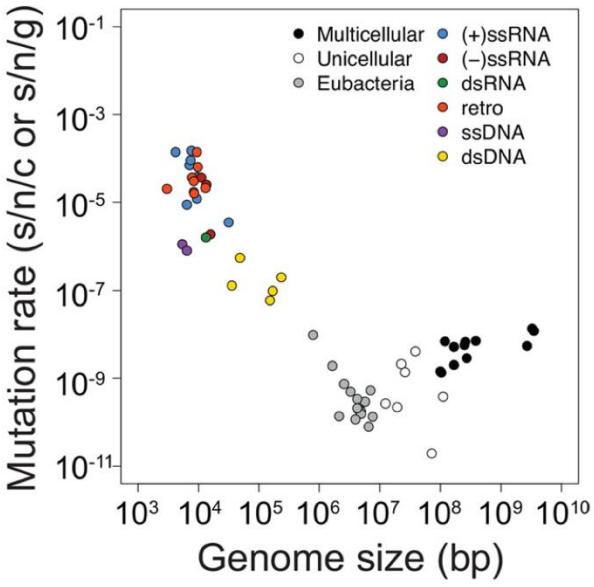


Figure 2.1: RNA viruses mutate at least a hundred-times faster than their hosts. Compared with single-stranded RNA viruses, double-stranded RNA viruses (a single green dot for reoviruses) are at the conservative end of the RNA spectrum. Mutation rate of genomes in substitutions per nucleotide site per cell infection [s/n/c] and genome size in base pairs (Peck & Lauringa, 2018).

In some cases in plant virology, there is so much starting material easily available that the virus just can be purified, and then the viral dsRNA extracted and sequenced (Thapa *et al.*, 2012; Tatineni *et al.*, 2014; Thapa *et al.*, 2015).

Instead of looking for the viral genome directly, methods have been developed to look for intermediates in viral replication (meta-transcriptomics) or the products of the host defence system, small RNAs as the result of RNA interference, which are enriched in the cell during the process of infection (Zheng *et al.*, 2017; Alleyne *et al.*, 2019; Xu *et al.*, 2020). To reduce computational requirements and to increase sensitivity, k-mer analysis of RNA-sequence data has been proposed (Baizan-Edge *et al.*, 2019). K-mer profiles of sequences make alignment-free similarity analyses between sequences possibly that allow binning if sequences for further viral genome assembly. The disadvantage of these methods is that they require either fresh material, cell cultures, or material that has been very carefully preserved to keep single stranded RNA intact, conditions that hardly can be met with alcohol preserved material. More importantly, the known *Totiviridae* viruses in single-cell eukaryotic parasites do not seem to undergo constant replication but seem at a dormant or chronic state which means little in terms of replication intermediates and low copy number of viral genomes (Robinson & Beverley, 2018). As far as RNA interference is concerned, this is a case of hit and miss in the family *Trypanosomatidae*, see Figure 2.2. Loss can occur even within a genus, for example, The New World species *Leishmania braziliensis* has maintained RNA interference, whereas the Old World species *L. major* has lost RNAi.

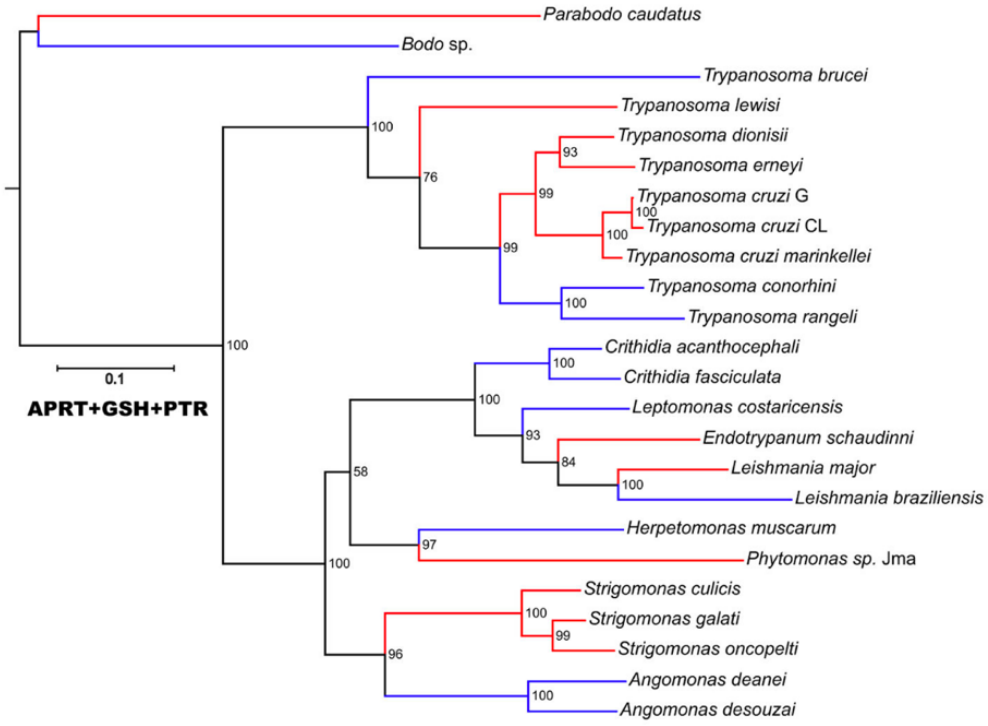


Figure 2.2: Frequent loss of RNA interference in lineages of parasites belonging to the Trypanosomatida. The tree is based on three catenated housekeeping genes: APRT: adenine phosphoribosyl transferase, GSH1: γ-glutamylcysteine synthetase, PTR1: pteridine reductase 1. Assuming the ancestral state is functional RNAi, lineages in blue have

remained RNAi proficient, lineages in red have lost functional RNAi. The clades leading to Leishmania braziliensis have lost RNAi twice (Matveyev et al., 2017).

Antibodies against nucleic acids are rare, in general (Hu *et al.*, 2014). Nucleic acids make poor immunogens. However, some autoimmune diseases such as systemic lupus erythematosus are characterised by high titres of autoantibodies against dsDNA (Ivanova *et al.*, 2014; Wang & Xia, 2019). Sera of systemic lupus erythematosus patients also have auto-antibodies against dsRNA in small amounts (Podgorodnichenko *et al.*, 1980).

A few attempts were made to generate sequence-independent antibodies against dsRNA, see table 2.2. In the table, only the first use of a method is recorded. Of these, four antibodies were commercialised. These are:

J2 recommended for dsRNA detection and quality control of in vitro transcribed (m)RNA using enzyme-linked immunosorbent assay (ELISA), Immunofluorescence staining in microscopy (IF), fluorescence-activated cell sorting (FACS), immunohistochemistry (IHC), immunoprecipitation (IP), dot blot assay, chromatin immunoprecipitation (ChIP), affinity purification, and immunoelectron microscopy

K1 recommended for Poly I:C detection using the methods above

K2 recommended as an IgM alternative for ELISA, sandwich-ELISA, IHC, and dot blot assay

J5 recommended for ELISA, flow cytometry, immunohistochemistry, fixed immunohistochemistry, immunoprecipitation, dot blot assay, electron microscopy, ChIP, affinity purification, dsRNA isolation; no longer commercially available

Using the antibody J2, the first dot blot assay for a totivirus in *Leishmania* was reported by Zangger *et al.* (2013). Zangger *et al.* (2013) used J2 for immunofluorescence, ELISA, and dot and slot blot assays for various strains of *Leishmania guyanensis* of known infection status.

Here I will be testing the suitability of this methodology for detecting the presence of dsRNA using dot blot assays.

| Name | Immunogen | Animal | Type | Reported reactivity | Source | Reference |
|---|---|---|---|---|---|---|
| Antibodies | | | | | | |
| Anti-dsRNA | poly (rA):poly (rU)<br>poly (rI):poly (rC) | rabbit | antisera | dsRNA | authors | Lacour *et al.* (1968) |
| 68-170a | poly (rA):poly (rU) | rabbit | antisera | dsRNA<br>less DNA:RNA<br>not ssDNA | David Stollar | Schwartz & Stollar (1969);<br>Son et al. (2015) |
| Anti-dsRNA | poly (rI):poly (rC) | rabbit | antisera | dsRNA<br>not DNA, ssRNA | authors | Francki & Jackson (1972) |
| 24-3 A8 | poly (rI):poly (rC) | mouse | monoclonal | dsRNA<br>not DNA, ssRNA | authors | Benhamou *et al.* (1987) |
| Anti-dsRNA | poly (rI):poly (rC) | guinea pig | polyclonal<br>IgG | dsRNA | authors | Mackenzie *et al.* (1996) |
| J2 | undisclosed dsRNA | mouse | monoclonal<br>IgG2a, kappa | dsRNA<br>not DNA, ssRNA, DNA:RNA | Scicons, Jena | Schönborn *et al.* (1991) |
| J5 | undisclosed dsRNA | mouse | monoclonal<br>IgG2b, kappa | dsRNA<br>not DNA, ssRNA, DNA:RNA | Scions, biolinks | Schönborn *et al.* (1991) |
| K1 | undisclosed dsRNA | mouse | monoclonal<br>IgG2a, kappa | dsRNA<br>not DNA, ssRNA, DNA:RNA | Scions, biolinks | Schönborn *et al.* (1991) |
| K2 | undisclosed dsRNA | mouse | monoclonal<br>IgM, kappa | dsRNA<br>not DNA, ssRNA, DNA:RNA | Scions, biolinks | Schönborn *et al.* (1991) |
| 9D5 | CBV-3(M) | mouse | ascitic fluid<br>IgG | dsRNA<br>not DNA, ssRNA | David Schnurr | Yagi *et al.* (1992); Son *et al.* (2015) |
| 2G4 | *Palm Creek virus* | mouse | monoclonal<br>IgM | dsRNA<br>not ssRNA, DNA:RNA | authors | O'Brien *et al.* (2015); Blouin *et al.* (2016) |
| 3G1 | *Palm Creek virus* | mouse | monoclonal<br>IgM | dsRNA<br>not ssRNA, DNA:RNA | authors | O'Brien *et al.* (2015); Blouin *et al.* (2016) |

| Name | Source | Reported reactivity | Source | Reference |
|---|---|---|---|---|
| Proteins | | | | |
| GST-DRB4 | *Arabidopsis thaliana* | dsRNA | authors | Kobayashi *et al.* (2009)<br>Atsumi *et al.* (2015) |
| IAV NS1 | *Influenza A virus* | dsRNA | authors | Cheng *et al.* (2015) |
| IAV B2 | *Influenza A virus* | dsRNA | authors | Cheng *et al.* (2015) |
| IAV VP35 | *Influenza A virus* | dsRNA | authors | Cheng *et al.* (2015) |
| MMV DRB | *Marburg marburgvirus* | dsRNA | authors | Cheng *et al.* (2015) |
| FHV B2 | *Flock House virus* | dsRNA | authors | Cheng *et al.* (2015)<br>Monsion *et al.* (2018) |

Table 2.1: List of antibodies and proteins developed against sequence-independent dsRNA. Undisclosed dsRNA means various synthetic homoplymeric RNAs of 4.3 kb size. Poly (rA) stands for polyriboadenylic acid; poly (rC): polyribocytidylic acid; poly (rI): polyriboinosinic acid; poly (rU): polyribouridylic acid; CBV-3(M): myocarditic strain of coxsackievirus B-3. David Schnurr: California State Department of Health, Richmond, CA; David Stollar: Rutgers, New Brunswick, NJ. Scions is trading as English and Scientific Consulting Kft. GST-DRB4: glutathione S-transferase (GST)-tagged dsRNA binding protein 4 (DRB4). Influenza A virus (Orthomyxoviridae, Articulavirales) is negative-sense ssRNA virus that encodes several dsRNA-binding proteins (DRBs). Marburg marburgvirus (Filoviridae, Mononegavirales) is negative-sense ssRNA virus. Flock House virus (Flaviviridae, Amarillovirales) is a positive-sense ssRNA virus of mosquitoes that replicates through a dsRNA intermediate. Flock House virus (Nodaviridae, Nodamuvirales) is a positive-sense ssRNA virus which encodes protein B2 that binds dsRNA and thereby inhibits the host's RNAi pathway. The table was inspired by Hu et al. (2014).

## *2.2* Materials and Methods

### *2.2.1 Samples*

Table 2.2 shows the samples for testing and control samples used in these assays.

| Virus Sample / Control Cells | Source / Control (positive or negative) |
|---|---|
| ***Virus Sample*** | |
| *Leishmania mexicana* | *L. mexicana* cultured cells obtained from Dr. Hamza and Dr. H. Price, Centre for Applied Entomology and Parasitology, School of Life Sciences, University Keele, United Kingdom. |
| *Leishmania donovani* | *L. donovani* cultured cells obtained from Dr. Hamza and Dr. H. Price, as above. |
| *Leishmania major* | *L. major* obtained from The Vector Infection Department, General Directorate of Health Affairs, Asir, Saudi Arabia. |
| ***Controls*** | |
| *Trichomonas vaginalis virus* | *T. vaginalis virus* (347) as infected T*richomonas vaginalis* obtained from Prof. M. Benchimol, The Federal University of Rio de Janeiro, University of Brazil. Positive Control. |
| Yeast | Yeast cells cultured at School of Natural Sciences, Bangor University, Bangor. Negative control. |
| *Schizosaccharomyces pombe (Yeast)* | *S. pombe* yeast obtained from Dr. Othman and Dr. Hussam Althagafi, North West Cancer Research Laboratory, Bangor University, Bangor. Negative control. |
| Human cancer cell K562 | Cultured Leukemia cell line NTERA2 cells line, obtained from Dr. Mishal Alsulami, North West Cancer Research Laboratory, Bangor University, Bangor. Negative control. |
| Human Blood sample | Human blood sample obtained from Dr. Mohammed Alshahrani, Braig laboratory, School of Natural Sciences, Bangor University, Bangor. Negative control. |
| *Sonchus yellow net virus* | *Sonchus yellow net virus* cultured (*Rhabdoviridae*, dsDNA), as infected lettuce. Negative control. |
| *Poinsettia latent virus* | *Poinsettia latent virus* (*Solemoviridae, ssDNA*) as infected *Euphorbia pulcherrima*). Negative control. |
| *Pseudomonas syringae phage phi6* | *Pseudomonas syringae phage phi6* (*Cystoviridae*, dsRNA) as infected *Pseudomonas syringae*. Positive control and to test sensitivity of the J2 primary antibody. |
| *Escherichia coli phage MS2* | *Escherichia coli phage MS2* (*Leviviridae*, ssRNA) as infected *Escherichia coli*. Negative control and to test sensitivity of the J2 primary antibody. |
| *Escherichia coli phage lambda* | *Escherichia coli phage lambda* (*Siphoviridae*, dsDNA) as infected *Escherichia coli*. Negative control and to test |

| Virus Sample / Control Cells | Source / Control (positive or negative) |
|---|---|
| | sensitivity of the J2 primary antibody. |

***RNA Extraction***

The successful isolation of intact RNA has four essential, generic steps:

h) effective disruption of cells or tissue;

i) denaturation of nucleoprotein complexes;

j) inactivation of endogenous ribonuclease (RNase) activity; and

k) removal of contaminating DNA and proteins.

The most important step is the immediate inactivation of endogenous RNases that are released from membrane-bound organelles upon cell disruption, commercially available RNA stabilising agents combined with cold storage were used to achieve this.

Three RNA extraction kits were used as follows.

*a) SV Total RNA Isolation System (Promega)*

SV Total RNA Isolation System is designed for cultured and white blood cells. It was used with 20 µL of the sample organisms: *Leishmania mexicana, Leishmania donovani* and *Leishmania major* and with 20 µL of controls: *Trichomonas vaginalis virus*, *Schizosaccharomyces pombe*, Yeast, human cancer cells, and human blood cells. Reagents used were as shown in Table 2.3, extraction was undertaken following the manufacturer's protocol as shown in Figure 2.3.

| Reagents | Volume (µl) |
|---|---|
| RNA Lysis Buffer (RLA) | 175 |
| RNA Dilution Buffer (RDA, blue) | 350 |
| Ethanol 95 % | 200 |
| RNA Wash Solution (RWA) | 600 |
| Yellow Core Buffer | 40 |
| $MnCl_2$, 0.09 M | 5 |
| DNase I | 5 |
| DNase Stop Solution (DSA) | 200 |
| RNA Wash Solution (RWA) | 600 |
| RNA Wash Solution (RWA) | 250 |
| Nuclease-Free Water | 100 |

Table 2.3 above:  Manufacturer's Reagents for the SV Total RNA Isolation System

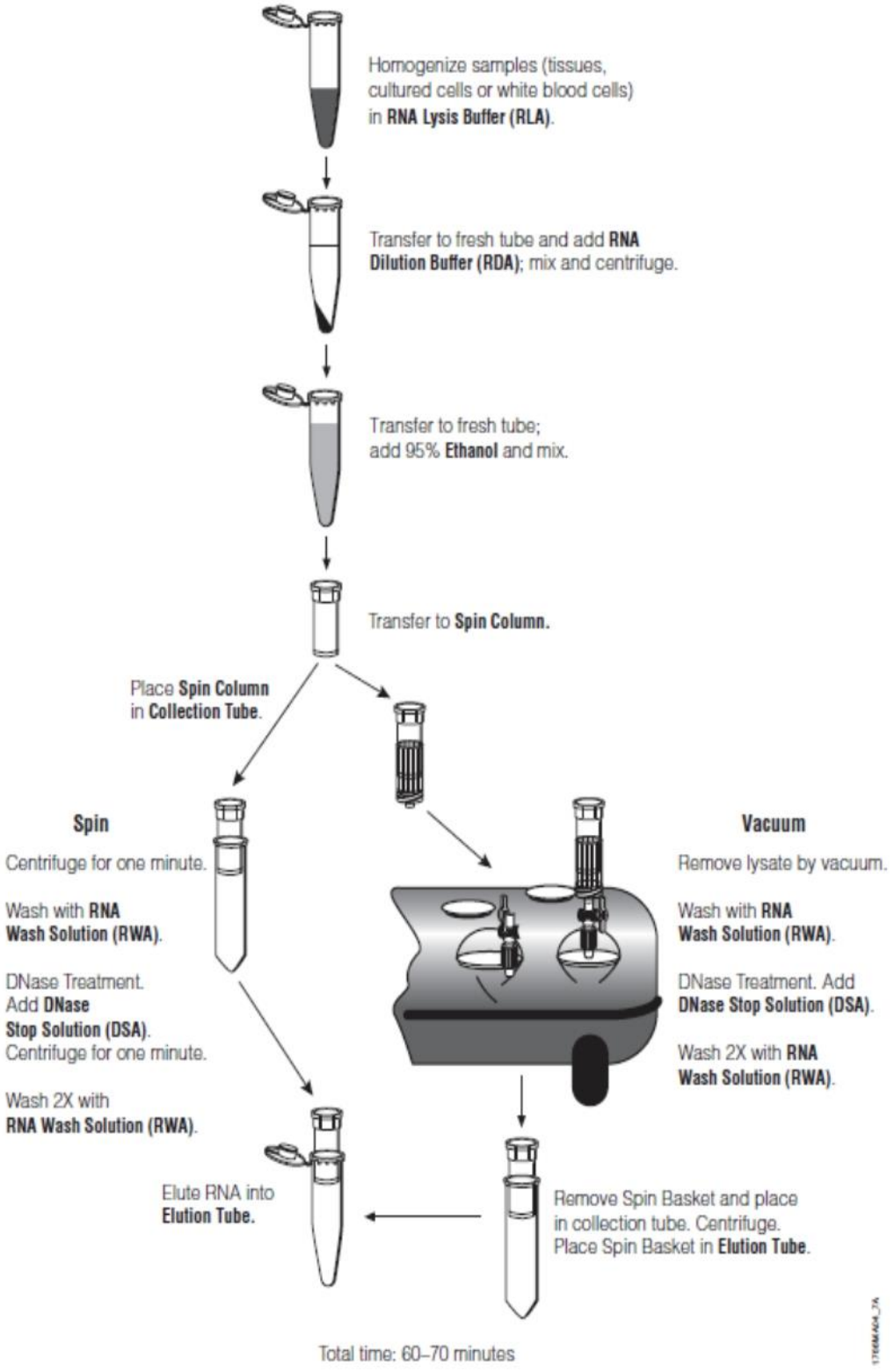


Figure 2.3: Figure 2.3:  SV Total RNA Isolation System, manufacturer's protocol. The 'spin path' was used.
Source: SV Total RNA Isolation System handbook.

*b) RNeasy Plus Micro (QIAGEN)*

The RNeasy Plus Micro Kit extracts and purifies up to 45 µg RNA, achieved by spinning cell or tissue lysates through gDNA Eliminator spin columns to remove the genomic DNA. All RNA molecules longer than 200 nucleotides are purified and most RNAs < 200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together make up 15 – 20 % of total RNA, are selectively omitted. The size distribution of the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently. It was used to extract *Sonchus yellow net virus* and *Poinsettia latent virus*. This kit was preferred for samples which yielded low volumes of RNA from SV Total RNA Isolation System extractions, especially *Leishmaniavirus* and *Trichomonasvirus*.

The manufacturer's protocol was followed for samples of *Leishmaniavirus* and *Trichomonasvirus* , as follows: a tissue sample of ≤ 5 mg was disrupted and homogenised using either TissueRuptor or TissueLyser. The lysate was centrifuged for 3 mins at maximum speed. Biological samples were first lysed and homogenised in a highly denaturing guanidine-isothiocyanate–containing buffer, to rapidly inactivate RNases. The lysate was then passed through a gDNA Eliminator spin column which removed genomic DNA. Ethanol was added to the flow-through to provide appropriate binding conditions for RNA and the sample was then transferred to a RNeasy MinElute spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA was then eluted in 14 µl of water. The supernatant was then processed according to the manufacturer's protocol. As shown in Figure 2.4, reagents used were as shown in Table 2.4

| **Reagents** | **Volume (µl)** |
|---|---|
| QIAzol | 5000 |
| gDNA Eliminator | 500 |
| Chloroform | 1000 |
| Ethanol 70 % | 3000 |
| Buffer RWT | 4000 |
| 2x Buffer RPE | 2500 |
| RNase-Free Water | 250 |

Table 2.4: manufacturer's protocol specification for RNeasy Plus Micro System

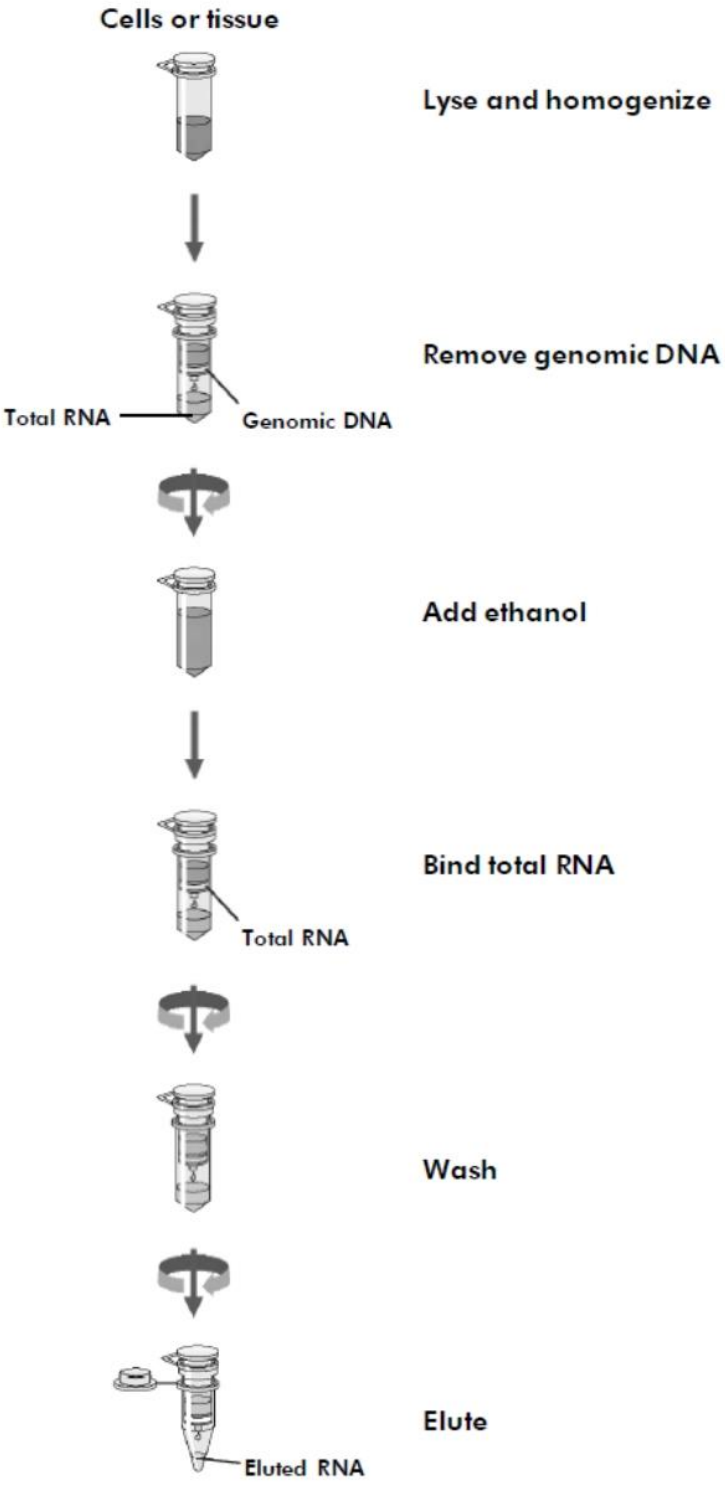


Figure 2.4: RNeasy Plus Micro, Manufacturer's Protocol.

Source: RNeasy Plus Universal Handbook, 2014.

*c) RNeasy Plus Universal Midi (QIAGEN)*

RNeasy Pus Universal Kit was used to extract *Pseudomonas syringe phage phi6* (dsRNA) and *Escherichia coli phage MS2* (ssRNA), both positive controls. This kit is designed to purify RNA from small amounts of a wide range of animal cells or tissues that are easy to lyse. Genomic DNA contamination is removed using a specially designed spin column. The purified extracted htis way is suited for downstream applications sensitive to low amounts of DNA contamination, such as quantitative RT-PCR. Extractions were performed following the manufacturer's specifications, as shown in Figure 2.5, reagents used, according to manufacturer's protocol, were as shown in Table 2.5.

| **Reagent** | **Volume (µl)** |
|---|---|
| RNA Lysis Buffer (RLA) | 350 |
| Ethanol 70 % | 350 |
| RNA Wash Solution (RW1) | 700 |
| Buffer RPE | 500 |
| Ethanol 80 % | 500 |
| RNase-Free Water | 14 |

Table 2.5: Manufacturer's Reagents for RNeasy Plus Universal Midi Kit

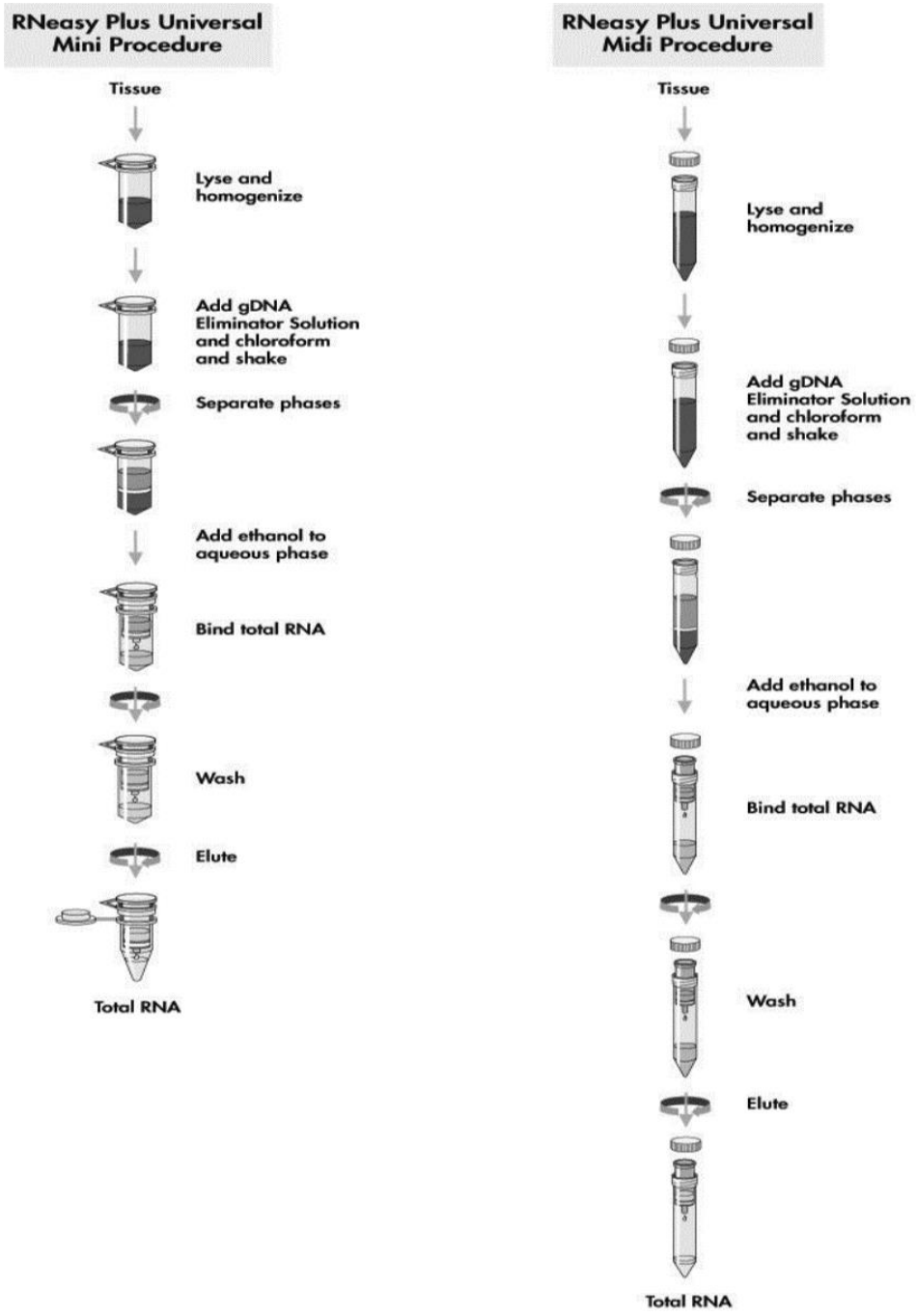


Figure 2.5: RNeasy Plus Universal Mini & Midi Kits, manufacturer's protocols.

Only the Midi kit was used.
Source: RNeasy Plus Universal Handbook, 2014

### *2.2.2 Dot Blot Manufacturer's Recommended Reagents and Protocols*

Dot blot manufacturer's procedures and reagents used are shown in Table 2.6.

| | |
|---|---|
| Reagents: | Tris-buffered saline, TBS: 20 mM Tris-HCl, 150 mM NaCl pH 8.0 TBS-T: 0.05 % Tween20 in TBS Blocking buffer, BSA/TBS-T: 0.1 % BSA in TBS-T or non-fat skimmed milk (see results) BCIP (5-bromo-4-chloro-3-indolyl-phosphate) in conjunction with NBT (nitro blue tetrazolium) for the colorimetric detection of alkaline phosphatase activity. Each vial of BCIP is supplied with a vial of NBT. |
| Membranes: | Two types of membranes were used, Nitrocellulose and polyvinylidene difluoride (PVDF) |
| PVDF Preparation: | Moisten in methanol for 1 – 3 seconds until the colour changes from an opaque white to a uniform translucent grey. Incubate in water for 1 or 2 minutes to elute methanol. Soak in transfer buffer for a few minutes to displace the water. The membrane is now ready for blotting. |
| Substrate Preparation: | Substrates are prepared to detect Alkaline Phosphatase as follows: 5 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.0, 150 mM $MgCl_2$) is added to 33 ml NBT (50 mg/ml) in 70 % dimethylformamide and 16.5 ml BCIP (50 mg/ml) in 100 % dimethylformamide. NBT is added mixed, BCIP is added and mixed again. Use within one hour and discard any unused solution. |
| Procedure: | Fit nitrocellulose or PVDF membrane in the rack. Spot 20 µl or 14 µl of samples onto the nitrocellulose or PVDF membrane at the centre of the grid. Allow to dry and incubate for 30 minutes at room temp. Add 20 µl from Block buffer like non-fat skimmed milk or 5 % BSA in TBS-T, then incubate for 30 minutes at RT (see results). Add 20 µl primary antibody J2, then incubate for 1 hour at room temp. Wash three times with TBS-T by adding 100 ml. Add 20 µl secondary antibody, then incubate for 30 min at room temp. Wash three times with TBS-T by adding 100ml. Colour the membrane with 33 µl NBT in RT first, and then with BCIP in RT, these components should be added in 5 ml alkaline phosphates buffer. |

Table 2.6: Manufacturer's Procedures and Reagents

### *2.2.3 Anti-dsRNA Antibody*

For details of the secondary antibody, please see lab book.

For concentrations of primary and secondary antibodies, please see lab book.

## *2.3* Results

Experiments were carried out to optimise dot blot assay conditions for detection of dsRNA viruses.

Zangger's *et al.*'s (2013) dot blot assay conditions were initially replicated. However, following initially unsuccessful results, dot blot conditions were varied, as shown in Figures 2.4 – 2.7 to find alternative conditions with higher levels of sensitivity and accuracy.

In these experiments, J2 was used as primary antibody and IgG as a secondary antibody. Bovine serum albumin has been used to block PVDF membranes, while fat-free skimmed milk has been used to block nitrocellulose membranes. Results are shown in Figure 2.6.


Figure 2.6: Dot blot, nitrocellulose membrane, milk blocking buffer.
Dots 1 and 8 positive controls (*Sonchus yellow net virus*); dots 2 to 7 water.

Results of the first dot blot test, Figure 2.6 show a signal for the positive controls, dots 1 and 8, but also difficult to interpret dots for the water samples. Consequently, the nitrocellulose membrane was swapped for a PVDF Membrane in subsequent tests.

In the second dot blot test, a PVDF membrane and bovine albumin serum-blocking buffer was used, as shown in Figure 2.7.

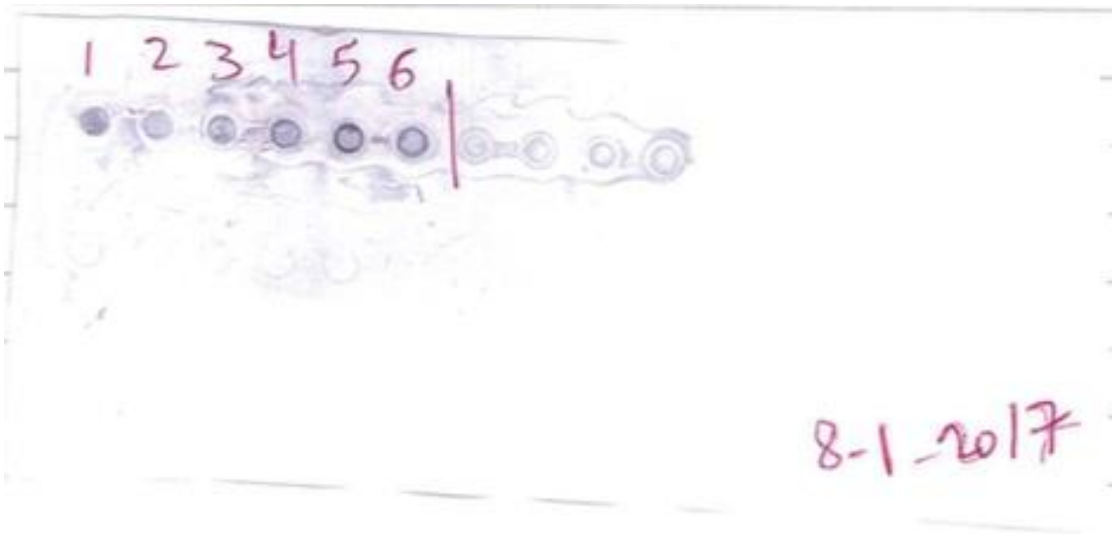


Figure 2.7: Dot blot PVDF membrane, bovine albumin serum-blocking buffer. Dots 1 and 4 positive controls, *Pseudomonas syringae phage phi6;* dots 2 and 5 negative controls (*Sonchus yellow net virus*); dots 3 and 6 water.

Results of the second dot blot test, Figure 2.7 show a signal for all four controls, positive and negative and water.

The third dot blot test was carried out to distinguish between positive and negative controls.


Figure 2.8: Dot blot, PVDF membrane, bovine serum albumin blocking buffer.

Dot 1 positive control, *Pseudomonas syringae phage phi6* (dsDNA); dot 2 negative control, *Poinsettia latent virus* (ssRNA); dot 3 water.

Results of the third dot blot test, Figure 2.8 show a signal for both positive and negative controls, but not water.

In the fourth assay, serial dilutions were spotted on a PVDF membrane using *Pseudomonas syringae phage phi6* (dsRNA), *Escherichia coli phage MS2* (ssRNA), and *Escherichia coli phage lambda* (dsDNA). Figure 2.9.

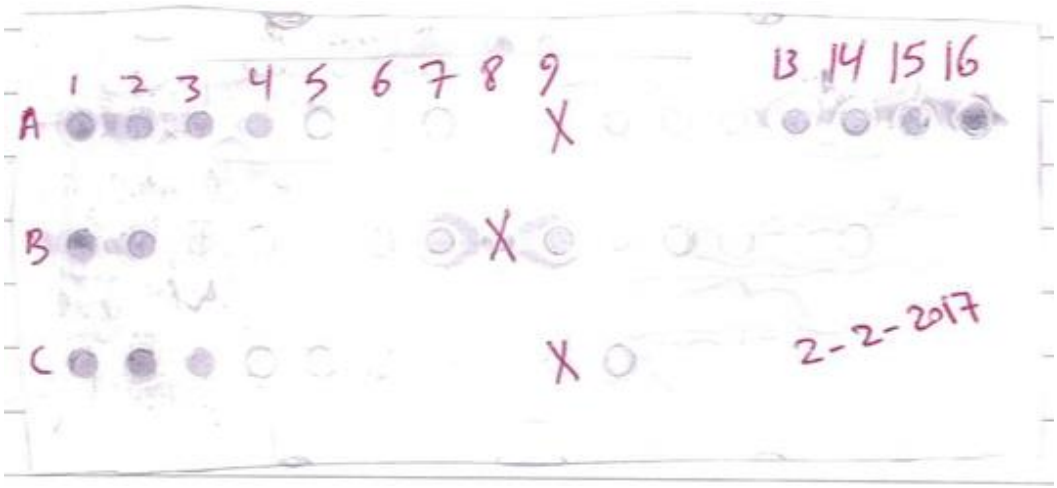


Figure 2.9: Dot blot, serial dilutions on a PVDF membrane.

Row A - *Pseudomonas syringae phage phi6*; Row B - *Escherichia coli phage MS2*; Row C - *Escherichia coli phage lambda.*

The results of the fourth dot blot test, Figure 2.9 show too many samples with positive signal. When dots should show signal for dsRNA virus (Row A) but not for negative controls (Rows B, C) ssRNA *Escherichia coli phage MS2*) and dsDNA *Escherichia coli phage lambda*), however, they actually show signal for both. Positive control samples were blotted in Row A dots 15 and 16 using dsRNA *Pseudomonas syringae phage phi6* and negative control samples were blotted negative in dots 13 and 14 ssRNA *Poinsettia latent virus*, however they all show positive signal.

## *2.4* Discussion

Anti-dsRNA antibodies do not work well for the *de novo* detection of dsRNA viruses in dot and slot blots. In this experiment, the reason is that the J2 antibody is not sensitive enough and the operational window in which the antibody is highly selective, is too narrow to be useful for exploratory dot blot assays. This does not come as a complete surprise. In the original publication advocating this method for field testing of totiviruses, the success rate of this assay under ideal conditions was only 20 %, or the failure rate under ideal conditions was 80 %, see Figure 2.10.

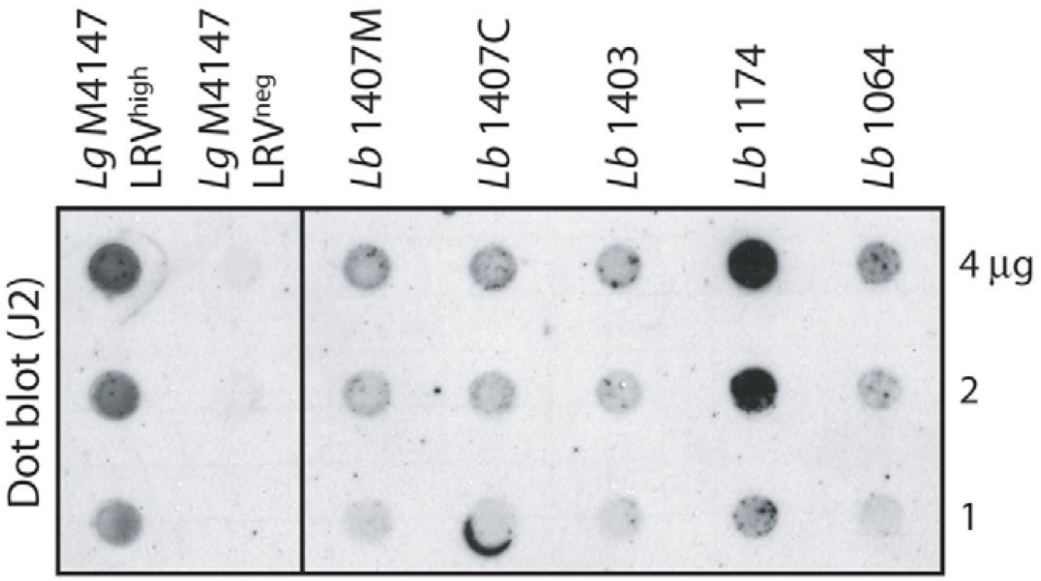


Figure 2.10: Only 1 in 5 dot blots for detecting the presence of dsRNA viruses work with real life samples at any concentration using antibody J2. On the far left, as positive control a highly virus-infected strain of human *Leishmania guyanensis* and next to it a non-infected strain as a negative control. On the right side, five virus-infected strains of human *L. braziliensis*. Between 1 and 4 µg of total protein were loaded per spot. Dot blot picture from Zangger *et al.* (2013).

What is surprising in the dot blot assay of Zangger *et al.* (2013) is the absence of a clear lysis step that will expose the dsRNA of the virus to the antibody. Here, three different RNA extraction methods were explored. The current method for detecting totiviruses in *Leishmania* and *Trichomonas* samples is by PCR (Margarita *et al.*, 2019; Abtahi *et al.*, 2020; Kariyawasam *et al.*, 2020; Parra-Muñoz *et al.*, 2021). The Fasel lab that originally promoted the J2 antibody method as the sequence-independent method to use for searching for viruses, is now recommending an agarose gel-based method without antibody application (Isorce & Fasel, 2020). This new method depends on culturing *Leishmania* for 10 days, and it starts with 5 ml live culture. It relies on culturing so much parasite that the viral dsRNA will become visible with the fluorescent dye Sybr green on a transilluminator; it is therefore of very little practical use.

Although the J2 antibody is commercialised as a sequence-independent antibody, studies using scanning force microscopy and site-directed mutagenesis showed that the antibody preferentially binds the ends of dsRNA molecules. Where the antibody binds to internal sites of dsRNA, it recognises adenosine residues on one side of the RNA double helix. This reveals a sequence specificity of the antibody as 5'-**AA**NNNNNNNNN**AAA**NNNNNNNNN**AA**-3' where N is any of the four nucleosides including A (Bonin *et al.*, 2000).

The group of monoclonal anti-dsRNA antibodies to which J2 belongs has been applied to the study of a large diversity of viruses. A literature review led to the identification of 156 different viruses subjected to one of these antibodies. These viruses are not, as expected, in a majority, dsRNA viruses but viruses with a positive-sense ssRNA genome (Table 2.7).

ssDNA viruses 1 virus

*Piccovirales* 1 virus

Minute Virus of Mice (MVM)

dsDNA viruses 14 different viruses

*Blubervirales* 1 virus

Hepatitis B Virus (HBV)

*Chitovirales* 4 viruses

Ectromelia (ECTV), Monkeypox Virus (MPXV), Myxoma virus (MYXV)

*Herpesvirales* 6 viruses

Cytomegalovirus (CMV), Herpes Simplex Virus (HSV), Kaposi's sarcoma associated herpesvirus (KSHV)

*Pimascovirales* 2 viruses

Frog Virus 3 (FV3), Tiger frog virus (TFV)

*Rowavirales* 1 virus

Adenovirus (AdV)

positive-sense ssRNA viruses 100 different viruses

*Amarillovirales* 19 viruses

Bovine Viral Diarrhea Virus (BVDV), Cell Fusing Agent Virus, Classical Swine Fever Virus (CSFV)

*Hepelivirales* 4 viruses

Beet Necrotic Yellow Vein Virus (BNYVV), Cutthroat trout virus (CTV), Hepatitis E Virus (HEV)

*Martellivirales* 16 viruses

Aura Virus (AURAV), Barley Stripe Mosaic Virus (BSMV), Barmah Forest Virus (BFV)

*Nidovirales* 20 viruses

Berne Virus, Coronavirus, Equine Arteritis Virus (EAV)

*Nodamuvirales* 2 viruses

Flock House Virus (FHV), Orange-spotted grouper nervous necrosis virus (OGNNV)

*Ortervirales* 5 viruses

Human Immunodeficiency Virus (HIV), Simian Immunodeficiency Virus (SIV)

*Patatavirales* 1 virus

Turnip Mosaic Virus (TuMV)

*Picornavirales* 22 viruses

Aichi Virus (AiV), Coxsackievirus A (CVA), Cricket Paralysis Virus (CrPV)

*Stellavirales* 2 viruses

Human Astrovirus (HAStV), Murine Astrovirus (MuAstV)

*Tolivirales* 7 viruses

Carnation Italian Ringspot Virus (CIRV), Groundnut Rosetta Virus (GRV), Providence Virus (PrV)

*Tymovirales* 1 virus

Potato virus X

*Wolframvirales* 1 virus

Leptomonas seymouri Narna-like virus 1 (NLV1)

negative-sense and ambisense ssRNA viruses 27 different viruses

*Articulavirales* 1 virus

Influenza A Virus (IAV)

*Bunyavirales* 15 viruses

Akabane Virus (AKAV), Bunyamwera Virus (BUNV), Dobrava Virus (DOBV)

*Mononegavirales* 11 viruses

Ebola Virus (EBOV), Human Parainfluenza Virus (HPIV), Measles Virus (MV)

dsRNA viruses 14 different viruses

*Birnaviridae* 3 viruses
Culex Y Virus, Infectious Bursal Disease Virus (IBDV), Infectious Pancreatic Necrosis Virus (IPNV)
*Durnavirales* 2 viruses
Beet Cryptic Virus 1 (BCV1), Beet Cryptic Virus 2 (BCV2)
*Ghabrivirales* 2 viruses
Leishmania dsRNA Virus (LRV), Trichomonasvirus (TVV)
*Reovirales* 7 viruses
Avian Orthoreovirus (ARV), Avian Reovirus (ARV), Blue Tongue Virus (BTV), Chum Salmon Reovirus (CSV), Piscine Orthoreovirus (PRV), Pteropine orthoreovirus 3 (PRV3M), Rotavirus (RV)

Table 2.7: Overview of taxonomic distribution of viruses investigated with anti-dsRNA antibodies. A total of 156 different viruses were identified. For non-dsRNA viruses, viruses were grouped into orders and only three examples given for each virus order. Vernacular names of the viruses were used for the examples. *Birnaviridae* has not yet been assigned to a viral order. For dsRNA, all available examples are listed. There are only two examples among the *Totiviridae* in the order *Ghabrivirales*.

The practical importance of anti-dsRNA antibodies like J2 lies in their use in cell biology, especially the unravelling of the various intermediate steps in viral replication. Initially, dsRNA intermediates were discovered in the replication of positive-strand ssRNA viruses as well as ssDNA and dsDNA viruses. However, dsRNA intermediates in the replication of negative-sense ssRNA viruses were beyond detection at that time (Weber *et al.*, 2006). Many negative-sense ssRNA viruses employ elaborative strategies to cover their dsRNA intermediates during replication with viral nucleoproteins to prevent detection (Ivanov *et al.*, 2011; Guu *et al.*, 2012). Later, using immunofluorescence, dsRNA intermediates were also confirmed for the replication of negative-sense ssRNA viruses in addition to an ambisense ssRNA virus, lymphocytic choriomeningitis virus (*Arenaviridae*, *Bunyavirales*) (Son *et al.*, 2015). The same authors compared the antibody J2 to another monoclonal antibody, 9D5, and to a polyclonal serum, 68-170A; for details, see Table 2.1. The J2 antibody was less sensitive than the 9D5 antibody and also suffered from more background staining. Anti-dsRNA antibodies aimed at dsRNA intermediates are now termed Monoclonal Antibodies to Viral RNA Intermediates in Cells: MAV–RIC (O'Brien *et al.*, 2015). Anti-dsRNA antibodies can have an important place in virus discovery by immunofluorescence and immunohistochemistry in archival tissue samples of, for example, tissues of the nervous system or the pancreas (Richardson *et al.*, 2010; Son *et al.*, 2015; Poynter & DeWitte-Orr, 2017; Richardson & Morgan, 2018).

dsRNA molecules are one of the key viral products by which the innate immune system recognizes viral infections using pathogen-associated molecular pattern (PAMP) receptors and protein kinase R (PKR) for dsRNA and leading to the production of type I interferons (Hur, 2019; Kim *et al.*, 2019;

Vaughn *et al.*, 2021). dsRNA might then also induce virus-specific RNAi (Schuster *et al.*, 2019; Das & Sherif, 2020). More and more roles are being discovered for dsRNA in the pathogenesis of viral infections (McGarry *et al.*, 2021; Vaughn *et al.*, 2021). Long dsRNA is not limited to viruses, cellular dsRNA is prolifically expressed in animal tissues. Most dominant of cellular dsRNA are introns and the double-stranded part of 3 prime untranslated regions of mRNA, now recognized as the cellular dsRNAome (Reich & Bass, 2019). Considerable amounts of long dsRNA resulting from transcripts from opposite strands of the mitochondrial genome have been detected in virus discovery experiments (Decker *et al.*, 2019).

While dot and slot blots do not work well in dsRNA virus discovery, the use of anti-dsRNA antibodies in various other techniques has been proposed. The simplest approach is using anti-dsRNA antibodies in immunoprecipitation or immunocapture of dsRNA. Protein L magnetic beads are coated with the anti-dsRNA antibodies to saturation for the capture of the dsRNA from a cleared homogenate (Blouin *et al.*, 2016). The amount of antibody needed in this procedure can be prohibitive. The same procedure, rebranded as dsRNA-Seq, has recently been used by Decker *et al.* (2019) using antibody J2 pre-bound to Protein A beads, see Figure 2.11.

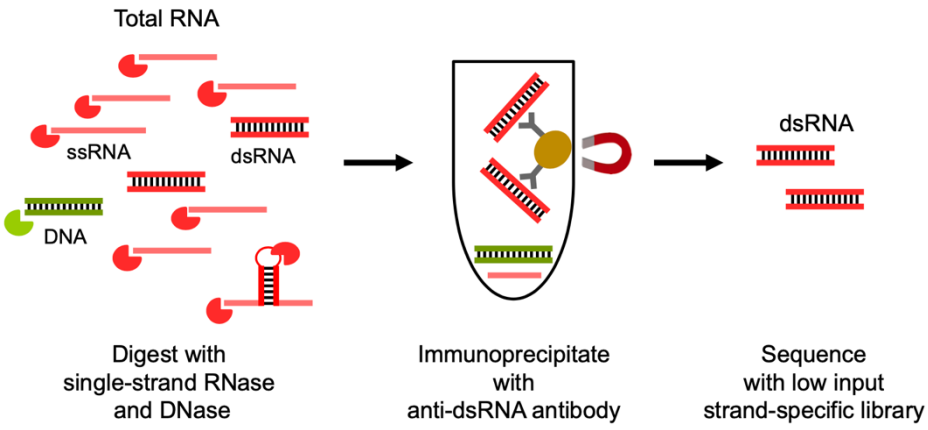


Figure 2.11: Immunocapture of dsRNA. Figure from Decker *et al.* (2019).

Ku *et al.* (2020) replaced the magnetic beads with a chemically modified surface to immobilize the anti-dsRNA antibodies. Although this might increase the sensitivity in a sandwich configuration, it does not improve the recovery of bound dsRNA.

Instead of antibodies, recombinant plant proteins binding dsRNA naturally have been proposed for the purification of viral dsRNA such as the glutathione S-transferase (GST)-tagged dsRNA binding protein 4 (DRB4) from thale cress; however, this is not commercially available (Kobayashi *et al.*, 2009; Atsumi *et al.*, 2015). Now this very procedure has seen a reincarnation as DECS: DsRNA isolation, Exhaustive amplification, Cloning and Sequencing analysis (Fujisaki *et al.*, 2018). The

very limited availability of the dsRNA-binding protein prevents its full characterization and a more widespread use of this technique.

Hunting for dsRNA viruses has the advantage that separating DNA from RNA is often just a question of increasing the acidity of a silicon spin column so that DNA no longer binds but RNA still does (Yang *et al.*, 2017; Shi *et al.*, 2018). This total RNA contains around 80 – 90 % ribosomal RNAs (cytoplasmic and mitochondrial), 10 – 15 % transfer RNAs, 3 – 10 % messenger RNAs, followed by cyclic RNAs, small nuclear RNAs, small nucleolar RNAs, micro RNAs, small interfering RNAs, piwi-interacting RNAs, and long noncoding RNAs, depending on whether or not cells are replicating. In mammalian tissue, only 0.01 – 0.1 % of total RNA is dsRNA. In chronic infections, the amount of viral dsRNA is very low as well. mRNA is easily removed through digestion with bovine pancreatic ribonuclease A (RNase A) or oligo (dT) cellulose column affinity chromatography. Ribosomal DNA can be removed with the help of rRNA deletion kits (Herbert *et al.*, 2018). These kits are available only for a small number of mammalian species, but none is available for invertebrate, protist or parasite species. Nevertheless, for a while rRNA depletion kits were used off-label. For example, Invitrogen's original RiboMinus Eukaryote Kit, developed with human ribosomes in mind, was successfully used for ribosome depletion from trypanosomes (Fadda *et al.*, 2013; Fadda *et al.*, 2014). The manufacture has changed the kits and no longer recommends the use for trypanosomes (Kraus *et al.*, 2019). Species-specific removal is possible but may be elaborate if a large number of host species is to be surveyed (Kraus *et al.*, 2019; Thompson *et al.*, 2020; Phelps *et al.*, 2021).

A great number of dsRNA viruses have been discovered in plants and in fungi (mycoviruses) infecting plants. Most of these have been detected with a rather straightforward method. One of the simplest methods to extract and concentrate viral dsRNA was developed by Morris & Dodds (1979). It uses chromatography or batch purification with CF-11 cellulose powder under 15 % ethanol. The cellulose was available from Whatmann or BioRad (Cellex N-1). Unfortunately, Whatmann stopped producing CF-11 cellulose and BioRad stopped offering Cellex N-1. This led to a lot of confusion, and the use of cellulose columns was interrupted. At that time, rumours circulated that Sigma Type 101 cellulose might be an alternative, but it was also unavailable for many years. Then Japanese researches started using cellulose powder D from Advantec, Japan (Okada *et al.*, 2015; Urayama *et al.*, 2015). Now, Sigma-Aldrich's (Merck) cellulose powder S6288 applied to microcentrifuge spin columns (NucleoSpin Filters, Macherey-Nagel) under 16 % (v/v) ethanol at a pH of 7.2 in a buffer composed of 10 mM HEPES, 0.1 mM EDTA, 125 mM sodium chloride (Baiersdörfer *et al.*, 2019).

Finally, to obtain sequences from terminal regions of viral dsRNA to assemble complete genomes, a method called Fragmented and primer-Ligated DsRNA Sequencing (FLDS) has been put forward (Urayama *et al.*, 2016; Urayama *et al.*, 2020).

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# *Chapter 3:* *LEISHMANIAVIRUS*

## *3.1* Introduction

According to World Health Organization figures, leishmaniasis is a group of vector-borne parasitic diseases endemic to South-East Asia, North and East Africa, the Americas, and the Eastern Mediterranean region. Worldwide, 700,000 to 1 million new cases of cutaneous leishmaniasis and 30,000 new cases of visceral leishmaniasis occur per year, but there are about one 350 million to 1 billion more individuals at risk of infection in around 93 countries; it causes 20,000 to 30,000 deaths each year due to visceral leishmaniasis (Alvar *et al.*, 2012; Curtin & Aronson, 2021; Mann *et al.*, 2021; Sasidharan & Saudagar, 2021).

Leishmaniasis is caused by an intracellular parasite transmitted by the bite of female sandflies of more than 90 species of the genera *Lutzomyia* and *Phlebotomus.* Here I adhere to the entomological rule that flies that belong to the order of true flies with only two wings, the Diptera, should be written in two words, such as sandflies , fruit flies, vinegar flies, bluebottle flies, bot flies, hover flies, robber flies, or tsetse flies; whereas flies with four wings belonging to different orders should be written as one word, such as mayflies or upwingflies (Ephemeroptera), damselflies or dragonflies (Odonata), whiteflies (Hemiptera), scorpionflies or hangingflies (Mecoptera), butterflies (Lepidoptera), caddisflies or sedgeflies (Trichoptera), sawflies or fairyflies (Hymenoptera), snakeflies (Raphidioptera), alderflies or dobsonflies or fishflies (Megaloptera), or owlflies (Neuroptera).

Leishmniasis is caused by around 22 distinct species or pathogenic types of flagellated unicellular protozoa of the genus *Leishmania*, although some of them have a contested taxonomic status. There are three main types of clinical disease due to *Leishmania*: cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis. Disease presentation relies on the *Leishmania* species involved and the host's immune response. Symptoms range from cutaneous reaction to the visceral form with potentially lethal effects. Asymptomatic cases are known to occur in endemic areas and may serve as effective reinfection reservoirs (Singh *et al.,* 2014). The parasite has two different transmission cycles: zoonotic, which includes dogs, which are, in addition to other animals, a particularly significant animal reservoir, and strictly human-driven, more common in densely inhabited areas of cities (Fiebig *et al.*, 2015; Bates, 2018; Burza *et al.*, 2018; Serafim *et al.*, 2018; Antonia & Ko, 2020).

The true burden of the disease may be higher than official WHO figures, particularly if the social-stigma and ostracization effects associated with certain clinical manifestations sufficiently affect reliable epidemiological data in a large number of endemic countries (Alvar *et al.,* 2012). In addition, HIV-*Leishmania* coinfection is a major complicating form of disease that has been systematically underreported in many endemic areas (WHO, 2007) and for which poor clinical guidelines have been

established (Diro *et al.,* 2015). Opportunistic infection with *Leishmania* is an AIDS-defining illness in endemic settings, and the immunosuppressive effects of the parasitic infection are compounded by HIV infection, often with irremediable consequences for the patient. Leishmaniasis is widely considered to be the second biggest parasitic killer after malaria, and it is thought that global warming, anthropogenic environmental changes and human migrations have led to an expansion of its geographic range (Desjeux, 2004).

Visceral leishmaniasis (VL) is a chronic disease caused by infection of the liver, spleen, and bone marrow by the parasite; also known as kala-azar, black fever, or dumdum fever. Untreated, within two years of the presentation of signs the disease, it is almost inevitably lethal. About 20 000 to 60,000 cases of VL occur annually, 90 % of which are concentrated in 6 countries, according to the WHO Global Health Observatory (GHO): Bangladesh, India, Ethiopia, Sudan, South Sudan and Brazil. An estimated 20 000 to 30 000 deaths per year are caused by VL, although the true number could be higher in many active transmission areas, due to inadequate epidemiological surveillance. A serious complication that may occur in cases of VL following a cure is post kala-azar dermal leishmaniasis (PKDL), a chronic syndrome characterised by the appearance, most notably on the face, of a multitude of papules, nodes, and patches, which require prolonged chemotherapy. In the Indian subcontinent, PKDL is rare, appears several years after successful therapy, and is particularly hard to treat. Conversely, in East Africa, PKDL is more frequent, is noted a few months after initial therapy for VL, and often resolves spontaneously. Patients with PKDL may act as important reservoir hosts (Bi *et al.*, 2018; Asfaram *et al.*, 2019; Abuzaid *et al.*, 2020; Guedes *et al.*, 2020).

Mucocutaneous leishmaniasis (MCL), also known as espundia, is a disfiguring illness usually spreading from the initial infection site to the mucosal membranes of the body, primarily around the nose and mouth, as a metastatic dissemination of the parasite. Timely diagnosis is frequently lacking resulting in total destruction of oropharyngeal tissues. Almost 90 % of all MCL cases occur in Bolivia, Brazil, and Peru (Handler *et al.*, 2015; Pinart *et al.*, 2020; Suqati *et al.*, 2020).

The disease is caused by *Leishmania* of the New World species group in the *Leishmania* subgenus Viannia, namely *L. braziliensis, L. panamensis. L guyanensis,* and, rarely*, L. amazonensis.* The first signs of *L. amazonensis* may not be identified for several months, or even years after primary skin lesion healing (e.g. recurrent nosebleeds). The pathogenesis of this form of the disease is poorly known and frequently related to the inability to treat skin damage properly (Martinez & Petersen, 2014; Aoki *et al.*, 2019).

The most common type of disease is the cutaneous leishmaniasis (CL) and is caused by both Leishmania New World and Old World species. The disease is manifested as ulcerative lesions that can slowly develop and fail to cure spontaneously in the sandfly bite. Such lesions are also associated with social stigma and handicaps in the vulnerable parts of the body such as the neck. These lesions can be very painful if contaminated with bacteria. Injury is often followed by

lymphadenopathy. Even if the disease is localised, numerous satellite lesions often occur and can last for months or years. The obvious marks that remain evident for life are not removed in

the positive treatment (Gurel *et al.*, 2020; Mohammadbeigi *et al.*, 2021). According to the World Health Observatory, about 700,000 to 1 million new cases of CL occur every year around the world as shown in Figure 3.1.

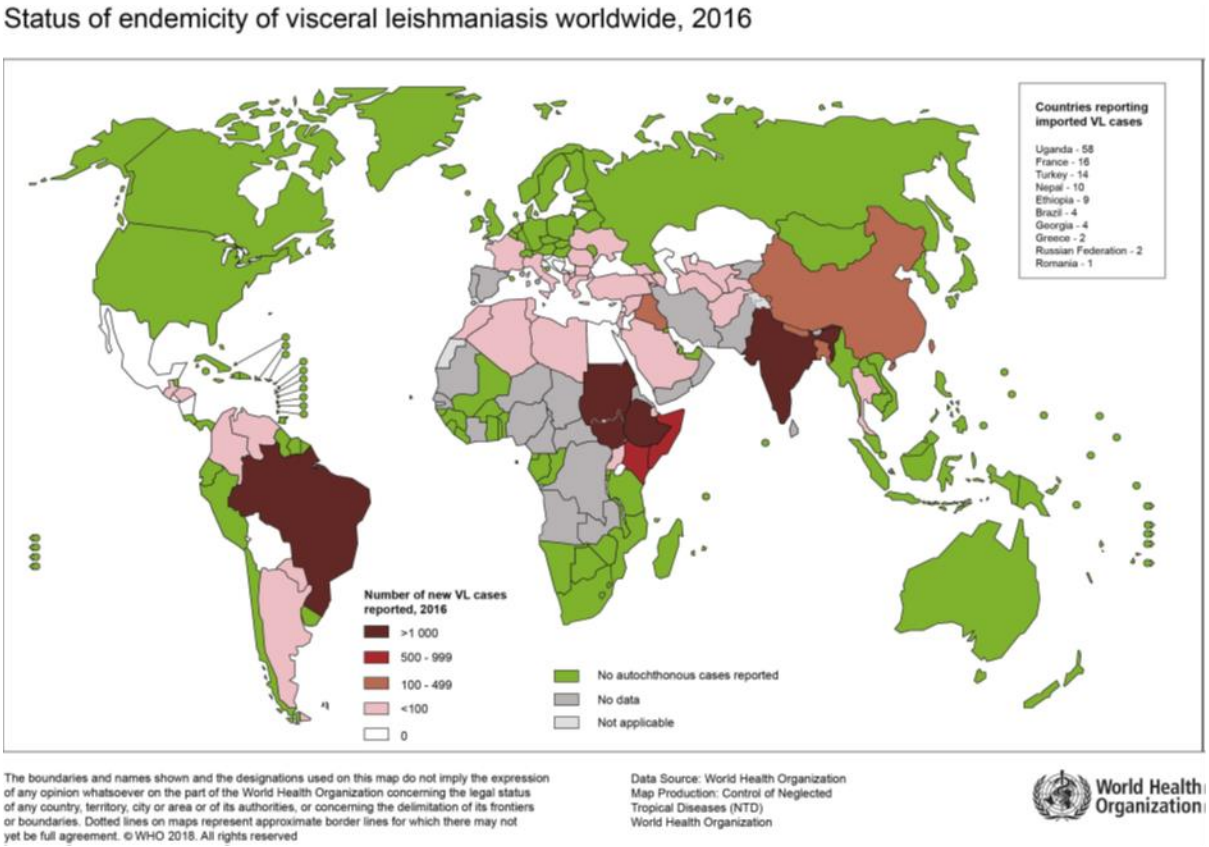



Figure 3.1: Distribution and endemicity of visceral leishmaniasis (VL) Distribution and endemicity of visceral leishmaniasis (VL) according to 2016 annual country reports. Countries in grey have no reliable epidemiological data or do not report disease incidence to the WHO Neglected Tropical Diseases (NTD) section. Countries in green had no autochthonous cases of VL reported in 2016 (Source: WHO Global Health Observatory).

This parasitic disease is one of the most neglected in the developing world and is mostly epidemic in areas with inadequate sanitation, limited access to medical care, and weakened health services due to war or social unrest (Beyrer *et al.,* 2007). A promising leishmaniasis elimination campaign has been championed by WHO since 2005 in Bangladesh, India, and Nepal, with the objective of reducing the incidence of VL to one case per 10 000 at the district or sub-district level by 2015. There were around 20 cases per 10 000 in the region in 2011, and the campaign has been making remarkable progress. The elimination of VL in this region is made achievable by the presence of a single sandfly vector species that is susceptible to insecticides, the distribution of cases in geographic clusters, and the fact that humans are the only reservoirs of infection in

that part of the country. The presence of asymptomatic carriers could complicate complete elimination in the region. Large scale control and global elimination of all clinical forms of disease associated with *Leishmania* infection is poised to be a significant challenge given the remarkable differences observed in clinical presentation in different patient populations, the presence of zoonotic reservoirs, and the number of different parasite species causing significant disease. An improved understanding of disease pathogenesis and the transmissibility of the parasite in each clinical presentation can inform prioritisation of different elimination strategies, as would the presence of an effective vaccine, the development of point of care diagnostics, and an affordable, easy to administer oral formulation for drug therapy (Matlashewski *et al.,* 2014).

Currently, leishmaniasis is treated with a variety of remedies. These remedies range from first line pentavalent antimony compounds, which are poorly tolerated in patients and to which many circulating parasite strains have developed resistance, to different regimens of amphotericin B, paromomycin, fluconazole, and the promising oral drug miltefosine, which recently received regulatory approval for use in India and the United States. No human vaccine is available, although there are several candidates in preclinical and clinical stages. The only truly successful way to achieve CL immunity is by the ancient method of "leishmanization" through the inoculation of live parasites in the skin in a cosmetically appropriate area of the human body. The fact that people who recover from VL may be immune to reinfection indicates that vaccines are able to gain tolerance to symptomatic visceral diseases as shown in (Table 3.1) (Costa *et al.,* 2011).

Table 3.1: *Leishmania* Species and Disruptions by Country and Vector with Disease Clinical Forms. Abbreviations. ACL: Anthroponotic Cutaneous Leishmaniasis; CL: Cutaneous Leishmaniasis; DCL: Diffuse Cutaneous Leishmaniasis; MCL: Mucocutaneous Leishmaniasis; VL: Visceral Leishmaniasis; ZCL: Zoonotic Leishmaniasis. L – Leishmania, Lu – Lutzomyia, P - Phlebotomus

| **Species name** | **Clinical form** | **Vector** | **Country of infection** |
|---|---|---|---|
| *'Ghana strain'* | CL | Unknown | Ghana |
| *'L. siamensis'* | CL, VL | Unknown | Germany, Myanmar, Switzerland, Thailand, and United States of America |
| *L. aethiopica* | CL, DCL | *P. sergenti, P. longipes, P. pedifer,* and *P. aculeatus* | Ethiopia, and Kenya |
| *L. amazonensis* | CL, DCL, MCL | *Lu. longipalpis, Lu. flaviscutellata, Lu. youngi, and Lu. reducta* | Argentina, Peru, Brazil, Bolivia, Colombia, Ecuador, French Guiana, Surinam, and Venezuela |
| *L. braziliensis* | CL, MCL | *Lu. Longipalpis , Lu. whitmani, Lu. migonei, and Lu. nuneztovari anglesi* | Argentina, Peru, Brazil, Bolivia, Colombia, Ecuador, French Guiana, Surinam, Mexico, Costa Rica, Honduras, and Venezuela |
| *L. colombiensis* | CL, VL | *Lu. gomezi, Lu. panamensis, and Lu. hartmanni* | Panama, Colombia, and Venezuela |
| *L. donovani* | VL, PKDL | *P. argentipes, P. alexandri, P. orientalis, and P. martini* | Cyprus, Saudi Arabia, Yemen, Turkey, Iraq, and Sudan |
| *L. guyanensis* | CL, MCL | *Lu. whitmani, Lu. shawi, Lu. anduzei, and Lu. umbratilis* | Argentina, Peru, Brazil, Bolivia, Colombia, Ecuador, French Guiana, and Ecuador |
| *L. infantum* | CL, VL | *P. neglectus, P. perfiliewi, P. tobbi, P. longicuspis, P. perniciosus, and Lu. longipalpis* | Afghanistan, Pakistan, Bosnia, Bulgaria, Central African Republic, Democratic Republic of the Congo, Jordan, Saudi Arabia, Greece, Italy, Romania, Tunisia, and Brazil |
| *L. lainsoni* | CL | *Lu. nuneztovari anglesi,* and *Lu. ubiquitalis* | French Guiana, Peru, and Brazil |
| *L. lindenbergi* | CL | Unknown | Brazil |
| *L. major* | ZCL | *P. papatasi, P. bergeroti,* and *P. caucasius* | Albania, Algeria, Arab Gulf countries, Egypt, India, Sudan, Morocco, Turkmenistan, and Uzbekistan |
| *L. martiquinensis* | CL, VL | Unknown | Thailand |
| *L. mexicana* | CL, DCL | *Lu. olmeca olmeca, Lu. flaviscutellata, Lu. colombiana,* and *Lu. shannoni* | Mexico, Venezuela, United States of America, Ecuador and Colombia |
| *L. naiffi* | CL | *Lu. ayrozai* and *Lu. squamiventris* | French Guiana and Brazil |
| *L. panamensis* | CL, MCL | *Lu. trapidoi, Lu. gomezi,* and *Lu.* | Panama, Costa Rica, and Honduras |

| Species name | Clinical form | Vector | Country of infection |
|---|---|---|---|
| | | *panamensis* | |
| *L. peruviana* | CL, MCL | *Lu. ayacuchensis, Lu. peruensis,* and *Lu. verrucarum* | Peru |
| *L. shawi* | CL | *Lu. whitmani* | Brazil |
| *L. tropica* | VL, ACL | *P. sergenti, P. saeveus, P. arabicus,* and *P. guggisbergi* | Saudi Arabia, Yemen, Turkmenistan, Uzbekistan, Turkey, and Iraq |
| *L. venezuelensis* | CL | *Lu. olmeca bicolor* | Venezuela |
| *L. waltoni* | DCL | Unknown | Dominican Republic |
| Unknown | Unknown | *P. kiangsuensis* | Taiwan |

### *3.1.1 The biology of the parasite*

*Leishmania* belongs to a controversial class of unicellular protists known as Kinetoplastida. The only kinetoplastid organisms known to cause disease in humans are approximately 20 different *Leishmania* species; one parasite species responsible for human African trypanosomiasis (HAT), or sleeping sickness, *Trypanosoma brucei*, and *T. cruzi*, the parasite species responsible for Chagas disease in the Americas. All kinetoplastids, in addition to being flagellated for at least part of their life cycle, also share a unique DNA-containing organelle known as the kinetoplast, situated in a mitochondrian-like structure. The kinetoplast contains multiple circular copies of kinetoplast DNA (kDNA), which serve the same function as the mitochondrial genome in other eukaryotes (de Souza *et al.*, 2009; Damasceno *et al.*, 2021).

Like *T. brucei* and *T. cruzi*, *Leishmania* has a digenetic life cycle, alternating between the sandfly vector and the mammalian host. When female sandflies blood feed on an appropriate host, the parasite is inoculated into the skin as metacyclic promastigotes, the infectious, extracellular, non-replicative stage (Bates, 2007). These metacyclic promastigotes are lodged near the stoma deal valve in the anterior gut of the sandfly, and are encased in a gel like “plug” created via secretion of PSG, or promastigote secretory gel. The sandfly is forced to regurgitate this plug into the skin as it takes its meal. Impaired uptake of blood leads to the sandfly attempting to feed with greater frequency, and thus increases the chance of parasite transmission as can be seen in Figure 3.2 (Rogers & Bates, 2007).

The parasite is thus inoculated in the skin along with pro-inflammatory salivary components, where it then taken up by neutrophils which later are phagocytised by macrophages of the host. Once inside the host cell, the parasite differentiates into the obligate intracellular, non-flagellated form called the amastigote. The parasite continues to replicate by mitotic cell division, escaping macrophages by exocytosis and re-invading macrophages as an intracellular amastigote, until it is taken up in the blood meal of the next sandfly (Loria-Cervera & Andrade-Narvaez, 2020).

Both parasite and host factors are thought to be important in determining whether the infection is symptomatic, and the type of pathology resulting from the infection. Tissue tropism of infecting parasites can vary, but VL is usually associated with heavy infections of the liver, spleen, and bone marrow. Different symptomatology and distribution in the host tissues may determine differences in transmissibility of the parasite. Unusual tissue tropism has been observed in HIV-*Leishmania* coinfections, such as parasites in the gastroendothelial mucosa (Afrin *et al.*, 2019; Elmahallawy & Alkhaldi, 2021).

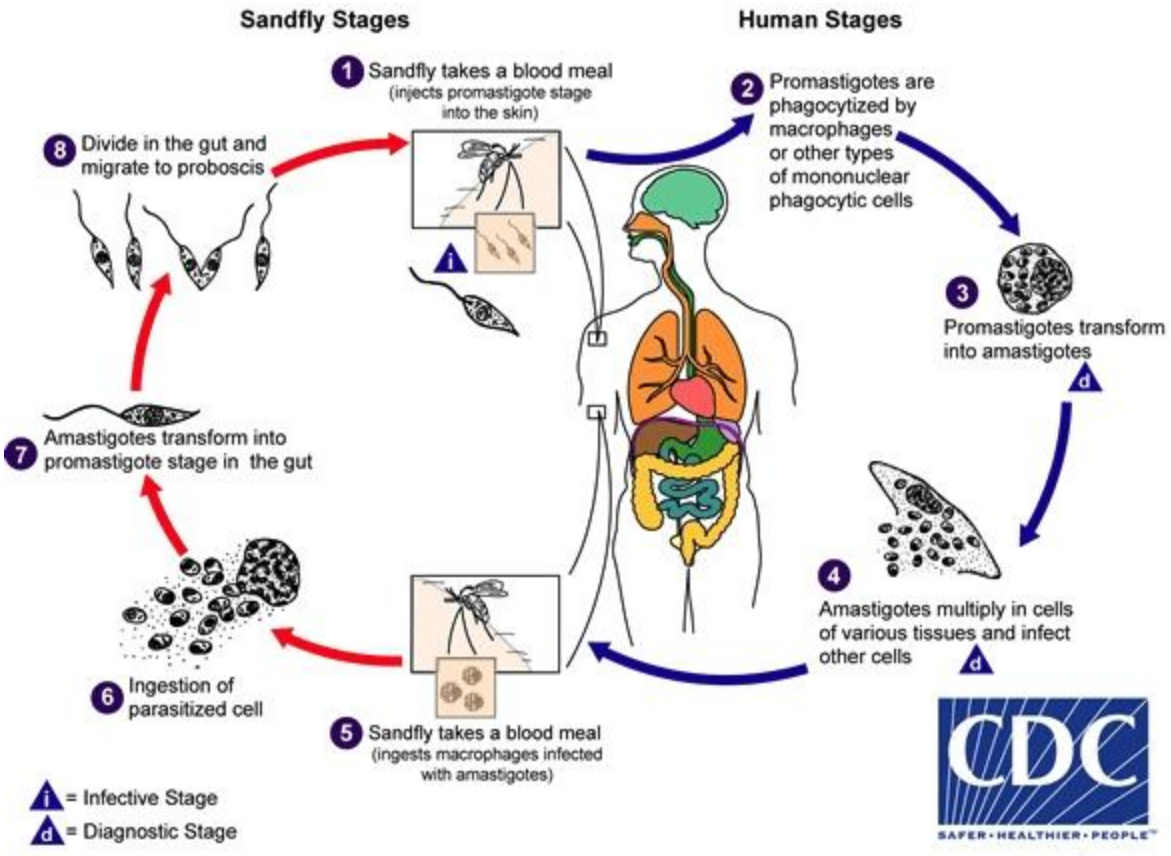



Figure 3.2: The *Leishmania* life cycle (Source: CDC)

(1) Upon blood feeding, the sandfly inoculates infectious met acyclic promastigotes into the host's skin; (2) once in the skin, promastigotes are ingested by phagocytic cells; (3) within the phagocytic cell the parasite differentiates into obligate intracellular amastigotes; (4) the parasite replicates intracellular through multiple rounds of mitosis, invading neighbouring cells; amastigote-infected cells may localise to the skin lesion, or spread to other sites in the body; (5) circulating amastigote infected macrophages are taken up in the blood meal of a sandfly; (6-7) amastigotes differentiate into extracellular promastigotes and attach to the midgut wall to survive excretion of the digested blood meal; (8) promastigotes migrate anteriorly and undergo a series of developmental transitions to form infectious met acyclic promastigotes, encased within a PSG plug that blocks normal feeding of the sandfly. (Source: US Center for Disease Control and Prevention, Division of Parasitic Diseases and Malaria).

Once in the sandfly midgut, the amastigote forms differentiate into early procyclic promastigote stages, which are multiplicative and increase in numbers by cell division, while attaching to the interior wall of the midgut. Parasite attachment to the midgut wall is mediated by lipophosphoglycan (LPG) covering the parasite cell surface. This molecule plays an important role in species-specific interactions between parasite and vector (Pimenta *et al.,* 1994, Sacks & Kamhawi, 2001). By attaching to the midgut wall, the parasite survives expulsion of the digested blood meal as sandfly excrement. The parasite then differentiates into non-replicating nectomonad promastigotes, and migrates to the anterior part of the midgut where it resumes replication as leptomonad promastigotes. This stage is also responsible for production of promastigote secretory gel (PSG), and immediately precedes differentiation into mammalian-infective metacyclic promastigotes (Bates and Rogers, 2004).

### *3.1.2 Leishmania pathogenicity*

The pathogenicity of leishmaniasis varies in humans (Chang *et al.*, 1990). Forms of *Leishmania* that cause self-healing skin leishmaniasis are considered less virulent than those that cause potentially fatal leishmaniasis. Although the environmental, genetic and immunological factors of their mammalian host may modulate *Leishmania* virulence (Blackwell,1996) as well as the saliva of the sandfly vectors (Titus *et al.*, 1988), *Leishmania* species' molecular determinants are the main components in pathogenesis. In the literature there is no evidence that *Leishmania* species produce toxins to induce clinical symptoms of leishmaniasis directly in the traditional context; so, how *Leishmania* causes leishmaniasis is a complicated question, which seems to involve many factors.

The first symptom of infection is a mild erythema at the site of the sandfly bite that develops after a variable period of incubation. The erythema become papules and then a nodule. The local skin leishmaniasis slowly becomes the hallmark lesion for two to six weeks (Reithinger *et al.*, 2007). The parasites must overcome a number of obstacles, including cell membrane proteins, before they develop a macrophage phagolysosomes infection (Lira *et al.*, 1996; McGwire *et al.*, 2003).

Clinical manifestations of cutaneous leishmaniasis varies with the host's immune response (Castes *et al.*, 1983; Pimerz, 1992; Lessa *et al.*, 2007; Sinha *et al.*, 2008).

Virulence factors associated with leishmaniasis in mammals are the lipophosphoglycan (LPG) mentioned earlier, *Leishmania infantum* virulence factor A2 protein, cysteine proteinases, metalloproteiase gp63, glicoinositolphospholipids (GIPLs), amastigote proteophosphoglycans (aPPGs), and the 11 kDa kinetoplastid membrane protein (KMP-11) (Handman & Goding, 1985; Späth *et al.*, 2000; Späth *et al.*, 2003; Matlashewski, 2001; Silva-Almeida *et al.*, 2012). Initially glycoinositolphospholipids of *L. major* had been proposed as virulence factors inhibiting nitric oxide synthesis by macrophages (Proudfoot *et al.,* 1995). Later it became clear that neither ether phospholipids nor glycosylinositolphospholipids are required for amastogote virulence or for inhibiting macrophage activation (Zufferey *et al.,* 2003).

Virulence factors acting in the sandfly have been identified as the mucin-like filamentous proteophosphoglycan (fPPG), which forms the promastigote secretory gel (PSG) (Stierhof *et al.*, 1999). Lipophosphoglycan biosynthetic protein 2 (LPG2) is a GDP-mannose transporter, supplying the substrate necessary for phosphoglycan synthesis (Azevedo *et al.*, 2020). Promastigotes require LPG2 for survival in sandflies but not LPG1 (Gaur *et al.*, 2009; Svárovská *et al.*, 2010).

The nuclear translocations of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in monocytes was reduced by *Leishmania* LPG leading to a subsequent decrease in the secretion of interleukin 12 (Argueta-Donohué *et al.,* 2008) and also to an effect on early immune responses by modulating dendritic cells by inhibition antigen presentation and encouraging an early IL-4 response (Liu *et al.*, 2009). On the one hand, PPR secreted by amastigotes results in the activation

of complement by prompting the mannan-binding lectin (MBL) pathway (Peters *et al.*, 1997a), and on the hand, PPR induces vacole formation in macrophages (Peters *et al.*, 1997b).

Finally, the mechanism(s) of KMP-11 as a virulence factor remain unexplained for the time being (Jardim *et al.*, 1995; Carvalho *et al*., 2005). However, the lack of any mammalian homologue has prompted its exploration as a vaccine candidate (Dalimi & Nasiri, 2020; Zhang *et al.*, 2020).

*Leishmania* infection may lead to cutaneous and mucocutaneous lesions or lethal, generalised, visceral infection. Species of the subgenus Viannia in the New World including *L. braziliensis*, *L. guyanensis,* and *L. panamensis* give rise to CL but are also responsible for MCL in up to 5 – 10 % of cases. Clinical MCL involves a hyper inflammatory response and parasite metastasis from the primary lesion to remote sites that leads in particular in the nasopharyngeal areas to destructive metastatic secondary lesions. The chronic, latent, and metastatic activity of MCL is clearly distinct from other dermal leishmaniases. MCL may appear severe, primarily in oral or nasopharyngeal areas, due to significant tissue destruction associated with high infiltration of immune cells, extreme inflammatory cell activation, and parasite presence even if only at low levels (Ronet *et.al*., 2010). MCL lesions are not self-healing and are more resistant than primary lesions to the treatment with antimony compounds. There were no known factors responsible for these re-occurrences. Both, antimony tolerance and discrepancies between infecting *Leishmania* species and their virulence were suggested (Arelavo *et al.,* 2007; Souza *et al.,* 2010). Reactivations may take place after local inflammation has been stimulated or immunosuppressed, and the question of how these factors interact with slow-growing or dormant parasites and the immune system in favour of the re-emergence of pathological conditions is raised. Little is known about MCL pathogenesis so far, especially factors which either contribute to the host's immune reaction, or to parasite diffusion or reactivation. Immune response includes tumour necrosis factor α (TNFα), tumour necrosis factor β, interleukin 6, C-X-C motif chemokine receptor 1, chemokine C-C motif ligand 2/ monocyte chemoattractant protein 1, and toll-like receptor 3 (Ronet *et al.*, 2011; Silveria *et al.,* 2009). Reduced responses to interleukin 10 (IL-10) and tumour growth factor β were identified (Bacellar *et al.*, 2002; Gomes-Silva *et al.,* 2007). MCL is related to persistent high levels of TNFα, C-X-C motif chemokine ligand 10, and chemokine C-C motif ligand 4, intra-lesional mixed Th1/Th2 response, and elevated cytotoxic T cell activity. Cells in MCL patients, however, have a deficit in immune response regulation because of a defect in their ability to respond to IL-10 (Faria *et al.,* 2005; Gaze *et al.,* 2006; Vargas-Inchaustegui *et al.,* 2010). Thus, immunological hyperactivity leads to MCL disease by these and possibly other mechanisms. A promising alternative or complement to traditional drug therapy may, in effect, be steps to decrease uncontrolled inflammation. It is noteworthy that in patients with MCL who did not respond to antimony therapy alone treatment with anti-inflammatory TNFα inhibitor pentoxyphylline in combination with antimony was successful (Lessa *et al.,* 2001).

### *3.1.3 Leishmaniavirus*

The discovery of the ability to induce virus-specific interferon response by fungi in the 1950s followed by the uncovering of virus like particles (VLPs) in the parasite *Entamoeba invadens* in 1959, scientists began to record similar structures in an ever-expanding list of unicellular eukaryotes. Table 3.1 gives examples for the diversity of hosts of virus-like particles and viruses of parasites and microbial eukaryotes in the early literature. The extensive distribution of VLPs in lower eukaryotes over an increasing number of eukaryotic supergroups suggests that the vast majority of, if not all, living systems might be susceptible to viral infection. Maybe surprisingly, a considerable number of virus-like particle findings have so far not been followed up and the putative viruses remain unidentified. The table also shows that in 1974, the first virus-like particles have been detected by electron microscopy in promastigotes of *Leishmania hertigi* from Panama, a parasites of the tropical porcubine, *Coendou rorhschildi* (Molyneux, 1974; Croft & Molyneux, 1979; Eley *et al.*, 1987; Grybchuk *et al.*, 2018).

### *3.1.4 Origin of Leishmania RNA Virus (LRV)*

Two surveys of New World parasites have detected LRV strains only in South America's Amazon River basin (Stuart *et al.,* 1992). This slim geographic distribution and the common nucleotide identity of greater then 90 % observed between the two independent LRV isolates may represent a recent origin of these viruses. However, the more recent discovery in the Old World parasite *Leishmania major* of a related virus along with the absence of an infectious process for these viruses indicates that LRV emerged prior to the separation between Old and New World parasites. Genetic recombination is unknown, since reproduction is primarily asexual in *Leishmania* species (Tibayrenc *et al.,* 1991). Comparative study of restriction fragment length polymorphisms (RFLPs) also indicates a long history of co-evolution between individual LRV isolates and their respective parasite host strains. The findings jointly support a current belief that LRV is an ancient virus.

Table 3.2: Examples for the diversity of hosts of virus-like particles and viruses of parasites and microbial eukaryotes in the early literature

| Host | Note | Habitat | Reference |
|---|---|---|---|
| Archaeplastida | | | |
| Chlorophyta (green algae) | | | (Lemke, 1976; Van Etten *et al.*, 1991) |
| *Coleochaete scutata* | | freshwater | (Mattox *et al.*, 1972) |
| *Mesostigma viride* | scaly green flagellate | freshwater | (Melkonian, 1982) |
| *Oedogonium* sp. | | freshwater | (Pickett-Heaps, 1972) |
| *Radiofilum transversale* | now *Parallela transversalis* | freshwater | (Mattox *et al.*, 1972) |
| *Stigeoclonium farctum* | | freshwater | (Mattox *et al.*, 1972) |
| *Uronema gigas* | | freshwater | (Mattox *et al.*, 1972) |
| Rhodophyta (red algae) | | | (Lemke, 1976; Reisser, 1993) |
| *Porphyridium purpureum* | | marine | (Chapman & Lang, 1973) |
| *Sirodotia tenuissima* | | freshwater | (Lee, 1971) |
| CRuMs: Collodictyonidae, Rigifilida, Mantamonas | | | |
| Collodictyonidae | | | |
| *Aulacomonas submarina* | now *Diphylleia rotans* | marine, freshwater | (Swale & Belcher, 1973) |
| TSAR: Telonemia, Stramenopila, Alveolata, Rhizaria | | | |
| Chrysophyta (golden algae) | | | (Short *et al.*, 2020) |
| *Chrysochromulina mantonii* | | marine, brackish | (Manton & Leadbeater, 1974) |
| *Coccolithus huxleyi* | now *Emiliania huxleyi* | marine | (Manton & Leadbeater, 1974) |
| Phaeophyta (brown algae) | | | (Muller *et al.*, 1998) |
| *Chorda tomentosa* | now *Halosiphon* | marine | (Toth & Wilce, 1972) |
| *Ectocarpus fasciculatus* | | marine | (Baker & Evans, 1973) |
| *Pylaiella littoralis* | | marine | (Markey, 1974) |

| | | | |
|---|---|---|---|
| Stramenopila | | | |
| *Blastocystis* sp | monkeys | animal parasite | (Stenzel & Boreham, 1997) |
| | sea snake | | (Teow *et al.*, 1992) |
| *Heterosigma akashiwo* | Japanese red tide | marine | (Nagasaki *et al.*, 1994) |
| *Thraustochytrium* sp. | | brackish, marine | (Kazama & Schornstein, 1972) |
| Rhizaria | | | |
| *Corythionella* sp | | marine | (Lipscomb & Riordan, 1995) |
| *Phaeodarian radiolarians* | | marine | (Gowing, 1993) |
| *Plasmodiophora brassicae* | club root | plant parasite | (Aist & Williams, 1971) |
| Alveolata, Apicomplexa | | | |
| *Eimeria stiedae* | | rabbit parasite | (Kaempfer & Kaufman, 1973) |
| *Leucocytozoon simondi* | | bird parasite | (Desser & Trefiak, 1971) |
| *Plasmodium berghei berghei* | | mouse parasite | (Davies & Howells, 1971; Davies *et al.*, 1971) |
| *Plasmodium berghei yoelii* | | mouse parasite | (Bird *et al.*, 1972) |
| *Plasmodium cynomolgi* | | primate parasite | (Garnham *et al.*, 1962) |
| *Plasmodium gallinaceum* | | bird parasite | (Garnham *et al.*, 1962; Terzakis, 1969) |
| *Plasmodium tropiduri* | | reptile parasite | (Scorza, 1971b, a) |
| *Plasmodium vivax* | | human parasite | (Bird *et al.*, 1972) |
| Alveolata, Ciliophora | | | |
| *Carchesium polypinum* | | freshwater | (Zagon, 1970) |
| *Ignotocoma sabellarum* | | annelid parasite | (Lorn & Kozloff, 1969) |
| Alveolata, Dinoflagellata | | | |
| *Gymnodinium uberrimum* | | freshwater | (Sickogoad & Walker, 1979) |
| Alveolata, Perkinsozoa | | | |
| *Labyrinthomyxa marina* | now *Perkinsus* | oyster parasite | (Perkins, 1969) |

Discoba

Euglenozoa

| | | | |
|---|---|---|---|
| *Endotrypanum* | | animal parasite | (Croft *et al.*, 1980) |
| *Leishmania hertigi* | porcupine | animal parasite | (Molyneux, 1974) |
| *Phytomonas* sp | coconut, oil palm | plant parasite | (Marche *et al.*, 1993) |
| *Trypanosoma melophagium* | sheep ked | sheep parasite | (Molyneux & Heywood, 1984) |

Percolozoa

| | | | |
|---|---|---|---|
| *Naegleria fowleri* | brain-eating amoeba | human parasite | (Maitra *et al.*, 1973) |
| *Naegleria gruberi* | | freshwater, soil | (Schuster, 1963) |

Amorphea

Amoebozoa

| | | | |
|---|---|---|---|
| *Acanthamoeba* sp. | | soil | (Vickerman, 1962) |
| *Entamoeba hartmanni* | | soil | (Hruska *et al.*, 1974) |
| *Entamoeba histolytica* | | human parasite | (Miller & Swartzwelder, 1960) |
| *Entamoeba invadens* | | reptile parasite | (Deutsch & Zaman, 1959) |

Obazoa, Opistokonta, Opisthosporidia

| | | | |
|---|---|---|---|
| *Aphelidium* sp. 215 | | parasite of green algae | (Schnepf *et al.*, 1974) |

Obazoa, Opistokonta, Fungi

Deuteromycetes

| | | | |
|---|---|---|---|
| *Penicillium funiculosum* | pineapple mould | plant pathogen | (Lewis *et al.*, 1959) |
| *P. chrysogenum* | penicillium mould | soil | (Lemke & Ness, 1970) |
| *P. stoloniferum* | mould | soil | (Kleinschmidt *et al.*, 1968) |

Ascomycetes

| | | | |
|---|---|---|---|
| *Saccharomyces cerevisiae* | baker’s yeast | soil | (Berry & Bevan, 1972) |

Basidomycetes

| | | | |
|---|---|---|---|
| *Agaricus hisporus* | common mushroom | fungal parasite | (Hollings, 1962) |

| | | |
|---|---|---|
| Blastocladiomycota | | |
| *Allomyces arbusculus* | freshwater | (Khandjian *et al.*, 1974) |
| *Blastocladiella emersonii* | freshwater | (Barstow & Lovett, 1975) |
| | | |
| Mesomycetozoea | | |
| *Paramoebidium arcuatum* | arthropod symbiont | (Kaempfer & Kaufman, 1973) |

The discovery that virus-infected parasites grow more readily in culture than their uninfected cohorts has raised concerns that LRV may potentially occur during laboratory manipulation due to a positive effect of virus-infection on *in vitro* development. Conflicting observations of at least one parasite strain's infection status when grown in various laboratories is consistent with the hypothesis (Tarr *et al.,* 1988) and (Widmer *et al.,* 1989). However, plus-strand RNA in human patients with cutaneous leishmaniasis in tissue biopsy material were recently detected, which shows conclusively that LRV is not an artefact of laboratory culture but exists naturally *in vivo* (Saiz *et al.*, 1999).

### *3.1.5 Genome Structure and Organization*

Stuart and collaborators were the first to publish a complete cDNA sequence for an LRV isolate's dsRNA genome (Stuart *et al.*, 1992). The prototype virus, called LRV1-1, was derived from a laboratory clone (1A) obtained from *Leishmania guyanensis*, a parasite of the New World. A second New World virus isolate (LRV1-4) and a diverged virus (LRV2-1) isolated from the *Leishmania major* are now available in full cDNA sequences. In various New World parasite species, more than 15 different LRVs have now been reported, corresponding to an estimated infection rate of about 20 % among the strains tested. Several of the isolates appeared to be deriving from parasitic infections outside the Amazon Basin, but no orderly study of Old World parasites has yet been attempted (Saiz *et al.*, 1999).

The entire nucleotide sequence of LVR1 from cDNAs was determine by Kenneth *et al.* (1992). Two large open reading frames (ORFs) were detected, the encoding of ORF3 for the RNA dependant RNA polymerase (RdRp), and the overlap of ORF2 which could encode a viral coat protein suggesting that + 1 transitional frameshift is a form of Gag-pol fusion protein.

The complete cDNA sequence for *LRV2-1*, known to infect an Old World parasite, *Leishmania major*, was published by Scheffter *et al.* (1995). Scheffter and his team show that *LRV2-1* is slightly different from the LRV1 in that it infects parasites of the New World. The findings support the view that LRV transmission is purely vertical, and as such, preserves of features in leishmaniavirus proteins could be established for the first time and form the basis for site-mutagenesis studies, which were followed by the divergence between the Old and New world parasites.

There is a great deal of interest in the role of leishmaniaviruses in *Leishmania* virulence and pathogenesis. MacBeth and Patterson (1995) found the short transcript of *LRV1-4*, 1995. Protein-induced endonucleases were only connected to intact viral particles and were responsible for the cleavage case. The viral capsid protein was identified in 1995 as a responsible endonuclease in a later study by MacBeth *et al.* (1997). The recombinant-expressed viral capsid protein showed the function of the endoribonuclease.

The full sequence of *LRV1-4* reveals that 71 nucleotides are overlapping ORF2 and ORF3, and that there is a lack in ORF3 of the possible initiator of translation indicating that viral polymerase can be synthesised with the virus capsid as 180 kDa-fusion proteins. Lee *et al.* (1996) have demonstrated the synthesis of a fusion protein by means of ribosomal frameshift, as well as the overlap of 71 nucleotides of ORF2 and ORF3 within the translation frameshifting area, which demonstrates the potential structure of a pseudoknot located within the 71 overlapping nucleotides. It has been confirmed that LRV1 capsid-RdRp protein can be synthesised through frameshifting at least *in vitro* and that the nucleotide sequences derived from the junction of the LRV capsid and polymerase genes simulate this event, which suggest a fusion protein is likely produced *in vivo*. Computer analysis of the 71 nucleotide ORF overlap sequence revealed a putative ribosomal slippery region (nt 2625 – 2630) and a downstream pseudoknot. The reading frame of the overlap sequence suggests a +1 or -2 frameshift model for the synthesis of the LRV1 capsid-polymerase fusion protein, which is different from -1 frameshifting found in GLV (Wang *et al.,* 1993), yeast viruses (Icho & Wickner, 1989; Dinman & Wickner, 1992) and retroviruses (Jacks & Varmus, 1985) and (Jacks *et al.,* 1988).

The results showed by MacBeth *et al.* (1997) that an in vitro cleavage assay with *LRV2-1* version can also be used on a single site to map a derived substrate of RNA. Precise RNA-site mapping confirmed that cleavage occurs in a LRV1 homology region. In previous studies, it was shown that an RNA substratum derived from LRV1-4 was susceptible to LRV1 cleavage but was resistant to the LRV2-1 endoribonuclease (MacBeth & Patterson 1995).

### *3.1.6 Parasite Phenotype Modulation*

Although genetic polymorphisms in the host or the parasite may be associated with disease outcome, our research may directly affect the diagnosis and treatment of MCL through the use or production of new medication. Such drugs will work through the blockage of *Leishmania RNA virus 1* (LRV1), thereby worsening the inflammatory response and failure of first line therapy including antimony. LRV1 is associated with a particularly active mucocutaneous syndrome caused by the double-stranded RNA (dsRNA) virus, infected by *Leishmania RNA Viruses LRV1*. The way *LRV1* is introduced to mammalian host cells, however, is unclear. Many viruses use the host exosome pathway to shape and spread cell to cell in higher eukaryotes. As a result, viral material or particles in exosomes originating from infected cell. Recent work has shown that LRV1 is leveraging the Leishmania exosome pathway for the extracellular environment in (Castelli *et al.*, 2019). Exosomes deriving from *LRV1*-infected *Leishmania*, which have been studied biochemically and electron microscopically, indicated that most dsRNA LRV1 co-fractionate from exosomes. The LRV1-containing exosome preparations have been transferred to show that a large number of parasites are infected rapidly and temporarily with LRV1. Such newly infected parasites are substantially more

severe than non-infected mice. In addition, parasite co-infected mice and exosomes carrying LRV1 have formed a tougher disease. In general, this work shows that *Leishmania* exosomes function as a viral shell, which promotes LRV1 transmission and increases mammalian host infectivity (Castelli *et al.*, 2019).

It has been shown that members of the *Totiviridae* family change their host's phenotype. *Yeast Virus L-A S. cerevisiae* was related to a killer toxin encoded by satellite dsRNA M 1, which is lethal to non-virus-infected strains. In protozoan virus systems where isogenic strains are available, the findings indicate that infection with the virus can, at least *in vitro*, affect parasite phenotype. Loss of the virus leads to a lack of phenotypical variability.

The incidence of *Trichomonas vaginalis virus* has been demonstrated to correlate with *Trichomonas vaginalis*' ability to undergo phenotypic variability through the up-regulated surface expression of a prominent cellular immunogen P 270 (Khoshnan & Alderete, 1993). Virus infected strains show qualitative and quantitative changes in the expression of other cellular proteins, often unidentified, as well as altered growth kinetics *in vitro*.

When developed at sufficiently high rates, *Giardia lamblia virus infection* can reduce the attachment of parasites to artificial surfaces and induce a cessation of division in cultured cells. The significance of these phenotypic changes in the disease and infection remains to be identified *in vivo* (Scheffter *et al.*, 1999). In conjunction with the mysterious pathophysiology of cutaneous leishmaniasis, the precedence of totiviruses altering the host phenotype, suggests an interesting possibility that LRV may confer a hypovirulence or hypervirulence on the host parasite. One explanation is that the reported variation in disease pathology at least partially reflects any intrinsic variations in the virulence of the parasites. The existence of LRV may alter the parasite phenotype in ways affecting virulence and ultimately pathogenesis of disease, as has been documented with simple eukaryotes with other ds RNA viruses. A method to assess whether the LRV presence and the phenotypic variability of the host associated with the existence of this virus, is to check biopsy samples and determine if the virus is connected with the mucocutaneous and the skin type of the disease. Early attempts to associate infection with virulence changes have led to inconclusive findings. Another approach to understanding the relationship between virus and parasite will be to use an infected and uninfected isogenic parasite in macrophage infectivity experiments to assess whether the virus plays a significant role in parasite entry. Unfortunately, LRV particles cannot produce an infection in uninfected parasites. Ro and his team have developed a *Leishmania* strain cured of its virus that could be used to circumvent this issue. It remains probable that the parasites infected with the virus do not show an altered capacity to reach macrophages, in which case cytokine profiles of macrophages extracted from infected bone marrow may be studied. A method for evaluating whether an association exists between the existence of LRV and the host's phenotypic variability will be to check biopsy samples for the existence of the virus and assess if the virus is related to either the mucocutaneous or cutaneous type of the disease (Ro *et al.*, 1997).

A lack of isogenic parasite strains has impeded a detailed review of the LRV-virulence relationship in *Leishmania* spp. Although early experiments showed the ability to transmit infection with whole virus particles, such infections were transient and after a short period in culture, the virus was quickly lost. Instability to generate a full genomic cDNA virus sequence in bacteria is an issue faced by many attempts to produce infectious RNA virus clones. However, LRV has recently been successfully extracted from a previously infected strain by developing the parasite in the culture medium that contains the translation inhibitor, hygromycin B (Ro *et al.*, 1997). Pairs may be tested on a parasite phenotype and disease pathogenesis role of an animal model for virus infection (Scheffter *et al.,* 1999).

Carrion, O'Halleron, and Patterson (2002) recently produced a Taqman detection assay. Preliminary findings of the analysis using this technique for virus detection in swabs of patients suffering from leishmaniasis in Brazil indicate a viral infection rate of more than 80 %. The association between LRV infection and virulence modulation should be possible at the end of this analysis (Carrion & Patterson, 2002).

### *3.1.7 Prevalence*

Nested reverse-transcription polymerase chain reaction was used to test for LRV1 in leishmaniasis lesions of Brazil (Pereira *et al.*, 2013). No LRV1, except with mucosal involvement, has been found in endemic areas of Rio de Janeiro (RJ). LRV1 was only observed in the northern area of the region in *L. guyanensis* skin lesions obtained from patients suffering from reactivation of their primary lesions after a surgical cure. Results suggested that leishmaniasis was not associated with *Leishmania LRV1* infection in some RJ areas, where *L. braziliensis* is the primary etiologic agent.

In South America, CL patients mainly infected by *L. braziliensis, L. panamensis* and *L. guyanensis* are at risk of developing mucosal (ML) or disseminated cutaneous leishmaniasis (DCL) (Santrich *et al.,* 1990; Weigle & Saravia, 1996; Banuls *et al.,* 2011; Guerra *et al.,* 2011). Complications of CL involving dissemination of primary lesion parasites in secondary locations, and lesions frequently associated with highly damaging inflammatory reactions (Faria *et al.,* 2005; Gaze *et al.,* 2006; Vargas-Inchaustegui *et al.,* 2010; Lessa *et al.,* 2012). Mucosal disease can be known for reduced responses, often complicating the secondary bacterial or fungal infections, to widely used therapies such as antimony. Very little is known about the pathogenesis and in particular the root of unregulated inflammatory response found in certain patients with metastatic and mucosal leishmaniasis.

Leishmaniasis represents a significant risk for people in French Guiana who are in contact with the forest. A study by Ginouves *et al.* (2016) revealed that most *Leishmania* infections are due to t, *L.*

*guyanensis* and *L. braziliensis*. The virus was present in 74 % of *Leishmania* species isolates with the highest prevalence in the country's internal areas.

Of the different species, *L. braziliensis* is considered to be one of the most common in North and South America due to its prevalence, the difficulty of treating the disease it causes and its significance for public health; and it is the most frequent cause of ML that begins as CL and progresses to ML in up to 10 % of cases (Reithinger *et al.,* 2007). The factors responsible for CL to ML progression are not well known and are likely to include host and parasite factors. No successful vaccine against *L. braziliensis* is yet available, and treatment is based on diagnosis and chemotherapy. At present the primary treatment is pentavalent antimony (SbV), usually sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime). SbV treatment, however, is characterised by a variable outcome in Latin America, with trewatment failure rates exceeding 39 % (Palacios *et al.,* 2001; Tuon *et al.,* 2008). Although SbV resistance has been associated with intrinsic changes in parasite susceptibility in some species of *Leishmania*, this does not appear to be the case in *L. braziliensis* in Peru (Yardley *et al.,* 2006; Croft *et al.,* 2006). The risk factors found to date include concurrent distant lesions and immune response factors (Llanos-Cuentas *et al.,* 2008; Valencia *et al.,* 2012). For example, the presence of high Interleukin 10 levels in lesions is related to a weak treatment response, and it is well recognised that immune responses significantly influence the effectiveness of antimony compounds (Croft *et al.,* 2006; Amato *et al.,* 2008; Maurer-Cecchini *et al.,* 2009; Castelluci *et al.,* 2014). Many factors leading to *L. braziliensis* ' relative insensitivity to SBV chemotherapy are likely. LRV1 is widespread among the *L. braziliensis* and *L. guyanensis* species with a prevalence of > 50 %, with an overall incidence of 20 % to 30 % (Salinas *et al.,* 1996; Ogg *et al.,* 2003; Pereira *et al.,* 2013; Bourreau *et al.,* 2016). LRV1 can act as an immunomodulator via the interactions between its dsRNA genome and the Toll-receptor 3 (TLR3). Two studies reported low rates of LRV1 interaction with cutaneous versus mucocutaneous presentation (Pereira *et al.,* 2013; Saiz *et al.,* 1998). So far, these studies have led to the thought that for a link between LRV1 and treatment success. Vanessa *et al.* (2016) performed a cross-sectional study of *L. braziliensis* isolate collections from patients in Peru and Bolivia displaying various types of tegumentary leishmaniasis (CL, ML, or both MCL). An important correlation was noted between the existence of LRV1 and SbV or amphotericin B therapeutic failure. Patients were treated with SbV or amphotericin B and the treatment outcome of 54 patients was tracked over the course of one year, and here are some major findings:

1. LRV1 is associated with significant increase in the risk of treatment failure;
2. LRV1 does not confer intrinsic parasite antimony resistance in infected macrophages;
3. LRV1 subtypes are not associated with treatment outcome; and
4. LRV1 is not preferentially associated with MCL or ML.

Although LRV1 was found decades ago, it has only recently been suggested that it may be a clinical and aggravating factor of CL (+ *et al.,* 2011). The first to study and endorse the potential pathogenic

significance of human LRV1 infection in the region were Bourreau *et al.* (2015). The prevalence of LRV1-positive *L. guyanensis* infection has been reported as 58 % for patients with primary localised tegumentary leishmaniasis diagnoses. All patients infected with LRV1-negative *L. guyanensis* were cured after one dose (71 %) or two doses (100 %) of pentamidine. In comparison, 12 of the patients with LRV1 (27 %) had chronic inflammatory infection and symptomatic relapse requiring intensive care and second-line medication. LRV1 activity was subsequently associated with a significant increase in intraregional inflammatory markers. LRV1 is shown to be substantially predictive of first-line failure of care and symptomatic relapse in *L. guyanensis* infection (P = .0009) and has the potential to contribute to therapeutic choices in tegumentary leishmaniasis.

*L. braziliensis* had been diagnosed in 30 cases, *L. guyanensis* in 5 cases, and a combination of the two were diagnosed in 2 cases (Marcos and al. 2015). While in mucosal lesions the virus titre is twice as high as in skin lesions of the same regions, a link between the clinical phenotype and the presence of LRV1 has yet to be found. The factors that influence the tropism of the parasite in the mucosal regions are not yet understood. LRV1 was recently identified as a key contributor to the seriousness of the disease. In a recent study (Cantanhêde *et al.,* 2015), a higher frequency of LRV1 was observed in 156 patients from the western Amazon in Brazil. The virus is associated with worsening of the disease, raising the risk of development of mucosal lesions by nearly 3-fold.

LRV was also detected in *Leishmania* isolated from a reservoir host (Hajjaran *et al.,* 2016). The virus was detected in *L. major* isolates originating from a great gerbil, *Rhombomys opimus*. The Iranian LRV sequences showed the highest similarity to LRV2, which was genetically distant from LRV1 isolates found in the parasites of New World *Leishmania*.

A research was conducted (Tirera *et al.,* 2017) to unravel the genetic diversity and phylogeny of LRV1 strains of *Leishmania* isolates that circulate in French Guyana. It was concluded that this is the first ever estimation of genomic diversity of LRV1 that occurs in *L. guyanensis*. This research is also the first to identify cases of multiple infections with LRV1. A total of 129 isolates were collected in French Guyana during the period 2011 – 2014, 19 tested positive for LRV1 in *L. guyanensis* and 1 for LRV1 in *L. braziliensis*.

Krauze *et al.* (2018) found LRV1 in *L. shawi*. The finding indicates that LRV1 strains are genetically diverse and that smaller variations between the virus sequences from the same parasite species have been observed. Phylogenetic analyses have shown that the LRV1 sequences cluster by parasite species and possibly by the population of the parasite identified.

Genetic clusters found for *L. braziliensis* strains correlate with the presence / absence of LRV1, as well as the phylogeny of the virus (Cantanhêde *et al.*, 2018). Studies have shown that *L. braziliensis* shows major intra-species variation that could explain its ability to adapt to different environmental conditions (Cupolilo *et al.,* 2003; Kuhls *et al.,* 2013). There are about 30 genome assemblies

available for *Leishmania*, six species for Olw World *Leishmania* and only two for New World *Leishmania*. These genomes were collected from long-term cultures and preserved using *in vivo* and *in vitro* conditions which have recently been shown to affect this organism's genomic characteristics (Dumetz *et al.,* 2017).

Krauze *et al.* (2018) were the first to record a *L. braziliensis* strain (IOC-L3564) genome sequence including LRV1.

The existence of the LRV was examined in Turkish *Leishmania* isolates from leishmaniasis cases diagnosed in various parts of Turkey (Nalcaci *et al.,* 2019). Results were compared with previously published data to strengthen the existing knowledge with a view to developing more successful leishmaniasis treatment strategies. A total of 29 *Leishmania* i*solates were used, 24 L. tropica, 2 L. infantum and 3 L. major. This study was the first to show LRV2* in *L. tropica* isolates. 14 *L. tropica* isolates collected from several countries (Namibia, Tunisia, Iraq, Saudi Arabia), tested by dot blot have been negative for virus (Hajjaran *et al.,* 2016). Hajjaran *et al.* (2016) also did not find LRV2 from CL samples collected in Iran in *L. tropica.* The virus may be lost during laboratory culture.

Leishmaniaviruses have so far only been reported from a very small number of *Leishmania* species. The leishmaniaviruses are assumed not to be infectious and are assumed to be vertically inherited only. The question arises whether these few *Leishmania* species testing positive for the virus represent the lineages of *Leishmania* that were originally infected or whether the ancestor of *Leihmania* was carrying the virus, and the virus was stochastically lost in many lineages. If only a few *Leishmania* lineages were originally infected, we would expect several occasions of horizontal infections into *Leishmania* lineages. A phylogeny of the virus should reveal these instances of horizontal infection. The low prevalence of virus in some *Leishmania* species strongly suggest a model of stochastic loss over evolutionary time.

### *3.1.8 Motifs*

RNA viruses evolve so fast that the automatic alignment of its nucleic acid sequences becomes very challenging over larger evolutionary distances. At some point, the more conserved amino acid sequences also become difficult to align. What is needed are references in a sequence by which the quality of an alignment either can be judged or, in the case were software solutions fail to provide consistent alignments, references in a sequence are needed that allow the anchoring of manual alignments. These highly conserved references in a sequence are protein motifs.

The word motif is used in structural biology in two ways. The first concerns a specific sequence of amino acids characterising a specific biochemical feature. An example is CXX(XX)CXXXXXXXXXX HXXXX, a finger motif found in several different DNA protein families (Figure 3.3).

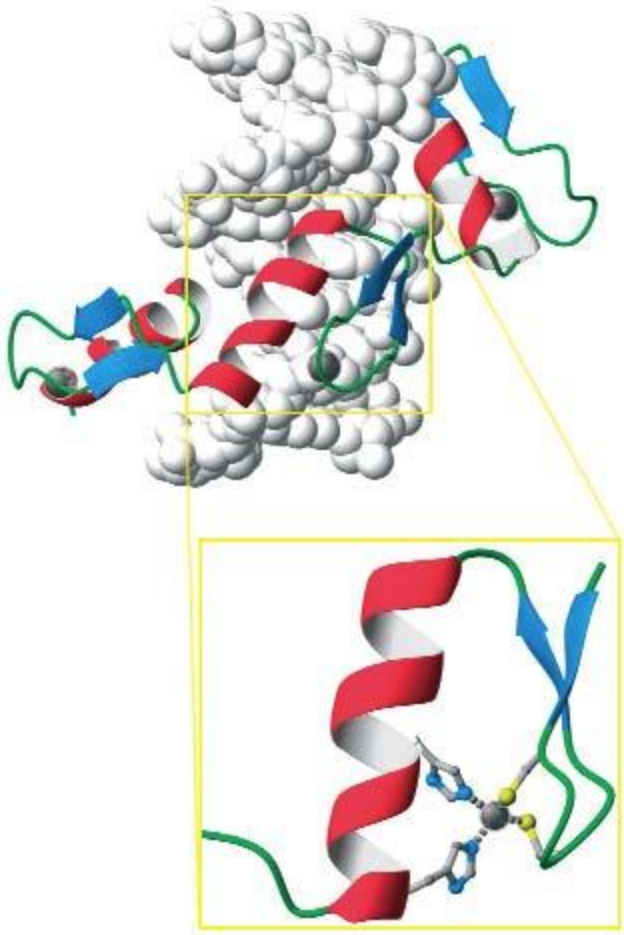


Figure 3.3: The Zinc Finger motif

The zinc finger motif displays a fragment derived from a mouse gene regulatory protein with three zinc finger fingers that are connected to a central groove of the RNA molecule. The insert shows the distribution of atoms with normally scattered cysteine and histidine residues in one zinc finger motif (Petsko & Ringe, 2004).

The retained cysteine and histidine residue motif forms ligands into a zinc ion in this sequence motif, whose coordination is necessary to stabilise a tertiary architecture. A class of residues is retained rather than a particular residue: one is ideally hydrophobic (e.g., leucine or phenylalanine) in the 12-residue loop between zinc ligands. Simple inspection of a protein amino acid sequence can also identify sequence motifs and provide solid evidence for biochemical activity, if detected. Being a protease of human immunodeficiency virus, the primary structure was the motif of the chain. Protease was first described as an aspartyl protease.

The second, equally common, use of the term motif refers to the collection of attached secondary structural elements, which have either a particular functional sense or are part of an unfolded area. Former motifs and functional sequence motifs are generally known as functional motifs. An example is the motif for helix-turn-helix in a number of RNA proteins (3.4).

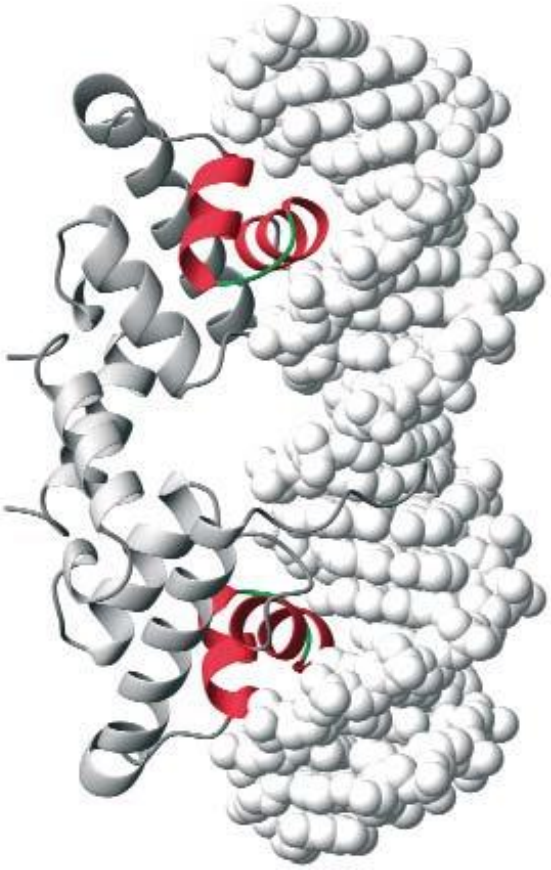


Figure 3.4: Two Colourful Helix-Return-Helix-motifs

Two Colourful Helix-Return-Helix-motifs seen in the bacterial gene regulatory protein repressor RNA-binding domain. Helix-turner-helix Helicals in the primary groove binding and the identification of special regulatory gene sequences of the RNA are the two closest to the RNA (Petsko & Ringe, 2004).

This basic design justification is not a stably folding identification, because it is found in a protein that is already expected to bind nucleic acids, when it is deemed distinct from the rest of its proteins. Examples of structural motifs, which are a major part of a stably folded area:

- the four-helix package of four alpha-helices which is embedded in various hormones and other proteins;
- Rossmann fold which is a twisting structure for alpha / beta that typically connects NAD cofactors; and
- the key Greek motif, the all beta scheme in several different proteins, is similar in topological terms to the pattern in ancient vases.

While these structural trends are often practical but not more frequently, the only case that explicitly has practical consequences here is the Rossmann fold.

Since sequence motifs also have functional implications, in the sequences of newly discovered genes, significant attention in bioinformatics is aimed at identifying these motifs. Practically this can

be difficult. In the example given, the pattern of the zinc finger is uninterrupted, making it relatively easy to align. But many other sequence motifs are discontinuous and the spacing can differ considerably between their constituent parts. The term sequence motif in these situations can be misleading, because not only the distance between the residues but also the order in which they occur can be entirely different. These are genuinely functional motifs in which their presence is identified not by sequence but by structure.

Using sequence information alone to identify structural motifs can present very significant challenges, for the following reasons.

- Firstly, several different sequences of amino acids may be associated with the same secondary structure; hundreds of different sequences can occur for four-helix bundles. Hence, sequence similarity alone cannot be used for absolute classification of structural motif patterns. Putting the sequence elements of the secondary structure must therefore only identify these motifs. Methods for prediction of secondary structures are not entirely accurate.
- Secondly, often structural regions are so large that several sections of additional polypeptide chain may be inserted in the motif without having a structural effect. The so-called TIM-barrel domain, containing a beta strand, followed by an alpha helix, repeated 8 times, is a typical example.

Many protein domains consist only of this group of secondary structural elements, while others have an additional structural motif added, and some are found where one or more additional whole domains disturb the sequence without interfering with the structure of the vessel.

Applied to RNA-dependent RNA polymerases (RdRp) of positive-sense ssRNA viruses, we see several motifs. Figure 3.5 shows a low resolution alignment based on RdRp motifs, Figure 3.6 shows a higher resolution where individual amino acids are visible, and Figure 3.7 shows 3D models for these viruses (Jia & Gong, 2019).

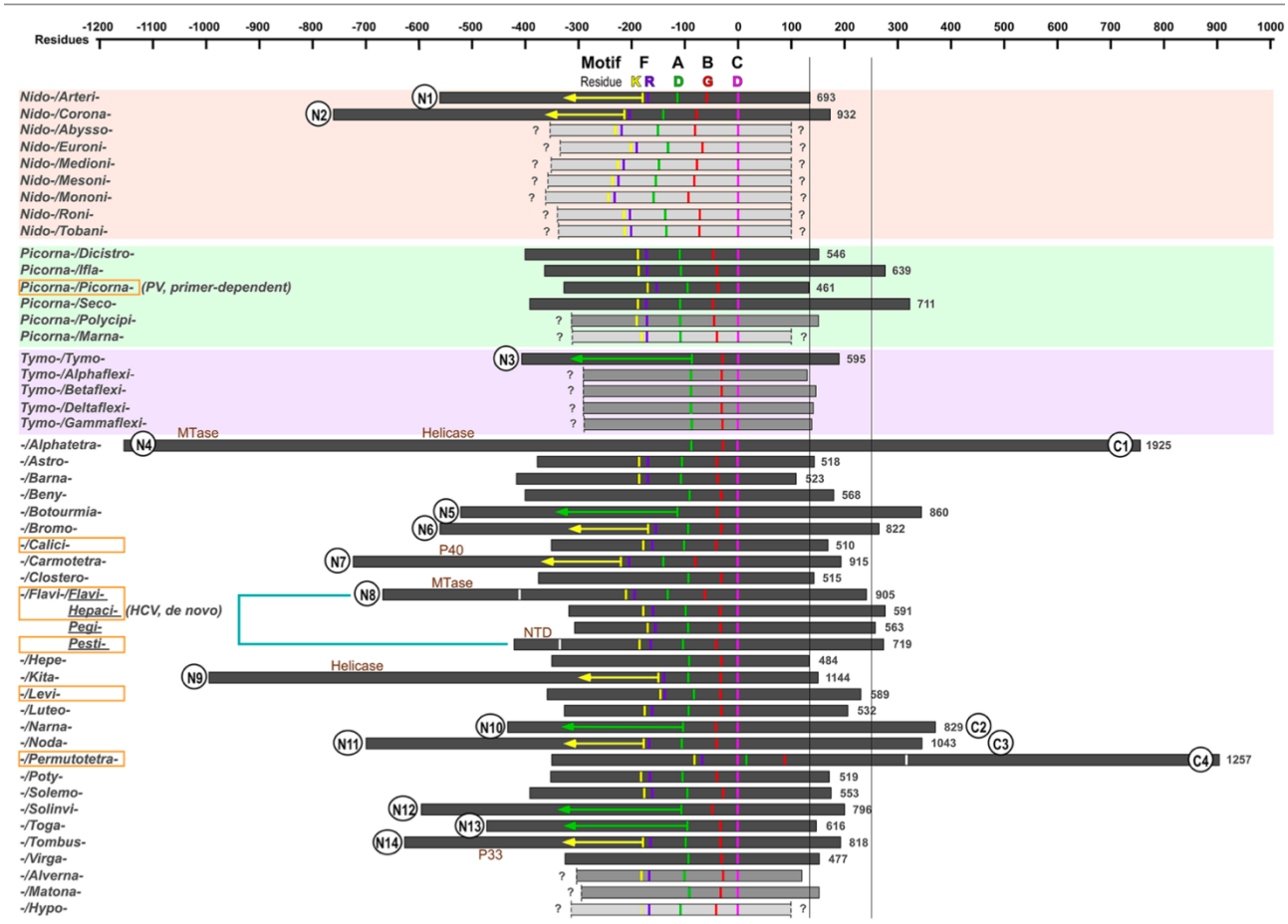



Figure 3.5: Primary structure of RdRp of representative positive-sense ssRNA viruse showing key protein motifs (Jia & Gong, 2019).

It is important to realize that such an alignment cannot be used for phylogenetic work, because many key motifs do not align with each other perfectly. This kind of alignment is missing gaps that secure that all amino acids (nucleotides) in all taxa a phylogenietic related. The virus species names are listed in alphabetical order giving the priority to virus order, then to virus family, and then to virus genus. The conserved motif C aspartic acid (magenta, corresponding to PV RdRP D328) is used as the origin in the scale bar. Conserved residues in motifs A, B, and F are also labelled: motif F lysine (corresponding to PV RdRP K159) in yellow; motif F arginine (corresponding to PV RdRP R174) in purple; motif A aspartic acid (corresponding to PV RdRP D233) in green; motif B glycine (corresponding to PV RdRP G289) in red. The orange rectangle indicates that 3D structures are available in that virus family (or virus genus in case of the Flaviviridae). The boundaries of the RdRP catalytic module defined by reported 3D structures are indicated by the white bars for the Flaviviridae and Permutotetraviridae RdRPs. Numbers on the right side of individual RdRP indicate the amino acids numbers for the full-length RdRPs. The question mark (?) indicates undefined boundaries of the RdRP proteins. The yellow and green arrows (150 and 230 residues in length, respectively) are used to estimate the N-terminal boundary of the RdRPs. The two long vertical bars (130 and 250 residues to the origin) indicate the C-terminal boundary of the RdRP catalytic module of the primer-dependent PV 3Dpol and the de novo HCV NS5B and are used to help predict additional functional regions. Wherever available in literature, the name of additional functional regions are labelled. Circles placed at the RdRP termini indicate predicted additional regions. The numbers following the “N” and “C” simply refer to the number of families possibly having additional regions at the RdRP N- and C-termini, respectively (Jia & Gong, 2019).

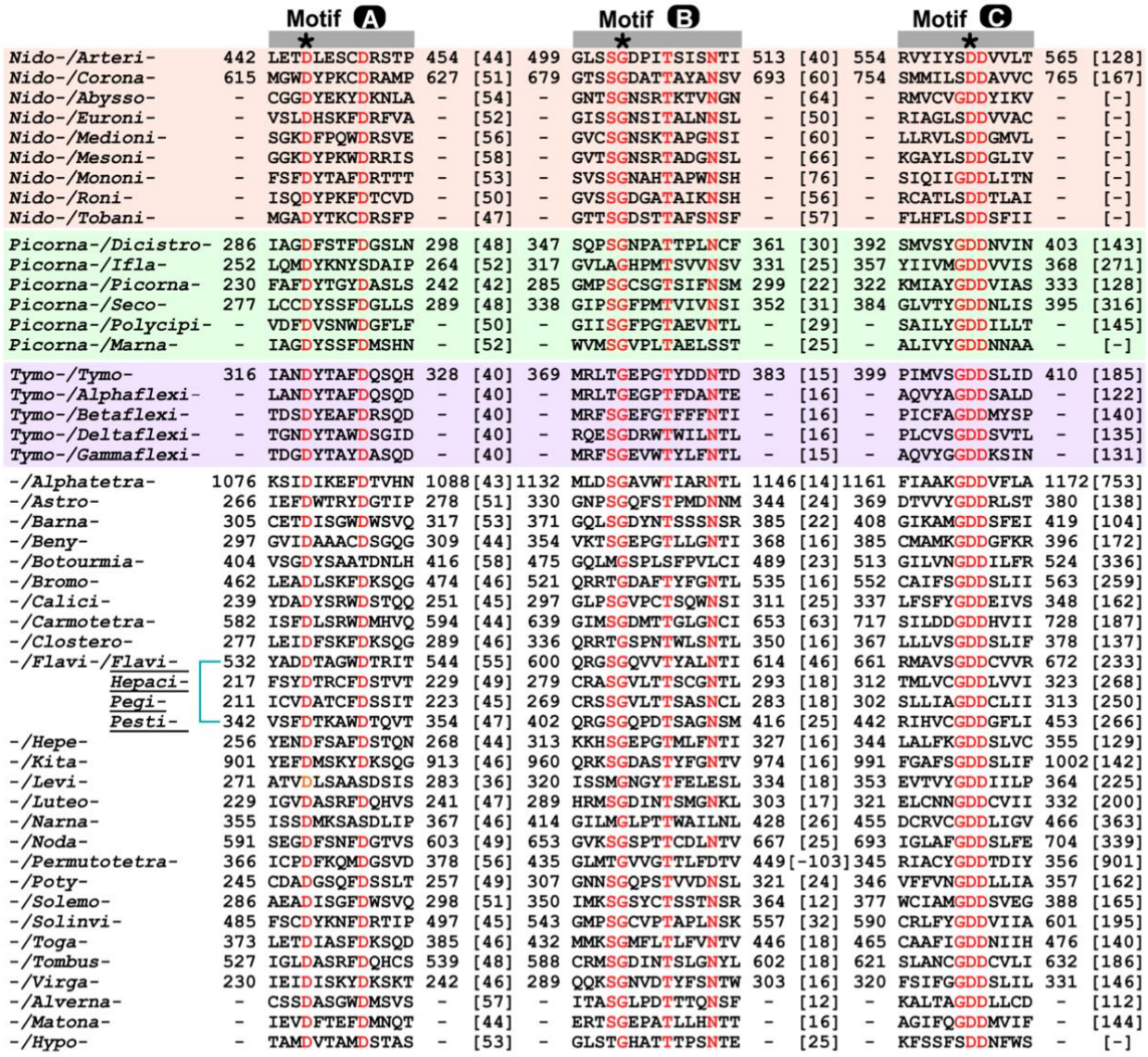



Figure 3.6: Higher resolution picture of Figure 3.5 (Jia & Gong, 2019).

Here the individual amino acid motifs are visible and perfect aligned useful for a phylogenetic analysis. The motifs are cut out of the sequences hiding the fact that the surrounding sequences need gaps to accommodate this alignment; see the numbers within the brackets indicating the number of residues not shown. Highly conserved residues, including three absolutely conserved residues (labelled by asterisks), are shown in red. Numbers within the brackets indicate the number of residues not shown (Jia & Gong, 2019).

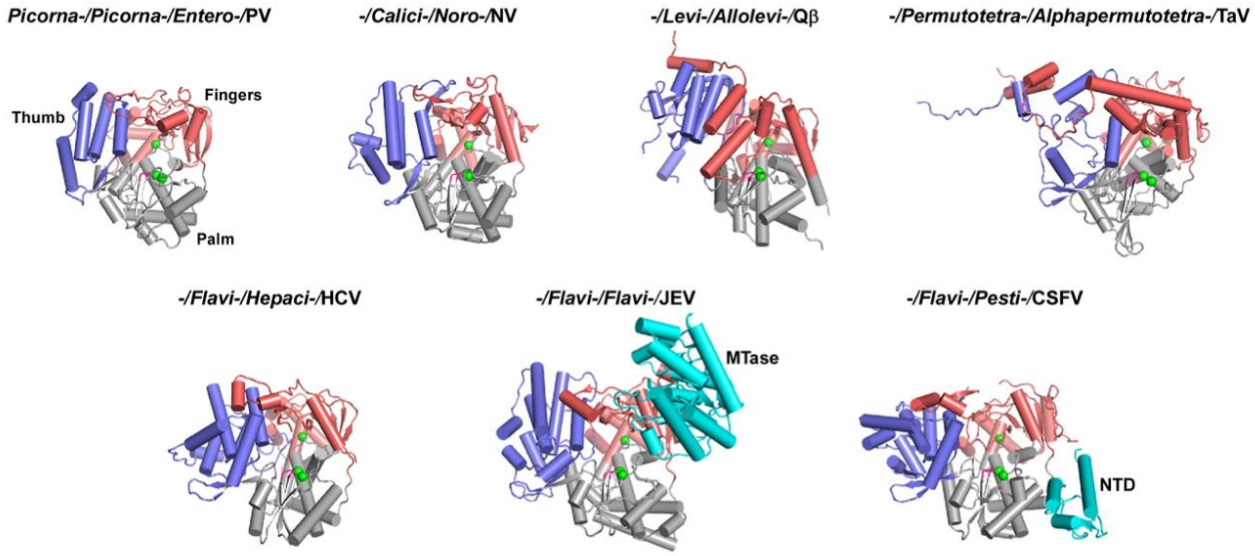



Figure 3.7: Variation in 3d structure RdRp of the seven virus groups represented in Figures 3.5 and 6 (Jia & Gong, 2019).

Order/family/genus/species assignments are shown on top of each structure. PV, poliovirus, PDB entry 1RA6 (chain A); NV, norovirus, PDB entry 1SH0 (chain A); Qβ, bacteriophage Qβ, PDB entry 3MMP (chain G); TaV, Thosea asigna virus, PDB entry 4XHI (chain A); HCV, hepatitis C virus, PDB entry 1C2P (chain A); JEV, Japanese encephalitis virus, PDB entry 4K6M (chain A); CSFV, classical swine fever virus, PDB entry 5YF5 (chain A). Colouring scheme: RdRP palm in gray, thumb in blue, fingers in pink, and signature sequence XGDD in magenta. The α-carbon atom of the three absolutely conserved amino acid residues (labelled by asterisks in Figure 3.6) are shown as green spheres (Jia & Gong, 2019).

## *3.2 Aims*

The aim is to search for new Leishmaniaviruses in *Leishmania* species that so far have not been surveyed to answer the question whether all Leishmaniaviruses have likely a common ancestor in the ancestor of all *Leishmania* species or whether Leishmaniaviruses originated from several independent events of horizonal infection.

The search for new viruses will depend on the quality of primers to be developed. To find appropriate conserved regions for primer design, existing Leishmaniavirus RdRp sequences will be aligned based on conserved protein motifs.

Already existing and newly acquired sequences of RdRp of Leishmaniaviruses will be used to build a robust alignment for use in a later chapter of the thesis that also will allow to apply time estimates for the evolution Leishmaniaviruses.

## *3.3* Method and Materials

### *3.3.1 Samples*

Samples were obtained from a variety of sources used to test for *Leishmaniavirus*.

*a) Canine Blood*

*Leishmania infantum* samples in canine blood were collected from kennel dogs suffering from canine leishmaniasis, sources included Brazil, Spain, Czech Republic, and Cyprus.

*b) Leishmania Trypanosomes*

*Leishmania donovani, Leishmania mexicana,* and *Trypanosoma brucei* were obtained from Keele University. *Leishmania hertigi, Leishmania infantum, Leishmania gerbilli, Leishmania panamensis, Leishmania major, Leishmania venezuelensis, Leishmania amazonensis, Leishmania chagasi, and Leishmania tarentolae* were purchased from The American Type Culture Collection (ATCC), USA.

All samples were tested for *Totivirus* in the first instance.

*c) Related species*

The following species were selected to test for cross-species infection with *Leishmaniavirus*: *Endotrypanum* sp. (Trypanosomatidae), *Herpetomonas megaseliae* (Trypanosomatidae), *Blastocrithidia culicis* (Trypanosomatidae), and *Bodo caudatus* (Bodonidae)

### *3.3.2 RNA Extractions*

Two proprietary kits were used with *Leishmania infantum* cultured cells to compare RNA extraction performance and final concentration of RNA.

*a) SV total RNA Isolation System, Promega*

The procedure followed is shown in 3.3.

Table 3.3: Reagents according to manufacturer's specifications

| Components | | Volume [µl] |
|---|---|---|
| BL + TG Buffer | | 100 |
| 100 % Isopropanol | | 35 |
| RNA Wash Solution (RWA) | | 500 |
| DNase I incubation mix | Yellow Core Buffer | 24 |
| | $MnCl_2$, 0.09M | 3 |
| | DNase I 3 | 3 |
| Column Wash Solution (DSA) | | 200 |
| RNA Wash Solution (RWA) | | 500 |
| RNA Wash Solution (RWA) | | 300 |
| Nuclease-Free Water | | 15 |

*b) RNeasy Plus Micro kit, QIAGEN*

The procedure followed is shown in Table 3.4.

Table 3.4: Reagents according to manufacturer's specifications

| Components | Volume [µl] |
|---|---|
| RLT Buffer | 350 |
| 70 % ethanol | 350 |
| RW1 Buffer | 700 |
| RPE | 500 |
| 80 % ethanol | 500 |
| RNase-free water | 14 |

The RNeasy Plus Micro protocol was used to extract total RNA from tissue or cultured sample.

The RNeasy Plus Micro kit was selected for the Leishmaniavirus isolation experiments, as it resulted in the highest concentrations of RNA in the final output.

### *3.3.3 Primer Design*

Oligonucleotide primers are needed to perform PCRs. A key feature of primers is that they have to fit the sequences in the template molecule, but do not necessarily have to completely suit the template strand. The length of the primer is also very important, short primers may lead to non-specific binding and long primers can lead to hairpins, self-dimers, primer dimers, and chimeras.

RdRp nucleic acid sequences were downloaded from GenBank, downloaded from the sequencing facility, or manually typed in for older publications, where sequences had not been uploaded to a database. Care was taken to download only the most up-to-date version of a given sequence. The nucleic acid sequences were translated to amino acid sequences in Geneious v10.1.3 which were aligned, despite the absence of stop codons, comparing several algorithms to find the highest degree of conservation. Primers were based on sequences obtained from the National Center for Biotechnology Information website. RdRp sequences of dsRNA viruses are notoriously difficult to align because of very little conservation of the primary sequence at the amino acid level (Rozanov *et al.*, 1992; Ilyina & Koonin, 1992; Bruenn, 1993; Butcher *et al.*, 2001; Sarin *et al.*, 2012). The best alignment of motifs was selected as the guide to appropriate alignment of the sequences for the detection of *Leishmaniavirus* in *Leishmania* species. In the absence of structural motifs, the most parsimonious alignment was chosen.

By way of example, amino acid alignments of RdRp for *L. aethiopica virus* and *Leishmania RNA virus 2* showing the selected motifs are shown below, Figures 3.8 – 3.9. A new suggested motif is labelled 'G' for the person detected the putative motif.

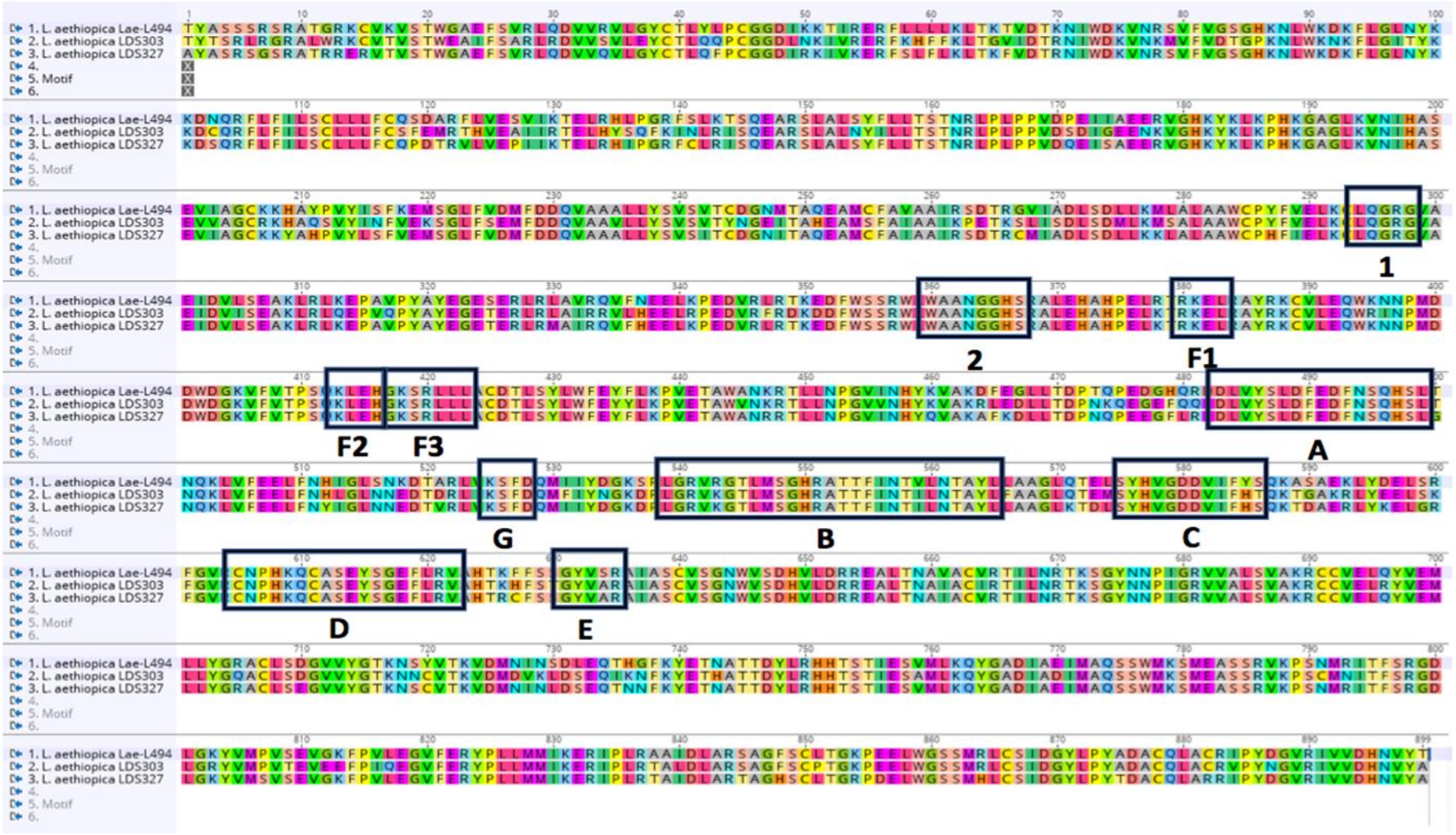



Figure 3.8: Amino acid alignment of RdRp of *Leishmania aethiopica virus 2* (LRV2) showing selected Motifs. Colour scheme is for contrast only. The new suggestion motif is called G

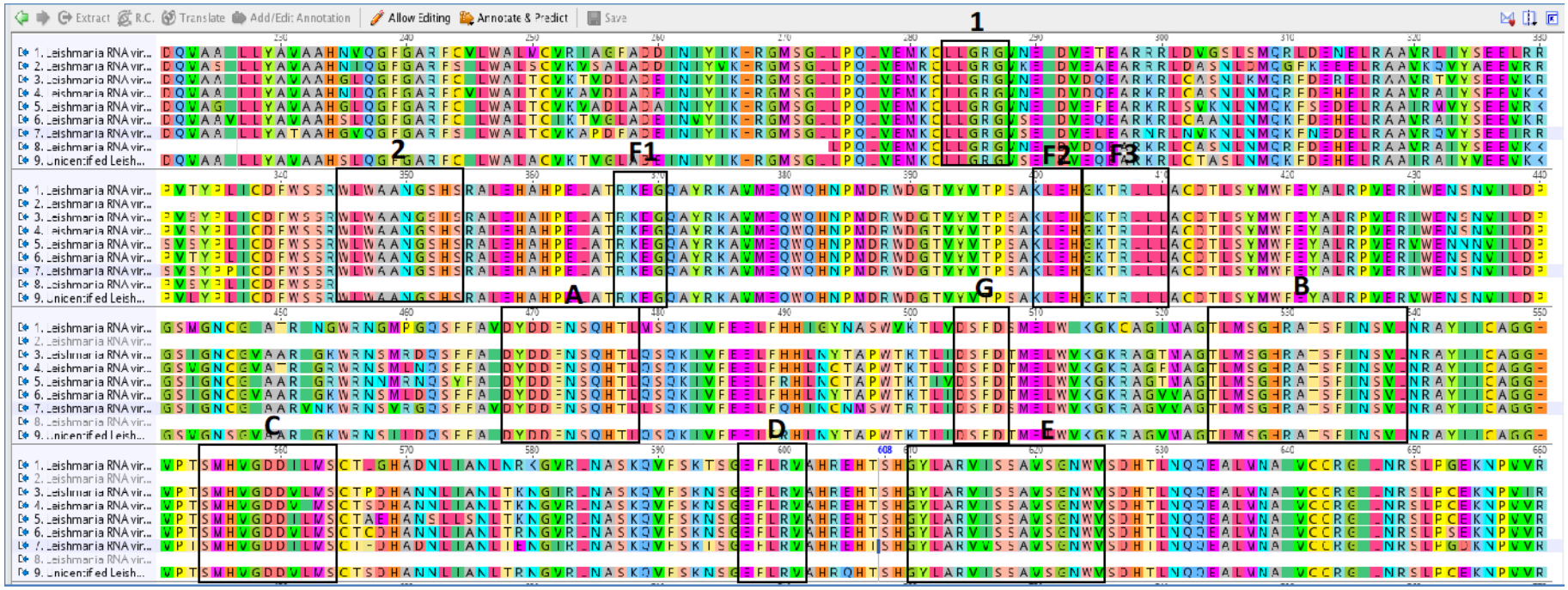
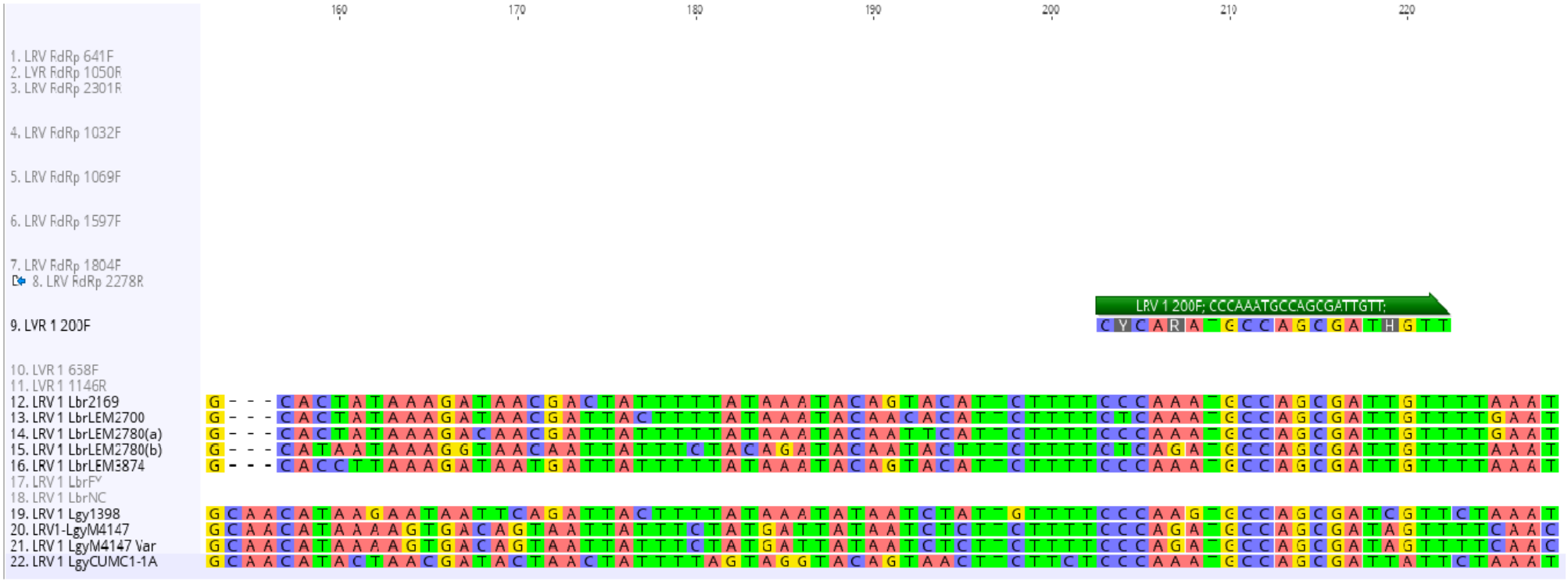



Figure 3.9: Amino acid alignment of RdRp of *Leishmania RNA virus 1* (LRV1) showing selected Motifs. Colour scheme is for contrast only. The new suggested motif is labelled 'G'.

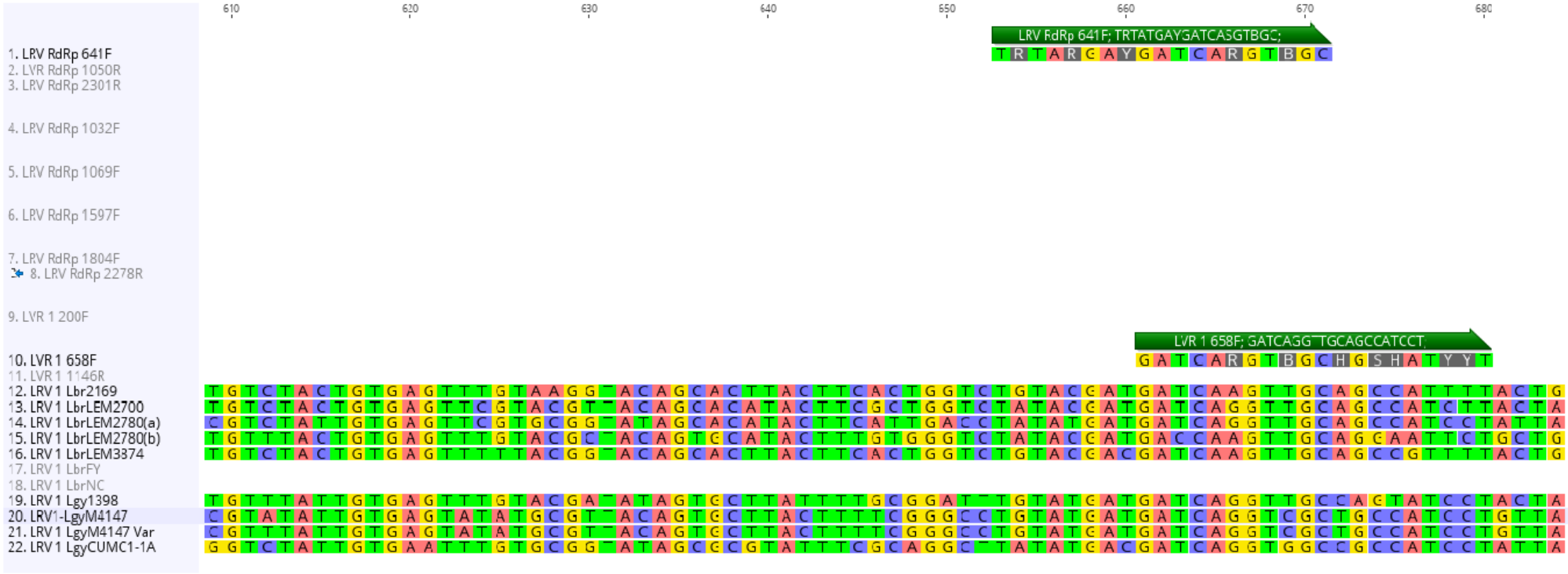
Unique primer sequences were generated using the algorithm implemented in Geneious. The aligned sequences and primers designed for the various types of nested PCRs, for both New World *Leishmania RNA virus 1* and Old World *Leishmania RNA virus 2* are shown in 3.10, 3.11 and 3.12 below.

**Figure 3.10 start: caption below**
0-~ 230




Primer legend: **LRV1 RdRp 200F**- 1146R nested outer

**Figure 3.10 continue 1: caption below**
~ 610-690

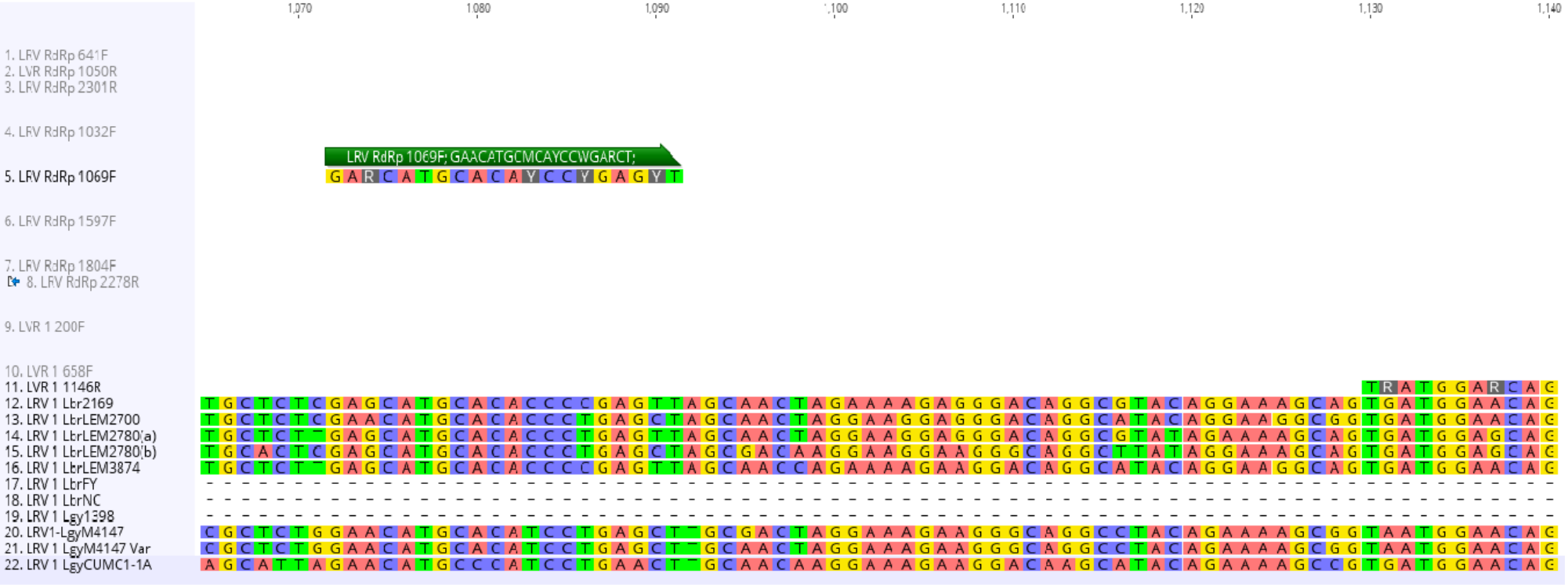



Primer legends: **LRV1 RdRp 641F**-1050R straight, **LRV1 RdRp 658F**-1146R nested inner

**Figure 3.10 continue 2: caption below**
~ 990-1,070

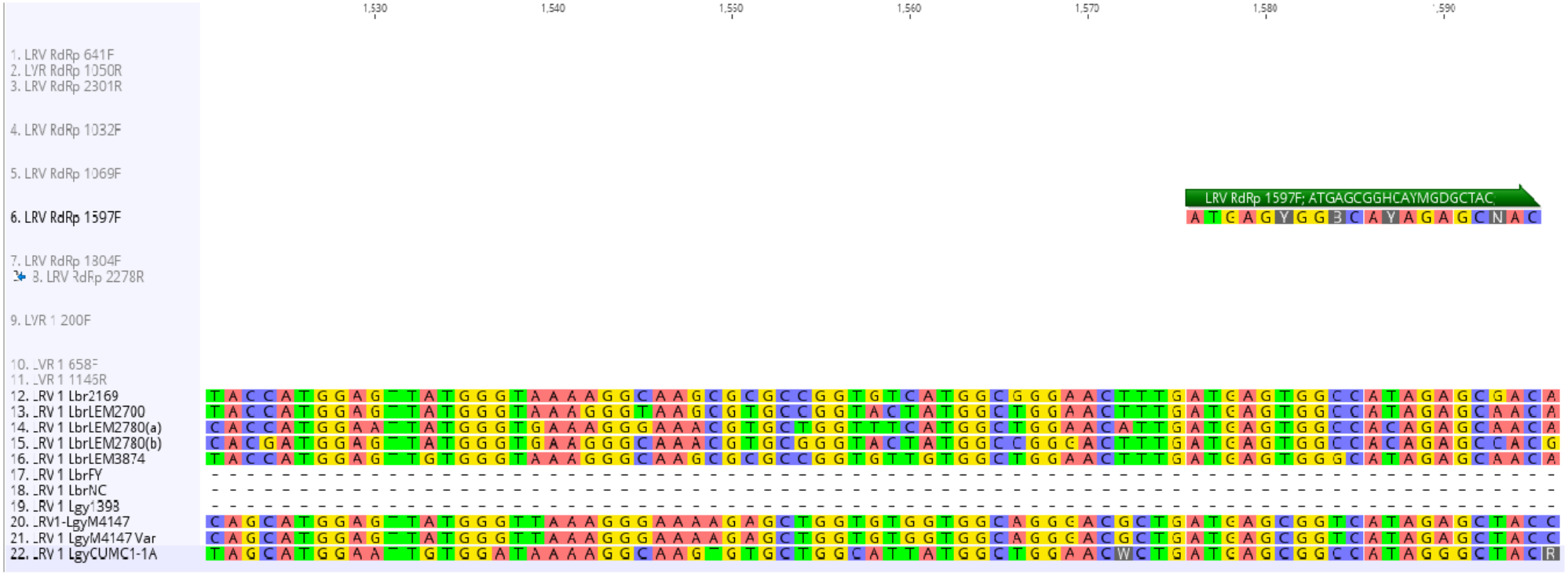

Primer legend: **LRV1 RdRp 1032F**- 2301R nested outer

**Figure 3.10 continue 3: caption below**
~ 1,060-1,140



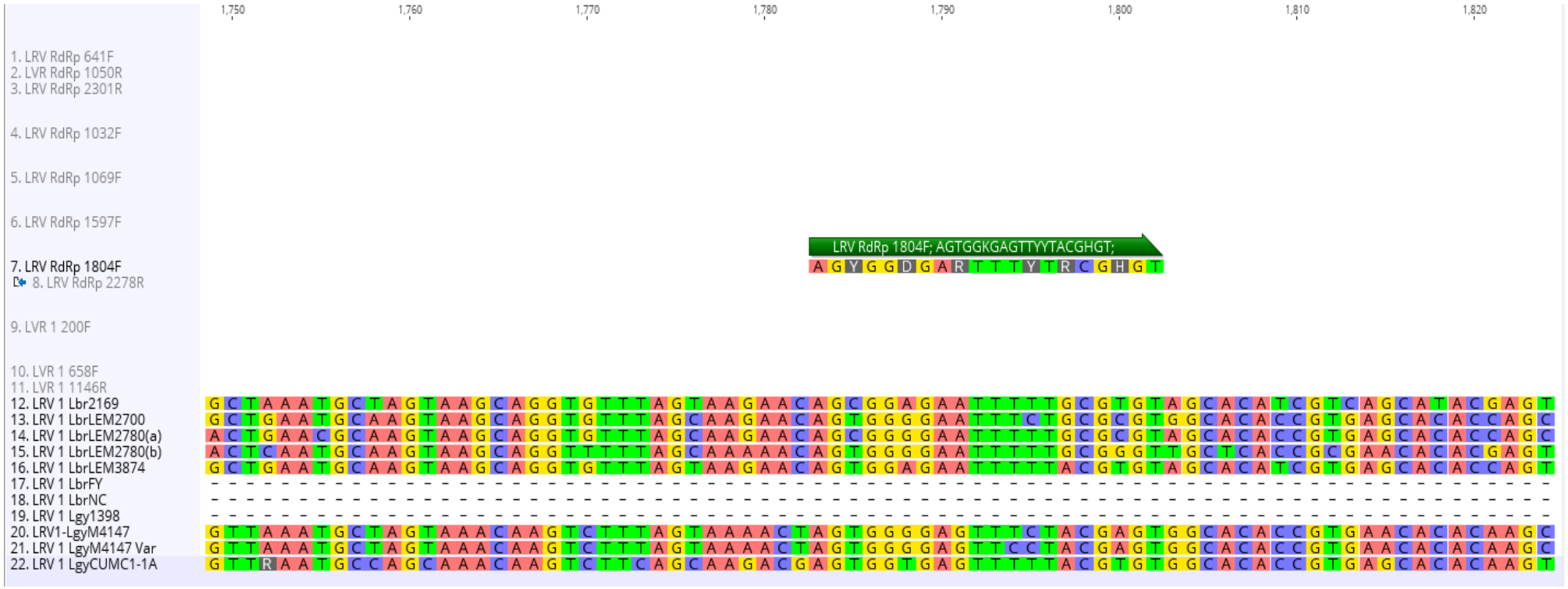

Primer legend: **LRV1 RdRp 1069F**- 2278R nested inner option 1

**Figure 3.10 continue 4: caption below**
~ 1,520-1,600




Primer legend: **LRV1 RdRp 1597F**- 2278R nested inner option 3

**Figure 3.10 continue 5: caption below**
~ 1,750-1,830




Primer legend: **LRV1 RdRp 1804F**- 2278R nested inner option 4

**Figure 3.10 continue 6: caption below**
~ 2,210-2,280

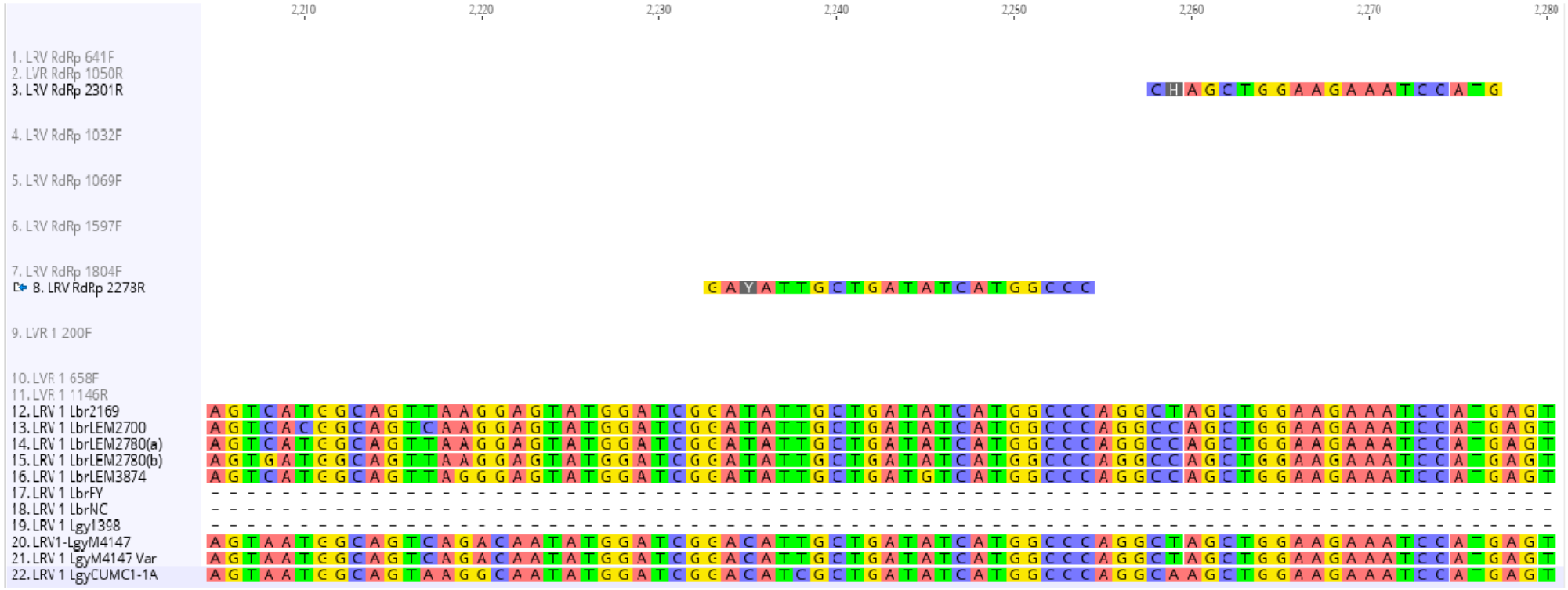



Figure 3.10: primer legends : LRV1 RdRp 200F- 1146R nested outer, LRV1 RdRp 641F-1050R straight, LRV1 RdRp 658F-1146R nested inner, LRV1 RdRp 1032F- 2301R nested outer, LRV1 RdRp 1069F- 2278R nested inner option 1, LRV1 RdRp 1597F- 2278R nested inner opt

**Figure 3.11 start: caption below**
0-~ 230

**Figure 3.11 continue 1: caption below**
~ 1,410-2,200

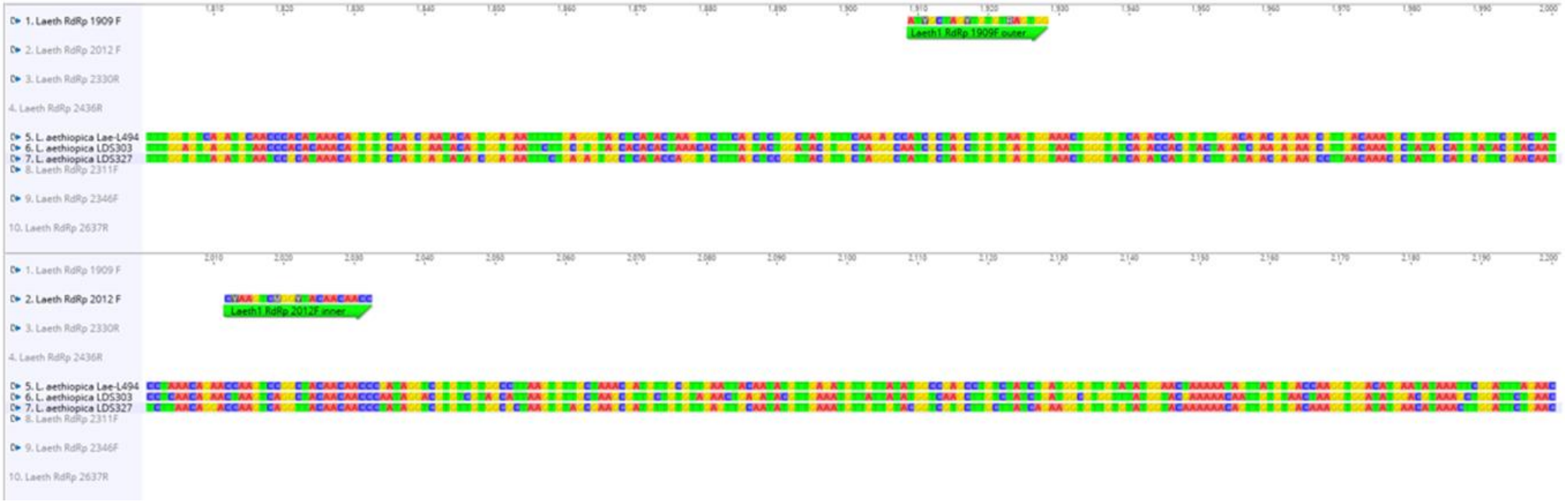



Figure 3.11 Primer legend: Laeth1 RdRp 1909F- 2436R nested outer, Laeth1 RdRp 2012F- 2330R nested inner

**Figure 3.11 continue 2: caption below**
~ 2,200-2,400

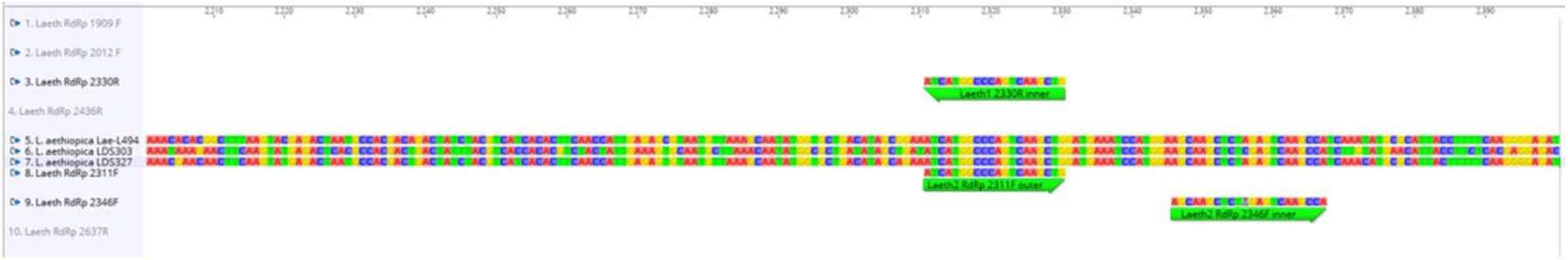



**Figure 3.11** Primer legend: Laeth1 RdRp 2012F- **2330R** nested inner, **Laeth2 RdRp 2311F**- 2637R semi-nested outer, **Laeth2 RdRp 2346F**- 2637R semi-nested inner

**Figure 3.11 continue 3: caption below**
~ 2,400-2,700

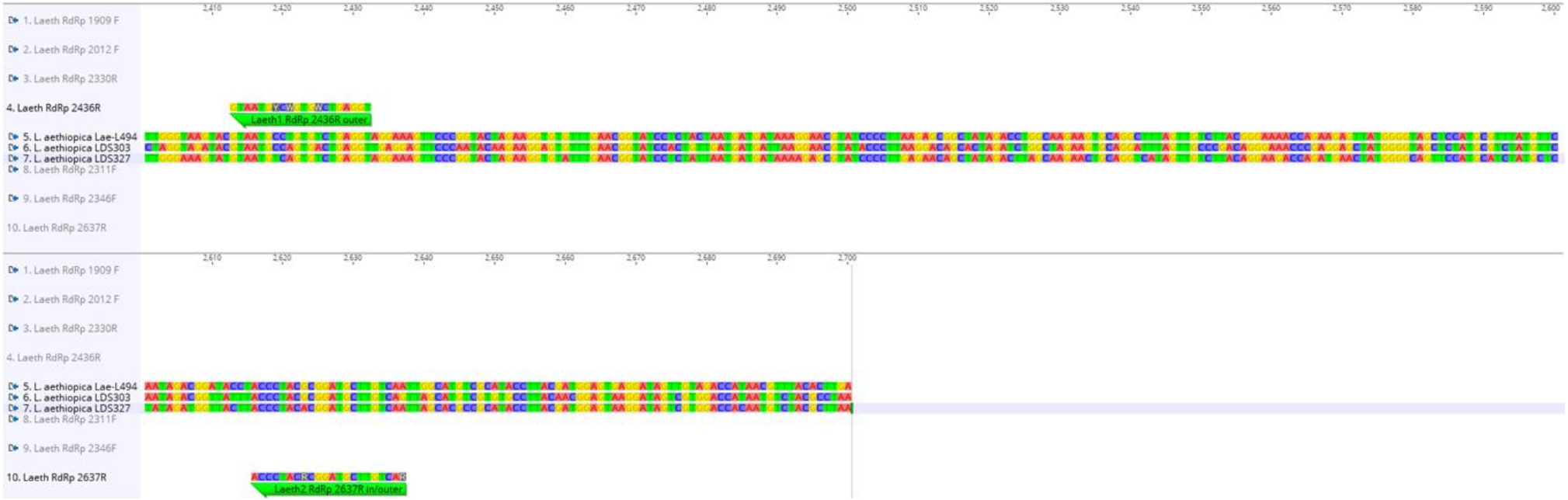



Figure 3.11: Primer alignment for RdRp of *Leishmania RNA virus 2* from divergent *L. aethiopica* only. primer legend: Laeth1 RdRp 1909F- 2436R nested outer, Laeth1 RdRp 2012F- 2330R nested inner option1, Laeth1 RdRp 2012F- 2330R nested inner, Laeth2 RdRp 231

**Figure 3.12 start: caption below**
~ 60-800

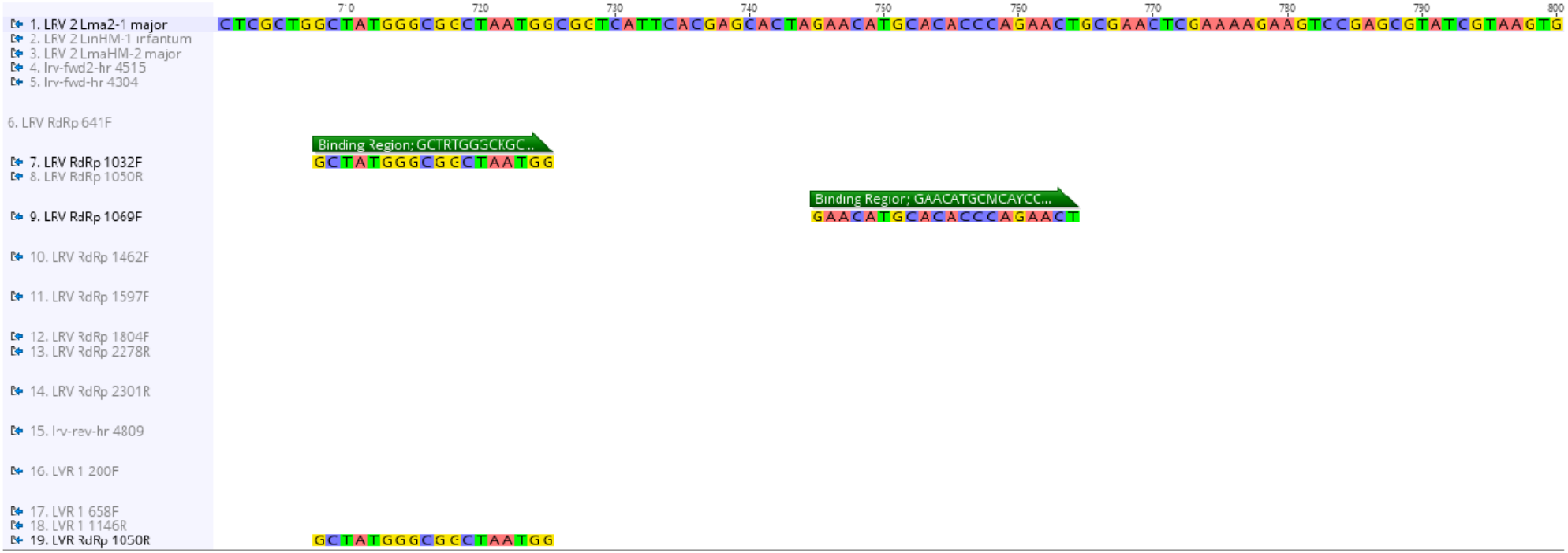


**Figure 3.12 continue 1: caption below**
~ 1,100 -1,300

**Figure 3.12 continue 2: caption below**
~ 1,400-1,500

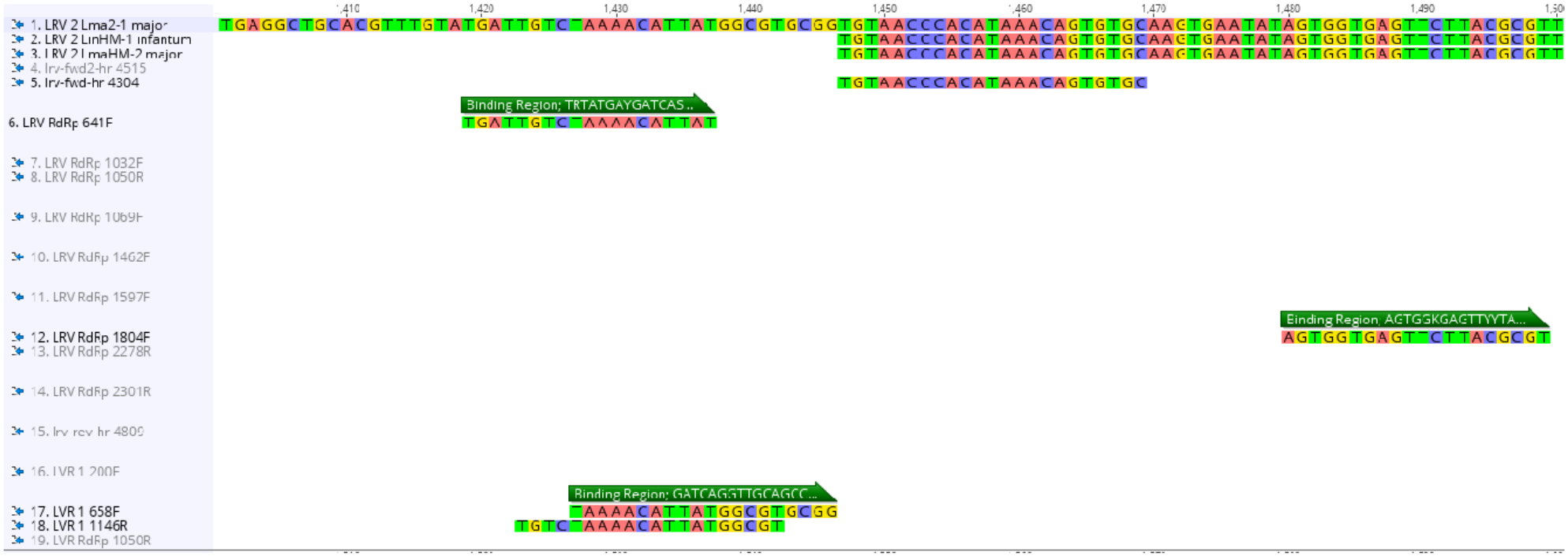


**Figure 3.12 continue 3: caption below**
~ 1,600-1,700

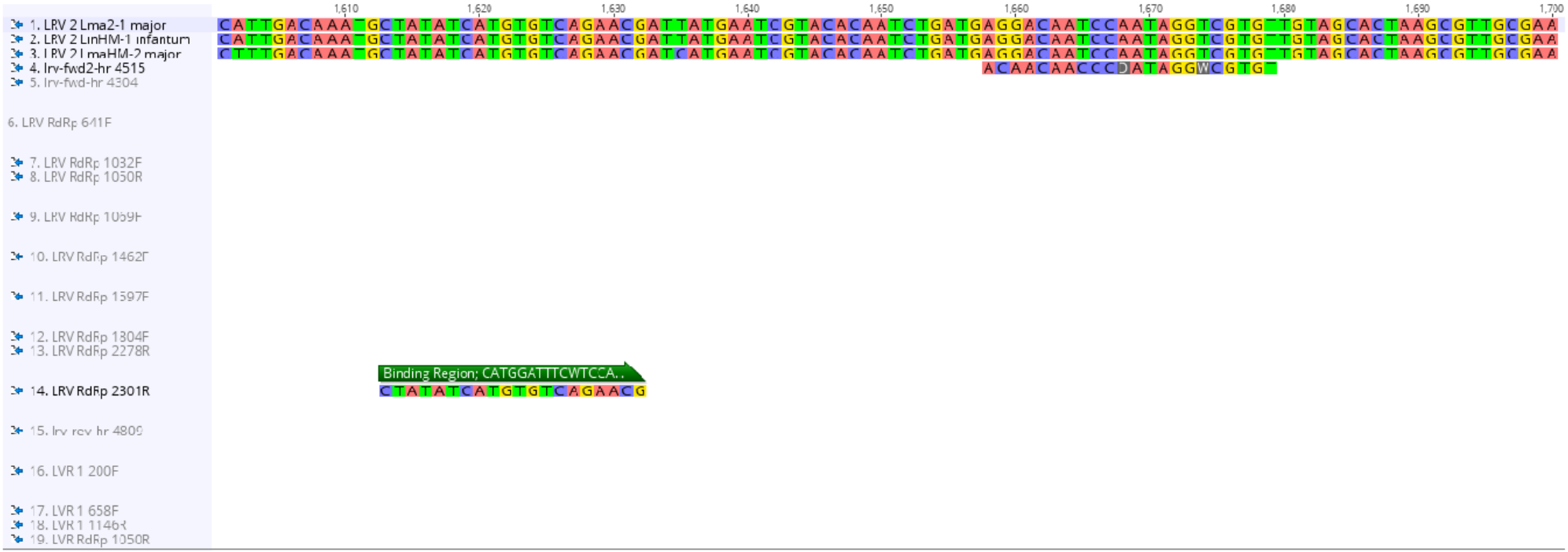


**Figure 3.12 continue 4: caption below**
~ 1,800-2,000

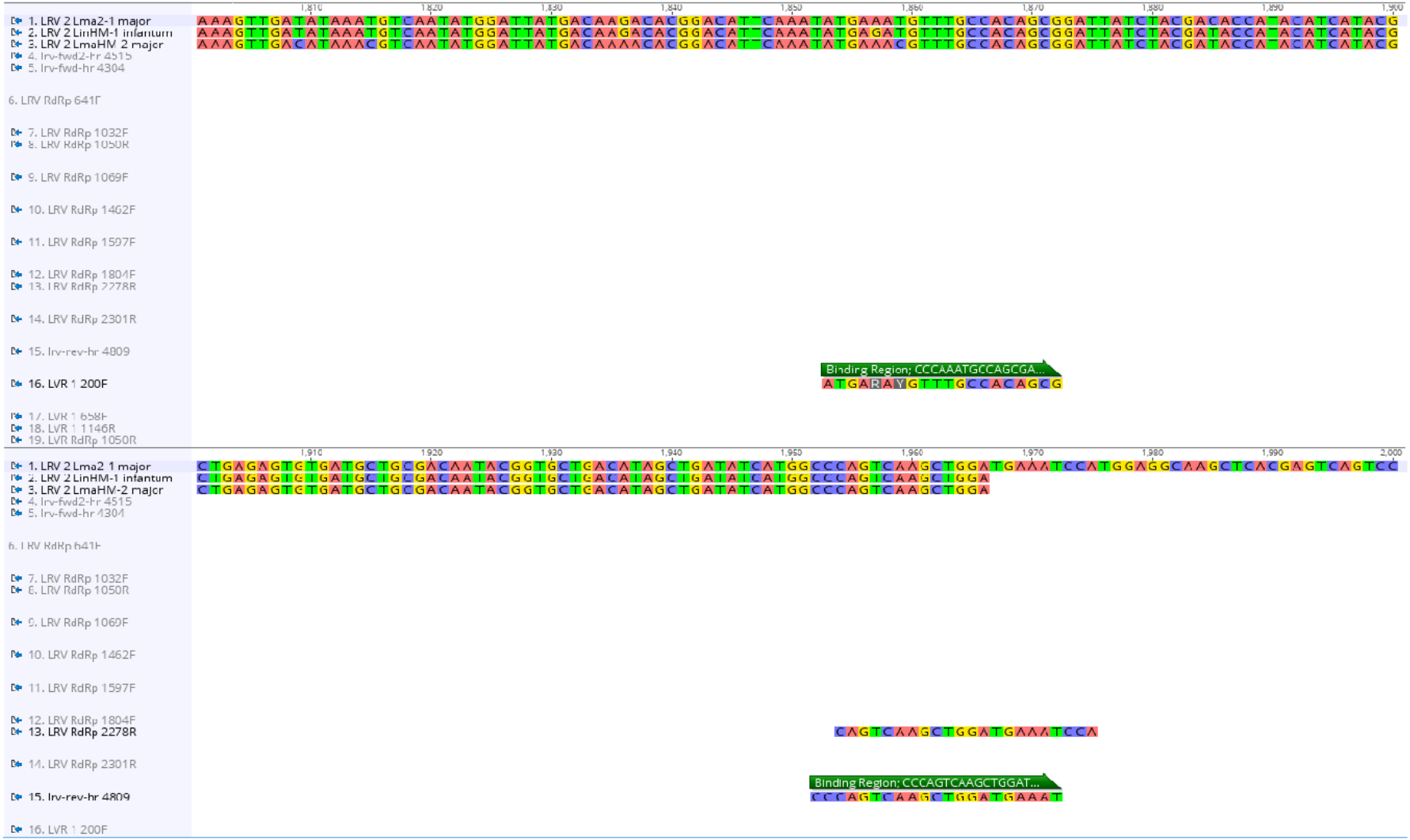



Figure 3.12: Alignment of PCR primers for RdRp of *Leishmania RNA virus 2* without *L. aethiopica*.

### *3.3.4 Primers*

Most conserved primers for RNA-dependent RNA polymerase of Leishmania RNA virus 1 and 2.

*a)* New World *Leishmania - Leishmania RNA virus 1*

Five different sets of primers were designed as shown in (Table 3.5).

Table 3.5: All Primer Sets used to detect *Leishmania RNA Virus 1*

| Set | Name | Sequence | Frag. size (bp) | Temp. (ºC) | PCR type |
|---|---|---|---|---|---|
| 1 | LRV1 RdRp 200F-1146R | CYC ARA TGC CAG CGA THG TT ATG CTG CCA CTG YTC CAT YA | 947 | 55 | Nested outer |
| | LRV1 RdRp 658F-1146R | GAT CAR GTB GCH GSH ATY YT ATG CTG CCA CTG YTC CAT YA | 487 | 55 | Nested inner |
| 2 | LRV1 RdRp 641F-1050R | TRT ARG AYG ATC ARG TBG C GYT RTG GGC TGC DAA YGG | 409 | 50 | Straight |
| 3 | LRV1 RdRp 1129F-2264R | GTR ATG GAR CAG TGG CAR CA CAG CTD GCC TGG GCC ATG A | 1136 | 53 | Semi-Nested outer |
| | LVR1 RdRp 1864F-2264R | GGS AAY TGG GTS AGT GAY CA CAG CTD GCC TGG GCC ATG A | 401 | 53 | Semi-Nested inner |
| 4 | LVR1 RdRp 1864F-2604R | GGS AAY TGG GTS AGT GAY CA DCC ACC ACA AGG AAG TTT TTG | 741 | 53 | Semi-Nested outer |
| | LVR1 RdRp 2239F-2604R | GCT GAT RTC ATG GCC CAG GC DCC ACC ACA AGG AAG TTT TTG | 364 | 53 | Semi-Nested inner |
| 5 | LRV1 RdRp 1032F-2301R | GYT RTG GGC TGC DAA YGG CAT GGA TTT CTT CCA GCA GCT DG | 1243 | 50 | Nested outer |
| | LRV1 RdRp 1069F-2278R | GAA CAT GCM CAY CCW GAR CT GAY ATT GCT GAT ATC ATG CCC C | 1183 | 55 | Nested inner |
| | LRV1 RdRp 1462F-2278R | GAR CAT GCA CAY CCY GAG YT GAY ATT GCT GAT ATC ATG CCC C | 814 | 50 | Nested inner |
| | LRV1 RdRp 1597F-2278R | ATG AGC GGH CAY MGD GCT AC GAY ATT GCT GAT ATC ATG CCC C | 681 | 50 | Nested inner |
| | LRV1 RdRp 1804F-2278R | AGT GGK GAG TTY YTA CGH GT GAY ATT GCT GAT ATC ATG CCC C | 474 | 50 | Nested inner |

*b)* Old World *Leishmania - Leishmania RNA Virus 2*

Three specific primers were designed as shown in (Table 3.6) below.

Table 3.6: All Primer Sets used to detect *Leishmania RNA Virus 2*

| Set | Name | Sequence | Frag. Size (bp) | Temp. (ºC) | PCR type |
|---|---|---|---|---|---|
| 1 | LVR2 RdRp 1451F-1964R | ACC CAC ATA AAC AGT GTG CA<br>CAG CTT GAC TGG GCC ATG A | 514 | 55 | Nested outer |
| | LVR2 RdRp 1587F-1923R | RGA CCG RCG AGA AGC WTT GA<br>TTG TCG CAG CAT CAC ACT CT | 337 | 57 | Nested inner |
| ***Leishmania aethiopica* primer sets** | | | | | |
| 1 | Laeth1 RdRp 1909F- 2436R | ATY GCT AGY TGT GTR AGT GG<br>ACC TCA GWC ACW GRC ATT AC | 524 | 52 | Nested outer |
| | Laeth1 RdRp 2012F- 2330R | CYA AGT CMG GYT ACA ACC<br>CAG CTT GAC TGG GCC ATG AT | 319 | 52 | Nested inner |
| 2 | Laeth2 RdRp 2311F- 2637R | ATC ATG GCC CAG TCA AGC TG<br>YTG ACA AGC ATC CGY GTA GGG T | 327 | 57 | Semi-Nested outer |
| | Laeth2 RdRp 2346F- 2637R | AGC AAG CTC TMG AGT CAA GCC A<br>YTG ACA AGC ATC CGY GTA GGG T | 292 | 57 | Semi-Nested inner |

### *3.3.5 Complementary DNA*

Prior to PCR amplifications it is necessary to generate first strand complementary DNA (cDNA) via reverse transcription.

SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase from Invitrogen was used for this process, according to the manufacturer's instructions as shown in Table 3.7 – 3.9.

PCR Reaction Mix to manufacturer's specifications SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase

Table 3.7: PCR Reaction Mix for SuperScript® III One-Step RT-PCR cDNA Reverse Transcription

| Components | Volume [µl] |
|---|---|
| 2 × Reaction mix | 12.5 |
| Primer ACTB-F | 1 |
| Primer ACTB-R | 1 |
| Template RNA | 1 |
| Ultra pure water | to 12.5 |
| SuperScript® III RT/Platinum® Taq Mix | 2 |

Table 3.8: cDNA Synthesis Procedure

| Steps | Temp [ºC] | Time [mins] |
|---|---|---|
| Initial Denaturation | 65 | 5 |
| Denaturation | 90 | 1 |
| Annealing | 50 | 50 |
| Extension | 85 | 5 |
| Final Extension | 4 | 1 |
| Final Step | 37 | 20 |

Table 3.9: PCR conditions for SuperScript® III One-Step RT-PCR

| Steps | | Temp. [ºC] | Time [sec.] |
|---|---|---|---|
| Initial Denaturation | | 95 | 60 |
| Denaturation | | 95 | 15 |
| Annealing | 35 cycles | 55 | 15 |
| Extension | | 72 | 10 |
| Final Extension | | 10 | Hold |

SuperScript™ IV First-Strand Synthesis System was then used to improve final cDNA product, according to the manufacturer's instructions Table 3.10 – 3.12.

PCR Reaction Mix to manufacturer's specifications, SuperScript® IV One-Step RT-PCR System with Platinum® Taq DNA Polymerase

Table 3.10: PCR Reaction Mix for SuperScript® IV First-Strand Synthesis cDNA Reverse Transcription

| Components | Volume [µl] |
|---|---|
| DEPC-treated water | 37.8 |
| 10 × high fidelity buffer | 5 |
| 50 mM $MgSO_4$ | 2 |
| 10 mM dNTP mix (10 mM each) | 1 |
| Forward Primer | 1 |
| Reverse Primer | 1 |
| Template RNA | 2 |
| Platinum™ *Taq* DNA polymerase high fidelity | 0.2 |

Table 3.11: cDNA Synthesis Procedure and Mixture

| Steps | Volume (µL) | Temp (ºC) | Time (mins) |
|---|---|---|---|
| **Tube 1** | | | |
| Random hexamers | 1 | | |
| 10 mM dNTP mix (10 mM each) | 1 | | |
| Template RNA | Up to 11 | | |
| DEPC-treated water | To 13 | | |
| Mix briefly and incubate | | 65 | 1-5 |
| **Tube 2** | | | |
| 5 × SSIV buffer | 4 | | |
| 100 mM DTT | 1 | | |
| Ribonuclease inhibitor | 1 | | |
| SuperScript® IV reverse transcriptase | 1 | | |
| Mix briefly and add tube 1 to tube 2 | | Room temp. | |
| Incubate | | 23 | 10 |
| Incubate | | 50 - 55 | 10 |
| Incubate | | 80 | 10 |
| *E. coli* RNase H incubate | 1 | 37 | 20 |

Table 3.12: PCR conditions for SuperScript® IV First-Strand Synthesis

| Steps | | Temp. (ºC) | Time (sec.) |
|---|---|---|---|
| Initial Denaturation | | 94 | 120 |
| Denaturation | | 94 | 15 |
| Annealing | 35 cycles | 55 | 30 |
| Extension | | 68 | 60 |
| Final Extension | | 4 | Hold |

### *3.3.6 PCR Amplification*

Two types of PCR were used, normal and nested. Normal PCRs are generally used for high concentrations of DNA, whereas nested PCRs perform better when the concentrations are low. Normal PCR uses only one set of primers and one amplification step. Semi-nested and nested PCR uses two different sets of primers. The first set amplifies a larger fragment but not to alevelwhere a band would be visible on an agarose gel. Then a small amount of the first amplification is used in a second PCR reaction that amplifies a smaller fragment within the larger fragment to a level where bands become visible on agarose gels. As concentrations were low, nested PCRs were preferred using the same proprietary kit, primers and conditions.

### *3.3.7 Gel Preparation and Electrophoresis*

A 2 % agarose gel was prepared by adding 2 g agarose in 100 mL to 1 × TBE buffer and dissolved by microwaving, then cooled to 50 – 55 ° C. Ethidium bromide (see lab book) was mixed in and the solution poured into a mould and left to set. When set, the gel was placed into an electrophoresis and the PCR product samples loaded. A current was applied across the gel at 150 V for ~35 minutes. This method was used for all PCR products.

### *3.3.8 Sequencing*

All gel positive bands were Sanger sequenced in both directions. If a single band was shown on the gel, a larger PCR reaction was set up, and then purified for sequencing. If other bands were visible on the gel, a larger PCR reaction was set up again, run on several lanes on a gel, then bands cut out, and the DNA extracted.

## *3.4* Results

### *3.4.1 Leishmaniavirus in New World Leishmania*

*Leishmaniavirus* was detected in species of *Leishmania* from the New World in which it has not previously been found. In *Leishmania panamensis* (Figure 3.13) and *L. mexicana* (Figure 3.14) it was successfully amplified by the New World primer set 5 (Table 3.5), designed to detect *Leishmania*

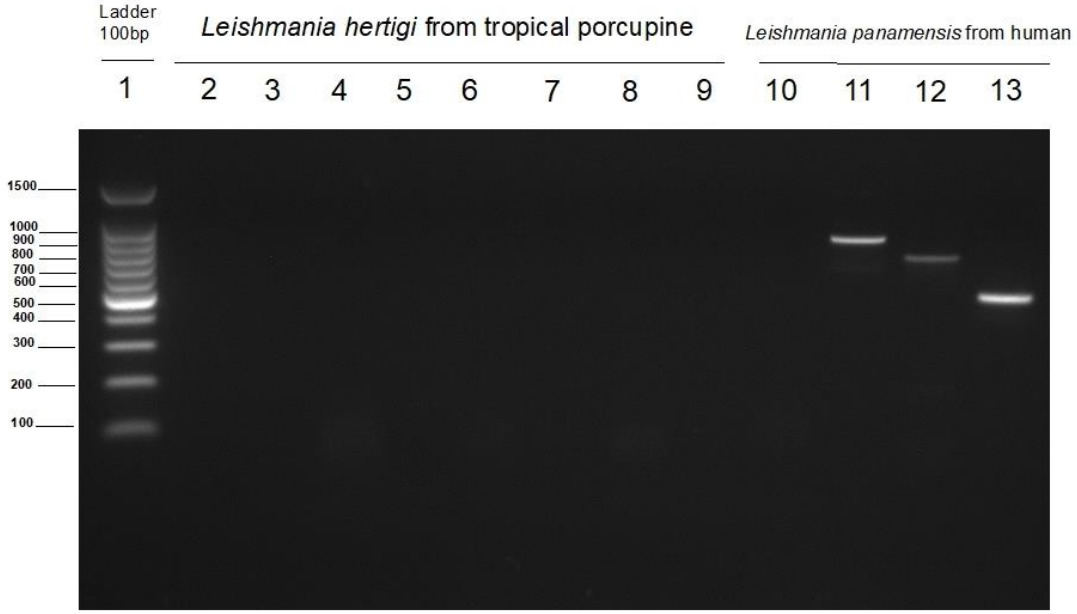



Figure 3.13: Electropherogram of *Leishmania panamensis and Leishmania hertigi*, LRV1 Primers. 2 % agarose gel electropherogram of PCR products. with primers: LVR1 RdRp 1069F- 2278R, LVR1 RdRp 1462F- 2278R, LVR1 RdRp 1597F- 2278R and LVR1 RdRp 1804F- 2278R respectively. Lane 1 - 100 bp Easy ladder. Lanes 2, 3, 4, and 5 *L. hertigi* no bands. Lane 6, 7, 8, and 9 *L. hertigi* no bands. Lane 2, 6 and 10 are LVR1 RdRp 1069F-2278R no bands. Lanes 11, 12, and 13 *L. hertigi* no bands *L. hertigi* no bands *L. hertigi* no bands bends showing *Leishmaniavirus* present

*RNA virus 1* in New World Leishmania. For two species, *Leishmania hertigi* (Figure 3.13) Old World primer set 1 was successful. These results were confirmed by Sanger sequences retrieved from GenBank using BLAST which showed expected matches to *Leishmania* viruses (Table 3.12 – 3.13).

Table 3.13: BLAST Output for Species in Figure 3.13 and 3.14

| Sample ID | BLAST output | Accession no. | RNA seq. |
|---|---|---|---|
| *L. panamensis* | *Leishmania RNA virus 2 - 1* isolate LRV2/EP97/TR/Lt07 | MK246759.1 | See Appendix 3.1 |
| *L. mexicana* | *Leishmania RNA virus 2-1* | U32108.1 | |
| *L. mexicana* | *Leishmania RNA virus 2-1* | U32108.1 | |

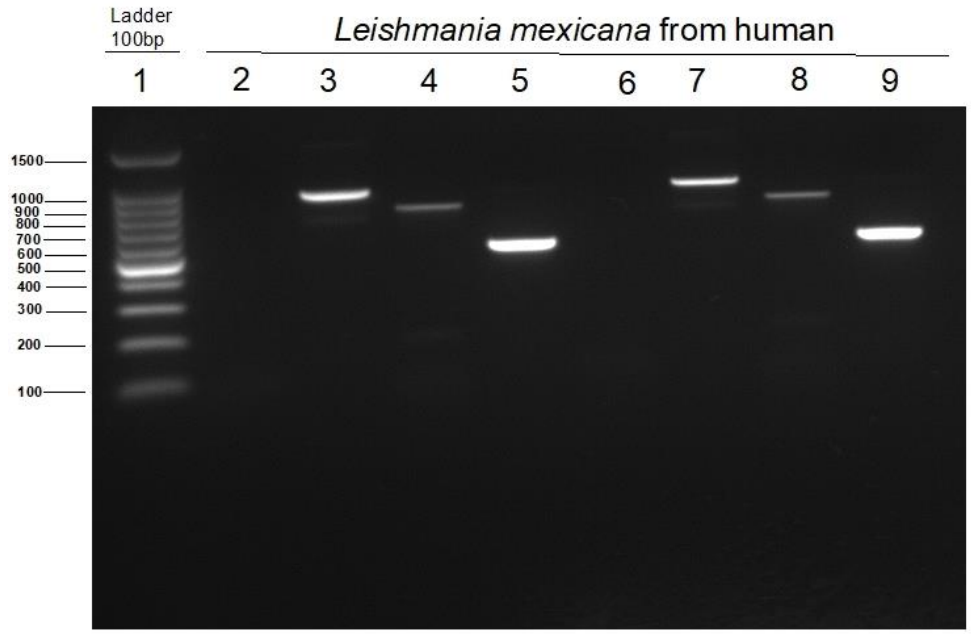



Figure 3.14: Figure 3.14: Electropherogram of *Leishmania mexicana, LRV1 Primers.* 2 % agarose gel electropherogram of PCR product with primers: LVR1 RdRp 1069F- 2278R, LVR1 RdRp 1462F- 2278R, LVR1 RdRp 1597F- 2278R and LVR1 RdRp 1804F- 2278R respectively. Lane 1 - 100 bp Easy ladder. Lane 3, 4, 5, 7, 8, and 9 *L. mexicana*, bands showing *Leishmaniavirus* present. Lane 2 and 6 are LVR1 RdRp 1069F- 2278R no bands.

Table 3.14: BLAST Output for Positive Species in Figure 3.14

| Sample ID | BLAST out put | Accession no. | RNA seq. |
|---|---|---|---|
| *L. hertigi* | *Leishmania RNA virus 2 - 1* isolate LRV2/CBU33/TR/Lm01 | MK246760.1 | See Appendix 3.1 |

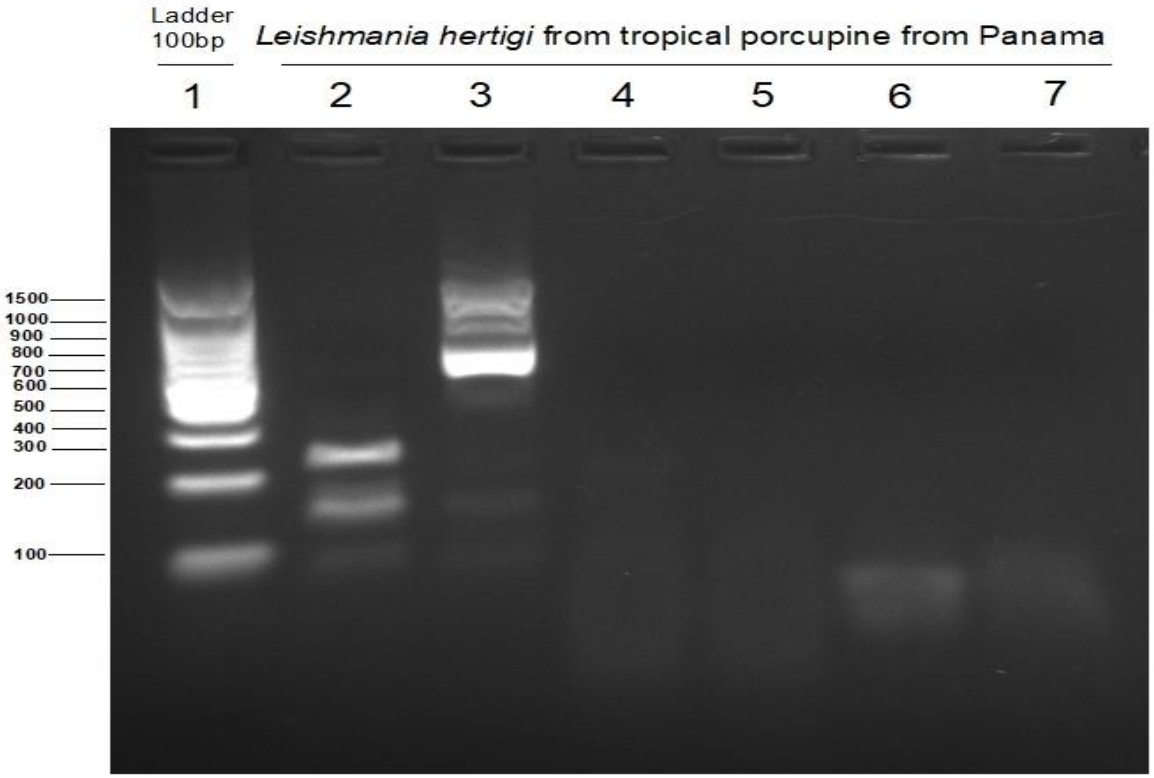



Figure 3.15: Electropherogram of *Leishmania hertigi*, LRV1 Comparing Primers. 2 % agarose gel electropherogram of PCR products. with primers: LVR1 RdRp 1069F- 2278R, LVR1 RdRp 1462F- 2278R, LVR1 RdRp 1597F- 2278R and LVR1 RdRp 1804F- 2278R respectively. Lane 1 - 100 bp Easy ladder. Lanes 2 and 3 primers LVR1 RdRp 658F-1146R bands show *Leishmaniavirus* present. Lane 4 and 5 primers LVR1 RdRp 1864F-2264R, no bands. Lane 6 and 7 primers LVR1 RdRp 2239F-2604R no bands.

### *3.4.2 Leishmaniavirus in New and Old World Leishmania, Old World Leishmania Primers*

*Leishmaniavirus LRV1* was detected in some species of New World Leishmania using Old World Leishmania primers. It was present in *Leishmania hertigi* as shown in (Figure 3.15 and 3.16). LRV2 was also detected with the same primers in the Old World host species *L. major*. Other species in which *Leishmaniavirus* was detected for the first time were *L. amazonensis*, *L. venezuelensis*, and *L. chagasi*, as shown in and the related *Endotrypanum* sp. (Figure 3.17), LRV2 was detected in all 4 species with Old World primers, whereas LRV1 with New World primers was only detected in *L. amazonensis* and *L. chagasi*. These results were confirmed by Sanger sequences retrieved from GenBank using BLAST which showed expected matches to Leishmaniaviruses (Table 3.15)

Table 3.15: BLAST Output for Positive Species in Figure 3.16

| Sample ID | BLAST out put | Accession no. | RNA seq. |
|---|---|---|---|
| *L. hertigi* | *Leishmania RNA virus 2 - 1* isolate LRV2/CBU33/TR/Lm01 | MK246760.1 | See Appendix 3.1 |
| *L. major* | *Leishmania RNA virus 2-1* | U32108.1 | |

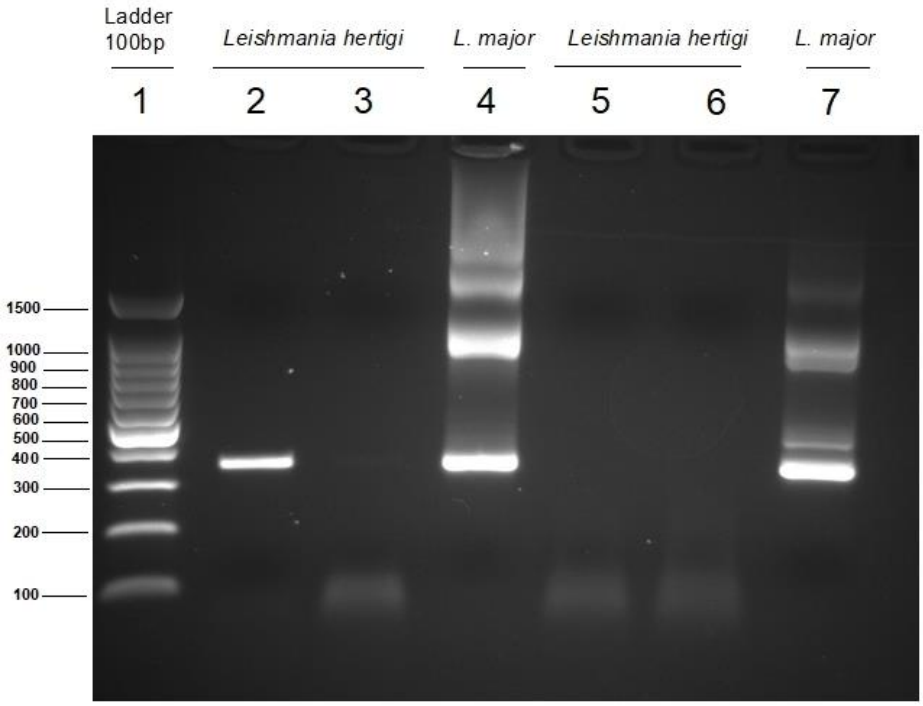



Figure 3.16: Electropherogram of Leishmania major and L. hertigi, LRV1 Primers. 2 % agarose gel electropherogram of PCR product with primers LVR2 RdRp 1587F-1923R, lanes 2 - 4 annealed at 58 °C, lanes 5 - 7 annealed at 60 °C. Lane 1 - 100 bp Easy ladder. Lane 2: L. hertigi (ATCC® 30286™) bands showing Leishmaniavirus present. Lane 4 and 7 Leishmania major (ATCC® 50155™) bands showing Leishmaniavirus present. Lanes 3 and 6 Leishmania hertigi (ATCC® 50125™) no bands.

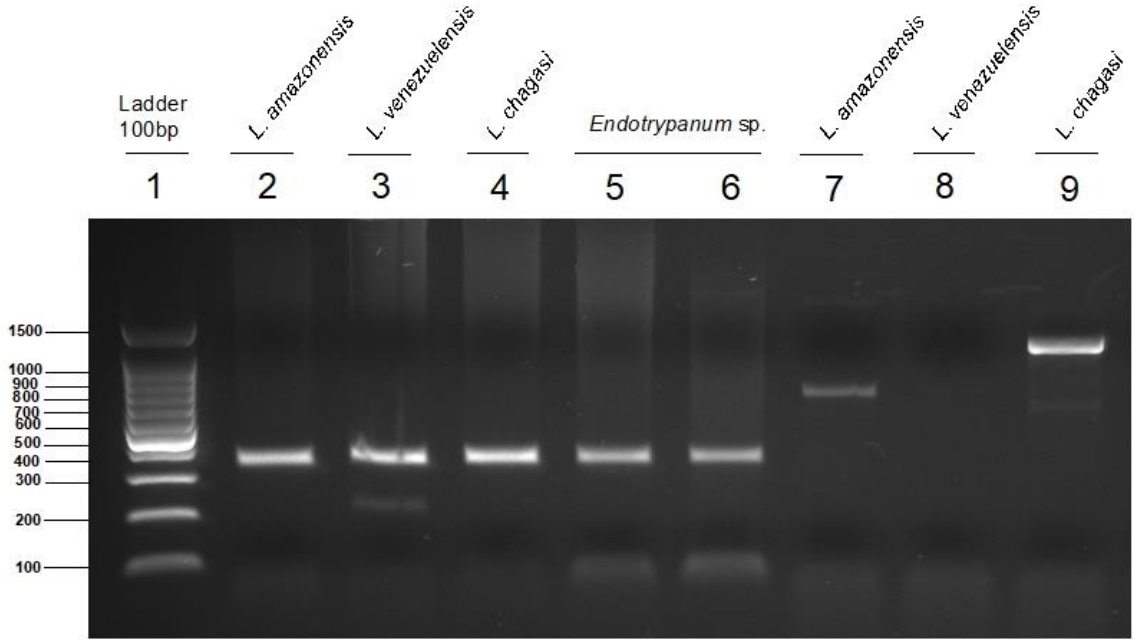



Figure 3.17: Figure Figure 3.17: Electropherogram of *Leishmaniavirus in various New and Old World Hosts.* 2 % agarose gel electropherogram of PCR product with primers: lanes 2 to 6 LVR2 RdRp 1587F-1923R, lanes 7 to 9 LVR1 RdRp 1864F-2264R. Lane 1 is 100 bp Easy ladder. Lane 2 *Leishmania amazonensis* (ATCC® 50131™). Lane 3 *Leishmania venezuelensis* (ATCC® PRA-350™). Lane 4 *Leishmania chagasi* (ATCC® 50133™). Lane 5 *Endotrypanum* sp. (ATCC® 30489™). Lane 6 *Endotrypanum* sp. (ATCC® 30507™) Lane 7 *Leishmania amazonensis* (ATCC® 50131™). Lane 9 *Leishmania chagasi* (ATCC® 50133™). Lanes 2-7 and 9 all have bands showing *Leishmaniavirus* present. Lane 8 *Leishmania venezuelensis* (ATCC® PRA-350™), no band.

Table 3.15: BLAST Output for Positive Species in Figure 3.17

| Sample ID | BLAST out put | Accession no. | RNA seq. |
|---|---|---|---|
| *L. amazonensis* | *Leishmania RNA virus 2 - 1* isolate LRV2/EP97/TR/Lt07 | MK246759.1 | See Appendix 3.1 |
| *L. venezuelensis* | *Leishmania RNA virus 2 - 1* isolate LRV2/EP94/TR/Lt06 | MK246758.1 | |
| *L. chagasi* | *Leishmania RNA virus 2-1* | U32108.1 | |
| *Endotrypanum* sp. | *Leishmania RNA virus 2 - 1* isolate LRV2/CBU33/TR/Lm01 | MK246760.1 | |

### *3.4.3 Leishmaniavirus in Old World Leishmania, Old World Leishmania Primers*

*LRV2* has previously been detected in *Leishmania aethiopica* from Ethiopia, in *L. major* from Turkmenistan, and in *L. infantum* from Iran. It was detected by our assays in *L. major* from Turkmenistan in human parasites (Figure 3.16 and 3.19). also, become positive with optimistion of annulling temprture (Figure 3.19 and 3.20) *L. aethiopica* primer sets successfully detected *LRV2* in *L. major* (Figure 3.21) but no other species. These results were confirmed by Sanger sequences retrieved from GenBank using BLAST which showed expected matches to Leishmaniaviruses as shown in (

Table 3.16).

Using Old World primers (Table 3.5 above), *LRV2* was detected in the genome of *L. infantum,* which has not previously been reported, from samples of blood of dogs obtained from Spain, Czech Republic, and Cyprus as shown in *L. donovani* was also found positive for *LRV2* in a sample of cultured cells from Keele University. *L. infantum* tested positive in samples from dogs obtained from Brazil and Spain Another two Old World *Leishmania* species, which were found, for the first time, to show positive for *LRV2,* are *L. gerbilli*, and *L. tarentolae.* Both species were obtained from a commercial laboratory.

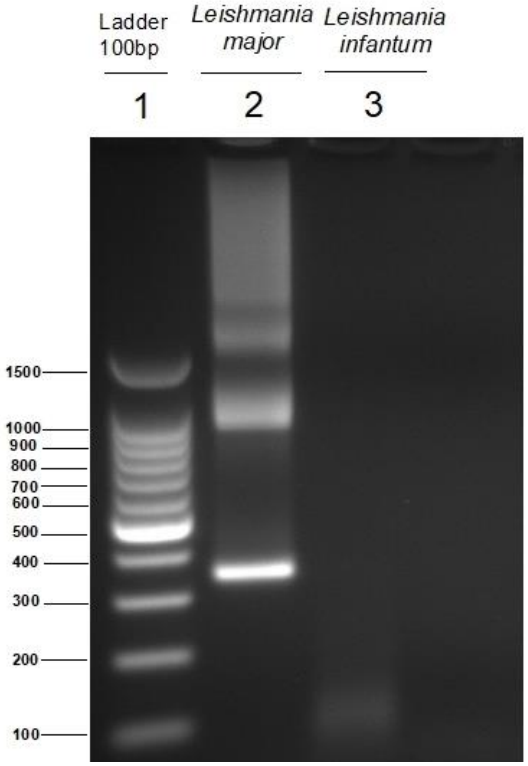



Figure 3.18: Electropherogram of *Leishmania major and L. infantum.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 RdRp 1587F-1923R annealed at 57 °C. Lane 1: 100 bp Easy ladder. Lane 2: *Leishmania major* (ATCC® 50155™) from human from Turkmenistan, the band shows *Leishmaniavirus* present. Lane 3 *Leishmania infantum* (ATCC® 58859213™) no band.

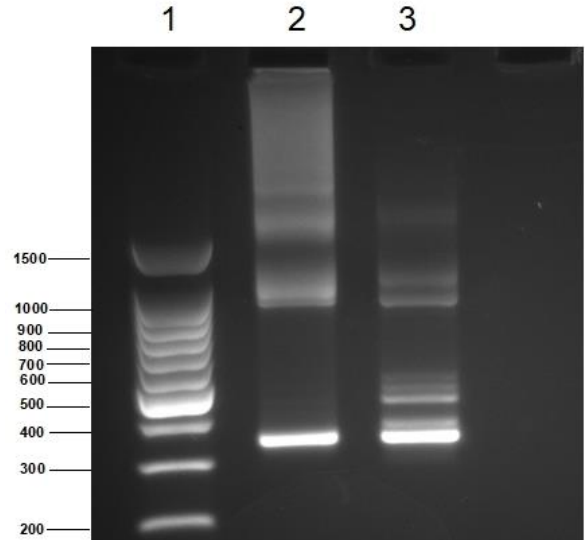



Figure 3.19: Electropherogram of *Leishmania major.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 RdRp 1587F-1923R annealed at 62 °C. Lane 1: 100 bp Easy ladder. Lane 2 and 3: *Leishmania major* (ATCC® 50155™), human samples from Turkmenistan, the band shows *Leishmaniavirus* present.

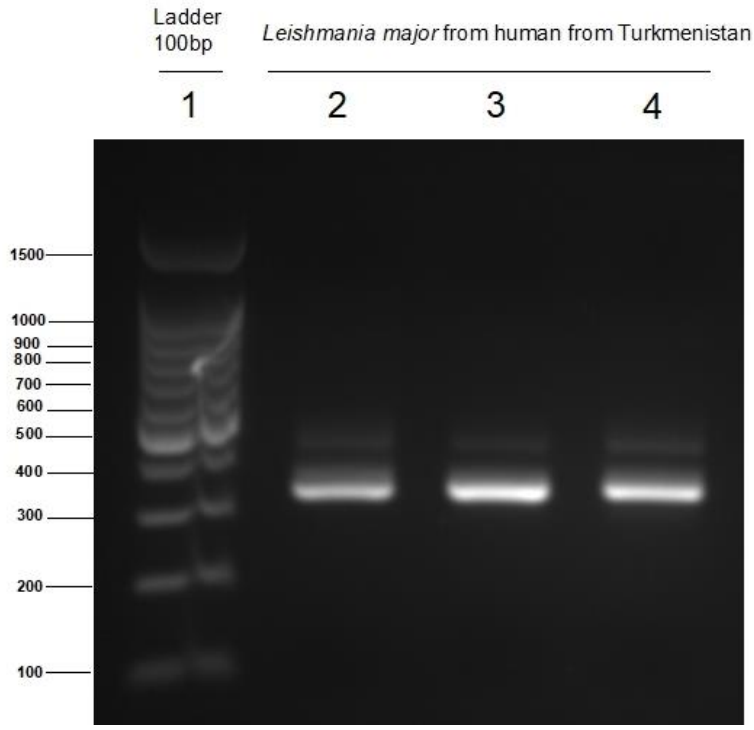



Figure 3.18: Electropherogram of *Leishmania major.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 RdRp 1587F-1923R, lane 2 annealed at 60 °C, lane 3 at 62 °C, lane 4 at 64 °C. Lane 1: 100 bp Easy ladder. Lane 2 - 4: *Leishmania major* (ATCC® 50155™), the band shows *Leishmaniavirus* present.

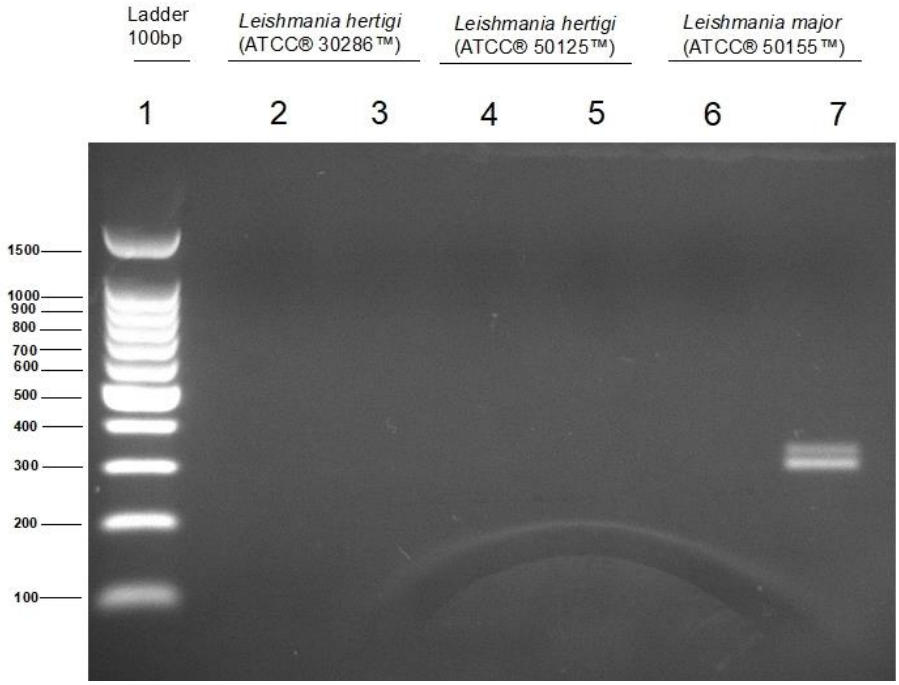



Figure 3.19: Electropherogram of *Leishmania major and L. hertigi.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 Laeth RdRp 1909F-2436R, lanes 2, 4, 6. LVR2 Laeth RdRp 2311F-2637R lanes 3, 5, 7. Lane 1: 100 bp Easy ladder. Lanes 2, 3, 6, 7: *L. major* (ATCC® 50155™), only lane 7 shows *Leishmaniavirus*. Lanes 4 and 5: *L. hertigi*, no bands.

Table 3.16: BLAST Output for Positive Species in Figures 3.18, 3.19, 3.20 and 3.21

| Sample ID | BLAST output | Accession no. | RNA seq. |
|---|---|---|---|
| *L. major* | *Leishmania RNA virus 2-1* | U32108.1 | See Appendix 3.1 |

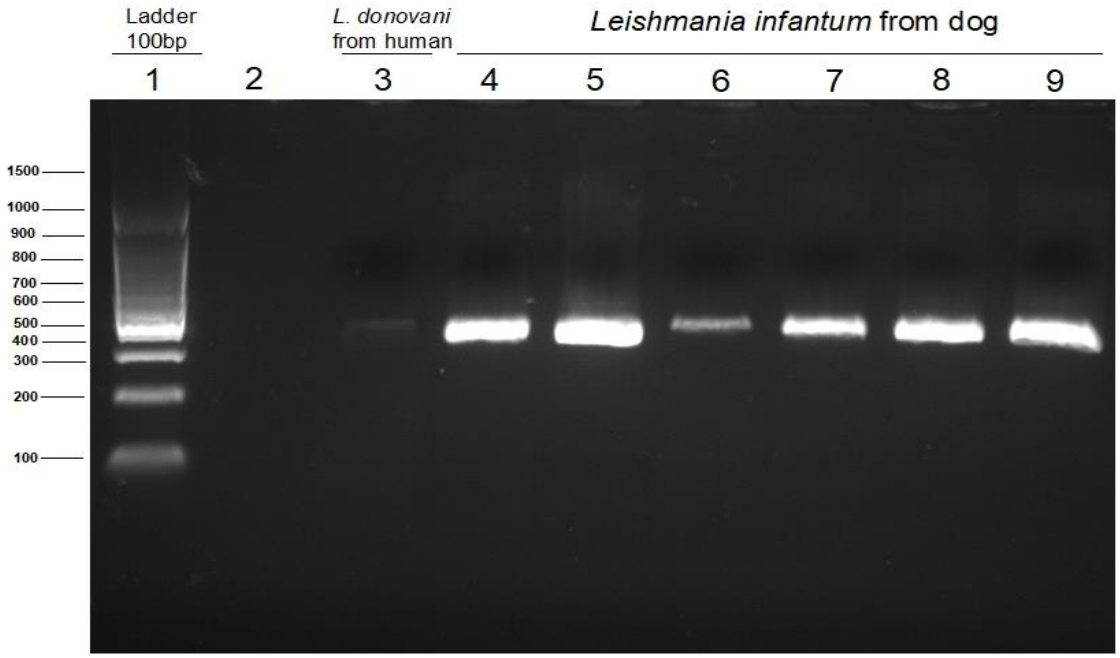



Figure 3.20: Electropherogram of *Leishmania donovani and L. infantum.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 RdRp 1587F- 1923R. Lane 1: 100 bp Easy ladder. Lane 2 void. Lane 3 *L. donovani,* human host, faint band shows *Leishmaniavirus* present. Lanes 4, 5, 6: *L. infantum*, dog host from Brazil, bands show *Leishmaniavirus* present. Lane 7: *L. infantum*, dog host from Czech Republic, band show *Leishmaniavirus* present. Lanes 8, 9: L. infantum, dog host from Cyprus, bands show *Leishmaniavirus* present.

Table 3.17: BLAST Output for Positive Species in Figure 3.22

| Sample ID | BLAST out put | Accession no. | RNA seq. |
|---|---|---|---|
| *L. donovani*<br>*L. infantum* | *Leishmania RNA virus 2 - 1* isolate LRV2/CBU33/TR/Lm01 | MK246760.1 | See Appendix 3.1 |

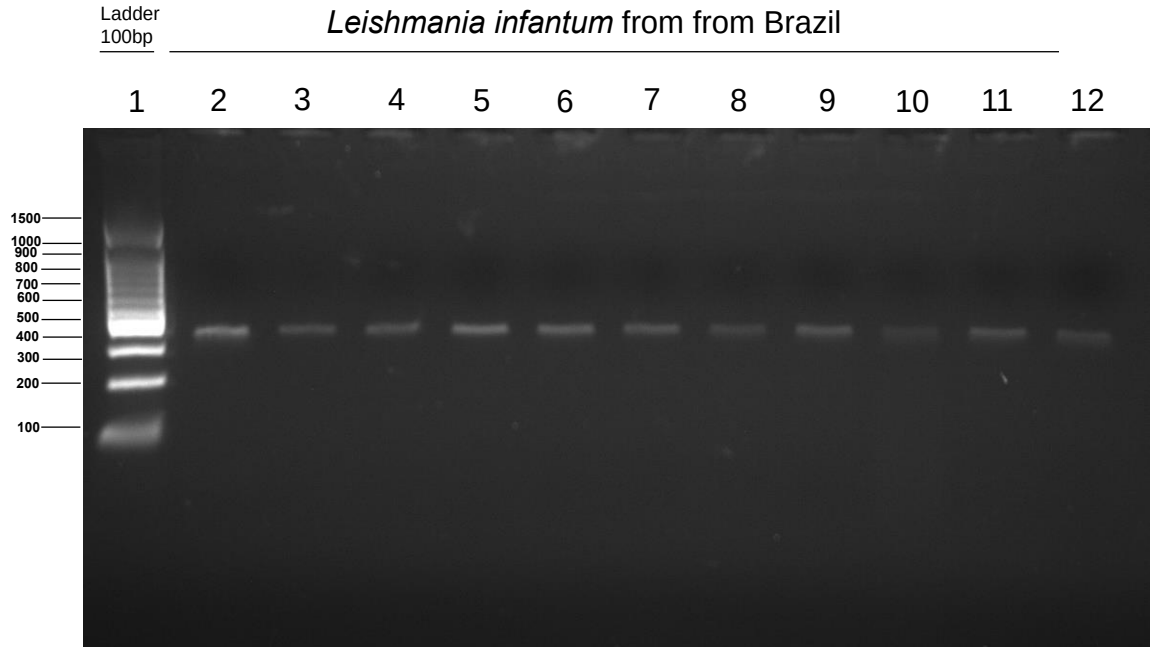



Figure 3.21: Electropherogram of *Leishmania infantum.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 RdRp 1587F-1923R. Lane 1: 100 bp Easy ladder. Lanes 2 – 12: *L. infantum,* dog host from Brazil, bands show *Leishmaniavirus* present.

Table 3.18: BLAST Output for Positive Species in Figures 3.23 and 3.24

| **Sample ID** | **BLAST out put** | **Accession no.** | **RNA seq.** |
|---|---|---|---|
| *L. infantum* | *Leishmania RNA virus 2 - 1* isolate LRV2/CBU33/TR/Lm01 | MK246760.1 | See Appendix 3.1 |

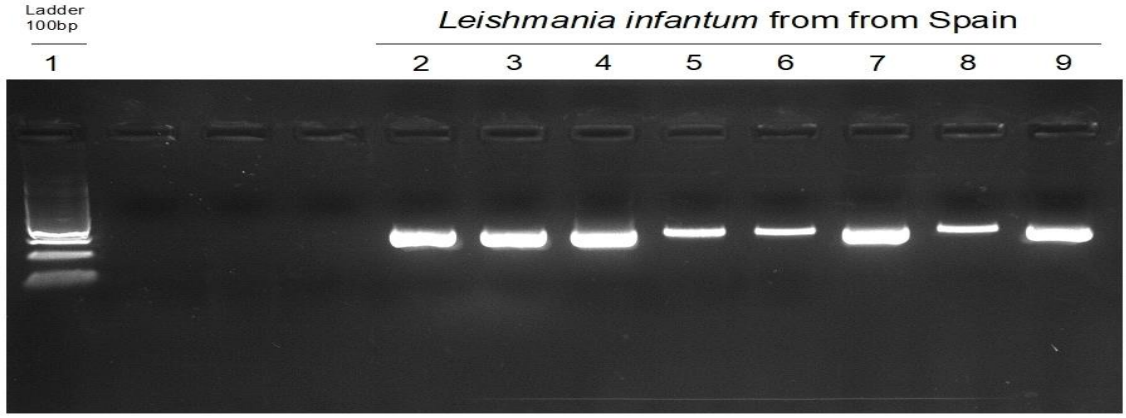



Figure 3.22: Electropherogram of *Leishmania infantum.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 RdRp 1587F-1923R. Lane 1: 100 bp Easy ladder. Lanes 2 – 12: *L. infantum,* dog host from Spain, bands show *Leishmaniavirus* present.

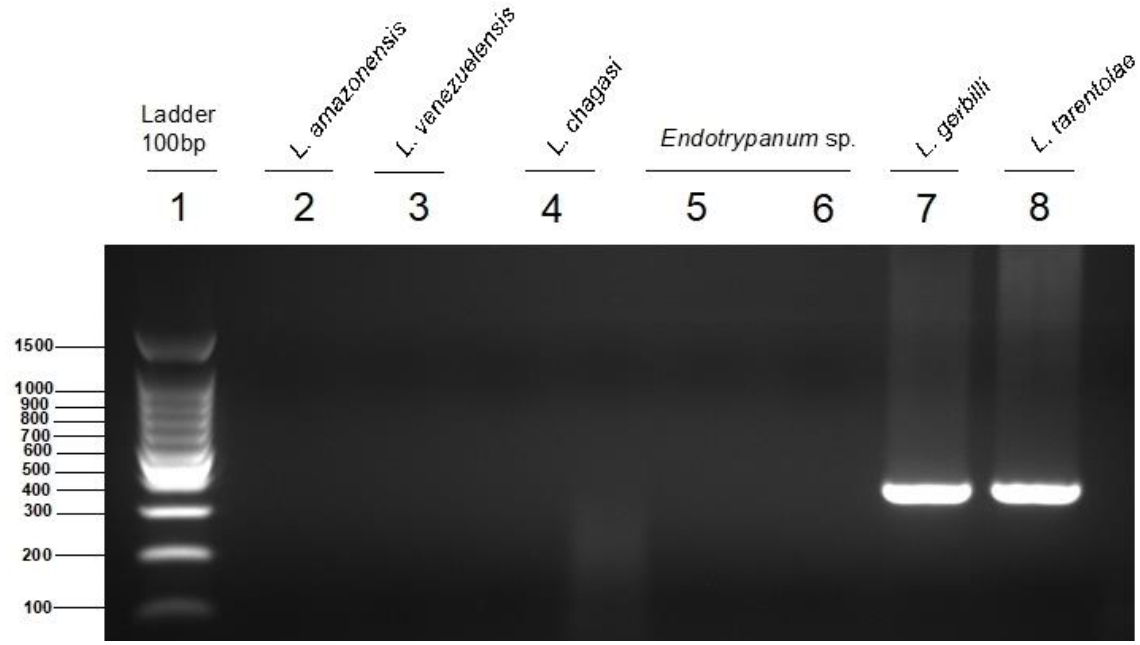



Figure 3.23: Electropherogram of *Leishmania species and related species.* 2 % agarose gel electropherogram of PCR product with primers: LVR2 RdRp 1587F-1923R. Lane 1: 100 bp Easy ladder. Lane 2 *Leishmania amazonensis* (ATCC® 50131™). Lane 3 *Leishmania venezuelensis* (ATCC® PRA-350™). Lane 4 *Leishmania chagasi* (ATCC® 50133™). Lane 5 *Endotrypanum* sp. (ATCC® 30489™). Lane 6 *Endotrypanum* sp. (ATCC® 30507™), Lanes 2-6 no bands. Lane 7 *Leishmania gerbilli* (ATCC® 50121™). Lane 8 *Leishmania tarentolae* (ATCC® 30143™). Lanes 7 and 8 bands show *Leishmaniavirus* present.

Table 3.19: BLAST Output for Positive Species in Figure 3.25

| Sample ID | Blast out put | Accession no. | RNA seq. |
|---|---|---|---|
| *L. gerbilli* | *Leishmania RNA virus 2 - 1* isolate LRV2/EP94/TR/Lt05 | MK246757.1 | See Appendix 3.1 |
| *L. tarentolae* | *Leishmania RNA virus 2 - 1* isolate LRV2/EP94/TR/Lt04 | MK246756.1 | |

### *3.4.4 Leishmaniavirus in Species Related to Leishmania, Old World Leishmania Primers*

Screening for LRV2 in *Trypanosomatidae* species related to Leishmania (sister species) using Old World primer sets resulted in positive detection in *Endotrypanum* sp., *Herpetomonas megaseliae*, *Blastocrithidia culicis* and *Bodo caudatus.* Results are shown below in Tables 3.20 and also above in Tables 3.16 and were confirmed for *Herpetomonas megaseliae* by Sanger sequences retrieved

from GenBank using BLAST which showed expected matches to Leishmaniaviruses (Table 3.20). see bands in (Figure 3.26 and 3.27)

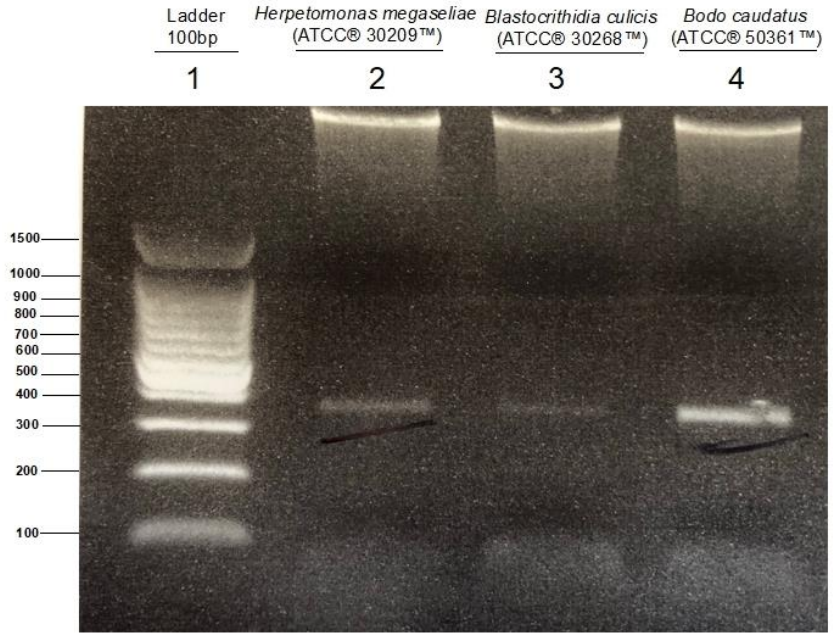



Figure 3.24: Electropherogram of *Herpetomonas megaseliae*, *Blastocrithidia culicis* and *Bodo caudatus.* 2 % agarose gel electropherogram of PCR products for LVR2 primers: LVR2 RdRp 1587F-1923R. Lane 1 - 100 bp Easy ladder. Lane 2 *Herpetomonas megaseliae* (ATCC® 30209™). Lane 3 *Blastocrithidia culicis* (ATCC® 30268™). Lane 4 *Bodo caudatus* (ATCC® 50361™). All host species have bands showing LRV2 present.

Table 3.20: BLAST output for positive species in figure 3.26 and 3.27

| Sample ID | BLAST output | Accession no. | RNA seq. |
|---|---|---|---|
| *Herpetomonas megaseliae*<br>*Blastocrithidia culicis*<br>*Bodo caudatus* | *Leishmania RNA virus 2 - 1* isolate LRV2/CBU33/TR/Lm01 | MK246760.1 | See Appendix 3.1 |

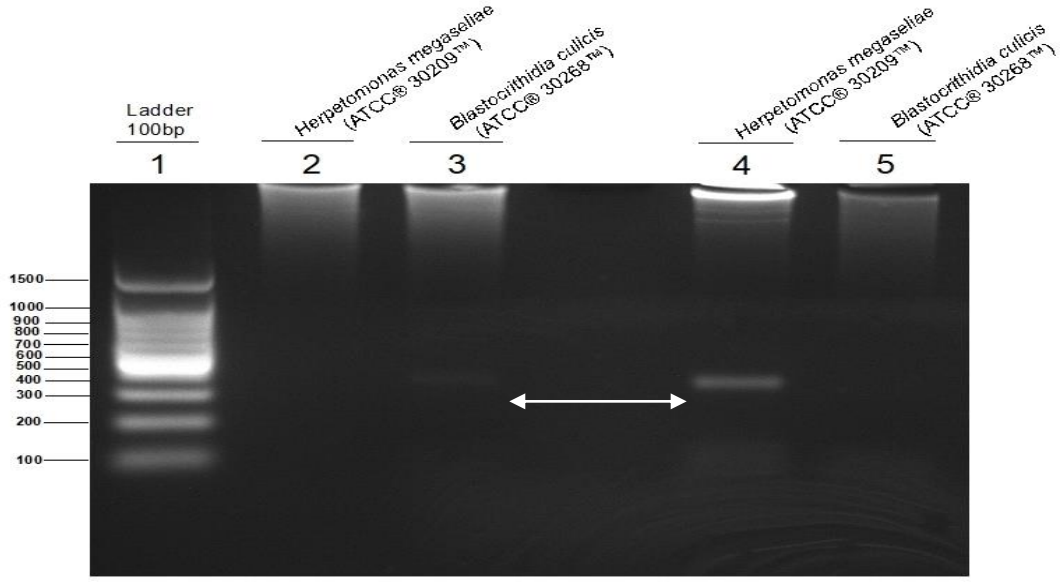



Figure 3.25: Electropherogram of *Herpetomonas megaseliae* and *Blastocrithidia culicis.* 2 % agarose gel electropherogram of PCR products for LVR2 primers: LVR2 RdRp 1587F-1923R. Lane 1 - 100 bp Easy ladder. Lanes 2: *Herpetomonas megaseliae* (ATCC® 30209™), no band. Lane 3 *Blastocrithidia culicis* (ATCC® 30268™), faint band showing LRV2 present. Lane 3 *Blastocrithidia culicis* (ATCC® 30268™) band showing LRV2 present. Lane 4 *Herpetomonas megaseliae* (ATCC® 30209™) band showing LRV2 present. Lanes 2 and 5 were no bands.

### *3.4.5 Leishmaniavirus not detected in Leishmania or Related Species*

Four New World primer sets were used in PCRS testing for *LRV1* in both New and Old World Leishmania host species and related species, no virus was detected in any sample (Figure 3.28).

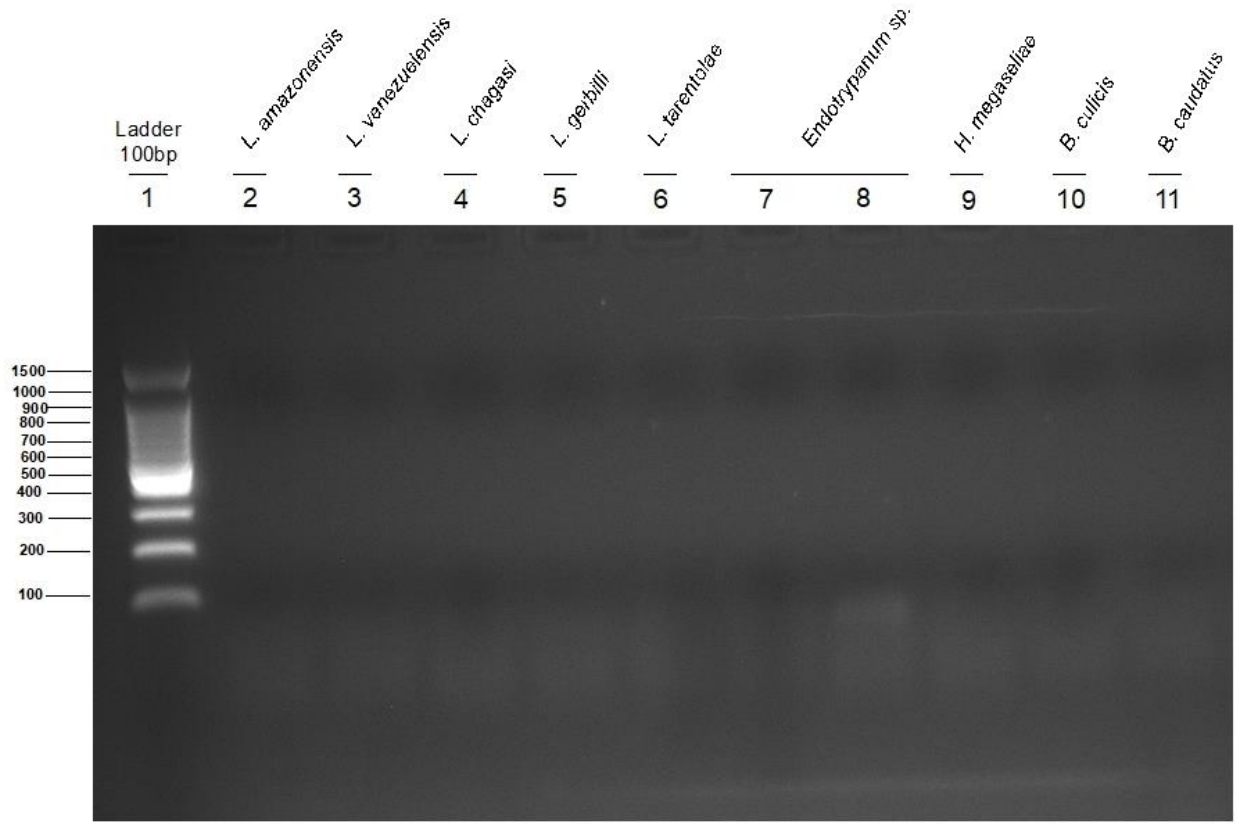



Figure 3.26: Electropherogram of several *Leishmania and Related Species* . 2 % agarose gel electropherogram of PCR products for LVR1 RdRp 1032F-2301R. Lane 1 - 100 bp Easy ladder. Lane 2 *Leishmania amazonensis* (ATCC® 50131™). Lane 3 *Leishmania venezuelensis* (ATCC® PRA-350™). Lane 4 *Leishmania chagasi* (ATCC® 50133™). Lane 5 *Leishmania gerbilli* (ATCC® 50121™). Lane 6 *Leishmania tarentolae* (ATCC® 30143™). Lane 7 *Endotrypanum* sp. (ATCC® 30489™). Lane 8 *Endotrypanum* sp. (ATCC® 30507™). Lane 9 *Herpetomonas megaseliae* (ATCC® 30209™). Lane 10 *Blastocrithidia culicis* (ATCC® 30268™). Lane 11 *Bodo caudatus* (ATCC® 50361™).

## *3.5 Discussion*

The detection of totiviruses in *Leishmania* species proved problematic as low concentrations of the virus inside the various parasites affected the reverse transcriptase PCRs (RT-PCRs) and PCRs. Consequently, a nested RT-PCR, optimised to detect low levels of target RNA was used (Pereira *et al.*, 2013).

Results showed many *Leishmania* samples tested positive for LRV1 or LRV2. All *Leishmania infantum* samples were positive for *Leishmaniavirus,* as shown in Figures 3.20, 3.21 and 3.22. *L. major* samples also tested positive for both viruses, the strongest signal was achieved when the PCR annealing temperature was optimised to 57 °C, 62 °C and 64 °C, as shown in Figures 3.16, 3.17 and 3.18 respectively. These PCR conditions were very effective for amplification of *Leishmaniavirus* in *L. major*.

The primers designed for *Leishmaniavirus* were more effective for long sequences than short ones, as was evident by their amplification of virus in *L. infantum* (Figures 3.11 to 3.16). The length of the sequence appeared to have a vital role in these tests, since both Old and New World *Leishmania* primers yielded positive results, particularly with *L. major* but did not have the same results with other

*Leishmania* species. Another credible factor can the divergence that happened before million years ago between the Old and New World *Leishmania* and transferred the virus from Old to New World *Leishmania*.

Primers, in cases where negative results were obtained, were optimised for virus detection as was evident in aforementioned figures. Figures 3.11 to 3.16 show negative results in several instances, indicating that virus was not present. However, since the same primers often amplified a different virus strain, in particular the primers designed with Old World sequences detected virus in some New World host species, it is likely that the primer optimisation was adequate. For instance, *L. hertigi* has not detected while screening with New World *Leishmania* primers but was detected with Old World *Leishmania* primers, similarly the New World hosts *L. amazonensis*, *L. venezuelensis*, *L. chagasi*, *L. gerbilli*, and *L. tarentolae*, were not detected by New World *Leishmania* primers, but were using Old World Leishmania primers. This also indicates that, although both Old and New World virus strains are clearly related, they are nevertheless independent entities, requiring separate methods of PCR detection.

New World *Leishmania* primers failed to perform with Old World *Leishmania*, yet the later proved capability of working with and detecting viruses in both Old and New World *Leishmania*. One likely explanation for this behaviour is that the virus originally infected Old World Leishmania and that the separation of South America from Africa (Fernandes *et al.,* 1993; Akhoundi *et al.*, 2016) led to the divergence of the Old and New World *Leishmania* species and in parallel with the leishmaniavirus strains. Hence, Old World primers match to conserved sequences in the two strains, but New World primers matched to a sequence in the New World hosts that was not present in Old World hosts.

*L. aethiopica* virus was detected especially in fresh isolations compared to stock isolations. Previous research (Zangger *et al.*, 2014) has shown that this virus is phylogenetically more closely related to LRV1 than to LRV2 and also came from Old World Leishmania. One recent study (Nalçacı *et al.*, 2019) demonstrated that of twenty-four sample isolations, seven were positive for LRV *Totivirus* in *L. tropica*, and three were positive for *L. major* from human and dog in Turkey. Another recent study (Kleschenko *et al.*, 2019) has reported the infection of two *L. major* with LRV *Totivirus*. All recent research work agrees concede the fact that *Totivirus*es are prevalent in Old World *Leishmania* regions and presence of *Leishmania* sp. in Old World *Leishmania*.

In this study, a total of 43 samples were analysed, 42 were positive isolates. This finding is in agreement with the overall high prevalence of *Leishmaniavirus* in both Old and New World *Leishmania* and differ from those reported by Hartley *et al.* (2012). In another study by Hajjaran *et al. (*2016), only two positive LRV2 were isolated from 50 test samples, one was identified as *L. infantum* and one as *L. major*, in comparison, our results, in showed 25 positive isolation samples of *L. infantum* and one of *L. major*. Sukla *et al.* (2017) tested 22 samples and were not able to isolate LRV in *L. donovani,* in comparison, in our results *L. donovani* was found positive for LRV2 in cultured cells.

The absence of *Leishmaniavirus* in some studies that showed negative result can be attributed to the following factors:

1. The low concentration and prevalence of the totivirus because genetic exchange that occurs then the virus transmits (Hartley et al. 2012).

2. These viruses might be lost in laboratory culture (Ronet et al., 2011).

3. Inaccurate non sensitive primer design that detect the totiviruses.

So, more research is needed to investigate the absence of totiviruses in all *Leishmania* species and when virulence is associated with prevelance of the totiviruses especially in Old World Leishmania.

One of the most important findings of this Chapter is the detection of *Totiviridae* for the first time in species related to *Leishmania,* specifically *Endotrypanum* sp., *Herpetomonas megaseliae*, *Blastocrithidia culicis*, and *Bodo caudatus*. This suggests that the virus is present across a broader range of hosts than has been previously considered and that further research is needed to establish just how widespread it is in species related to *Leishmania*.

In cases where LRV1 was involved, it has been shown (Ives *et al.,* 2011; Hartley *et al.,* 2012) that Old World *Leishmania* spp. do not cause mucocutaneous leishmaniasis, further, Zangger *et al.,* (2014) have reported that *LRV2* from *L. aethiopica* displayed similar immunological effects as LRV1 *in vitro*. This leads to the conclusion that LRV2 may affect the development of the visceral disease, a prevalent type of spread leishmaniasis in the Old World.

Hajjaran *et al.'s* (2016) results includes only one sample in which LRV2 in *L. major* was isolated. Although in this study a baby was isolated from the visceral leishmaniasis positive parent, the possible aetiology and disease progression as affected by the virus was not studied. Studies of this kind are further complicated due to the absence of any Old World Leishmania isogenic virus-free isolates (Zangger *et al.*, 2013), and, in addition, by the consideration there is no easy way to isolate LRV1 carried by *L. guyanensis* (Brettmann *et al.,* 2016) because the parasitic virus interferes with the functioning of RNAase (Lye *et al*, 2010; Matveyev *et al.*,2017).

It is clear that there remains much to learn and understand from further research into the prevalence, distribution, phylogeny and effects on virulence of the leishmaniaviruses as well as their relationships to the globally important, disease causing *Leishmania* and affected vertebrate hosts. While there has been considerable focus on their occurrence in some *Leishmania* species, nevertheless a characterisation of the different strains has yet to emerge and much further research is necessary. This Chapter serves to highlight the extent of the gaps in our knowledge by extending the virus' host genome range and highlighting the value of an experimental approach to primer design and PCR amplification.

## *3.6 References*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# Chapter 4: *TRICHOMONASVIRUS, GIARDIAVIRUS AND EIMERIAVIRUS*

## 4.1 Introduction

### 4.1.1 Trichomonasvirus

*Trichomonasvirus* are a relatively new genus of double-stranded RNA (dsRNA), endobiotic viruses in the *Totiviridae* family that is named after its host parasite, *Trichomonas vaginalis* a protozoan. *Trichomonas vaginalis virus 1* is the type species.

*Trichomonas vaginalis* is a sexually transmitted, flagellated protozoan that causes inflammatory conditions and related risk of reproductive problems in the human genitourinary tract, mainly vagina among women and in the urethra among men. *T. vaginalis* is the most common non-viral sexually transmitted infection in the world; its presentation known as Trichomoniasis. Trichomoniasis is more prevalent than syphilis, gonorrhoea and chlamydia and is also the most curable sexually-transmitted disease in the world (Gerbase *et al.,* 1998), at ~50 % of curable global infections (Schwebke & Burgess, 2004). There are ~170 million new cases per year (WHO 2001). A number of other clinical conditions including low birth weight and premature delivery have also been linked with this disease, as has the risk of transmission of human immuno-deficit viruses, human papillomavirus and cervical diseases, including pruritus (Laga *et al.,* 1994, Zhang & Begg, 1994). *T. vaginalis*, is difficult to treat and is resistant to the commonest medication, metronidazole (Schwebke & Burgess, 2004). The parasite's virulence is regulated by numerous factors, such as cysteine proteases, surface proteins, and main surface lipophosphoglycan; the latter is responsible for host cervical and vaginal cell selective over-regulation of inflammatory mediators (Fichorova *et al.,* 2006).

Long linear dsRNA molecules were first identified in 1985 and evidence of their interaction with virus particles in several varieties of *T. vaginalis* soon followed (Flegr *et al.,* 1987, Wang & Wang, 1985, Wang & Wang, 1986). Further analysis of strains of *T. vaginalis* revealed that viral dsRNA in *Trichomonasvirus* genome was relatively common and that up to three, equally long segments of dsRNA (4000 – 5000 bp), could be found in single isolates, indicating that either a multisegmented virus or several different strains of *Trichomonas vaginalis virus* (TVV) were present (Flegr *et al.,* 1988, Khoshnan *et al.,* 1994). The genomes of a number of TVVs were found to be homologic to monosegmented dsRNA viruses within the *Totiviridae* (Bessarab *et al.*, 2000; Su & Tai, 1996; Tai & Chui-Fun, 1995). TVVs were originally, tentatively assigned to the *Giardiavirus* genus, which is a well-characterised type of *Giardia lamblia virus* (Wang & Wang 1991, Wang *et al.,* 1993), on the basis of the relationship between their host species*, Trichomonas* and *Giardia*. However, further genome sequencing and phylogenetic analyses indicate that TVVs are not so closely aligned with *Giardia lamblia virus* (GLV) (Gabrial, 2008; Kim *et al.,* 2007) and recent phylogenetic analysis of the *Totiviridae* led to the establishment of *Trichomonasvirus* approved by the ICTV as a new genus.

There is strong evidence that TVVs are present as viral-like particles (VLPs) which are molecules that closely resemble viruses, but are non-infectious because they contain no viral genetic material (Zeltins, A. 2013). ranging in size from 30 – 200 nm. The VLPs are spherical, filamentous or variously cylindrical. Two, separate, major protein virus capsids were also identified in TVV isolates from *T. vaginalis*, with 75 and 85 kDa, a low-level protein of 160 kDa and an 86 kDa viral RNA polymerase. Their structure, with icosahedrons 33 nm in dimension, was further investigated with the viral capsid proteins. A recent 3D structural analysis found 120 subunits present in the icosahedral viral capsid forming filled-in channels that span the whole capsid. These channels may help to emit viral genetic material into the *T. vaginalis* cytoplasm. Viral proteins and VLPs were present in the cytoplasm of *T. vaginalis* in the close or budding vacuole- and in the endocyticised pits of the Golgi complex, a plasma membrane.

The association with the Golgi complex, vacuoles and endocytopic fossilises with TVV and their viral proteins indicates their possible function in transmitting TVV to *T. vaginalis*. *T. vaginalis* split in asexual fission, a type of mitotic cell division. The ability to infect trichomonads and machinery is missing in TVV. It was proposed that TVV transmits vertically, using *T. vaginalis*, during the copy and division cycle during binary fission (Graves, 2019).

### *4.1.2 Giardiavirus*

*Giardia duodenalis* (*Giardiida: Giardiidae*) (syn. *G. lamblia, G. intestinalis,* both in widespread use) is a microscopic parasite that causes a diarrheal disease of humans known as giardiasis, it is the only species in this genus to cause the disease and the commonest species present in primates (Kulda, 1978). It is found in soil, food, or water contaminated with human or animal faeces and thought to infect most mammals and many other animals, world-wide, being especially prevalent in warm climates. *Giardia* has an outer shell, which allows for longer periods of time outside the body and tolerates chlorine disinfection. Infected water (drinking and recreational) is the typical mode of transmission, although it may also be ingested in contaminated food (CDC, 2020). There are six species of *Giardia* which are morphologically identical (Robertson & Gjerde, 2004).

Giardiasis is an acute or chronic diarrhoeal gastrointestinal disease; it is seldom fatal, but mortality can be caused primarily in babies or undernourished children by severe dehydration. Outbreaks in people from polluted water or food or direct contact with other infected people are well documented. For example, in childcare centres, people are known as the most significant human giardiasis reservoir hosts. The predominant genetic forms of *Giardia duodenalis* differ between people and domesticated animals (livestock and pets). Zoonotic transmission is considered to be minor in human disease epidemiology, nevertheless, it is clear that certain genetic forms of *Giardia duodenalis* (assemblages A and B) are often shared between animals and humans and are, therefore, considered to be potentially zoonotic, the remaining 5 genotypes do not occur in humans. (CSFPH, 2012).

A detailed account of the biology, including life cycle, infectivity, pathology, geographic range and a comprehensive list of organisms infected, of *Guardia duodenalis* can be found in CSFPH (2012).

Wang & Wang (1986) were among the first workers to detect repetitive DNA bands in electrophoretic gels from *Giardia* isolates which were later identified as a double strand RNA (dsRNA) viroid. RNA viroids have subsequently been investigated in 38 *Giardia* isolates with axenic growth, from various geographical areas. Strains identified as *Giardia* have been isolated from people in the USA, England and Poland but not in human samples from Belgium or Israel. The isolated RNA viroids did not replicate in free media in the absence of a *Giardia* host and no link was found between them and *in vitro* resistance to anti-protozoal medicines nor did symptomatic or asymptomatic carriers necessarily cause infection. *Giardia* trophozoites were not infected with the RNA virus and did not affect repeated DNA isoenzyme patterns or endonuclease limitations.

RNA polymerase activity was detected in cultures known to contain *Giardiavirus* (GLV) infected cells, present in both crude entire cell lysates and GLV particle lysates cleansed from the culture media (Wang & Wang, 1986). The polymerase RNA operation synthesises RNAs consisting of a single strand of the GLV genome, but in the current experimental conditions the RNAs' reaction products are not full-length viral RNAs. RNA polymerase co-sediment *in vitro* products with saccharose gradients and viral particles of purified GLV has RNA polymerase activity. RNA polymerases acts closely in tandem with the number of viruses that occur during viral infection inside and without infected cells. The lysate level in the test and the reaction time and temperature determine this polymerase activity. This cation requires divalent cations and all four tribonucularosides and is inhibited by doubling-stranded nucleic acid-crossed pyrophosphates and the molecules interfering with the action of polymerase. The RNA product of this reaction is a GLV-genome strand, which has the same cytoplasm as a ssRNA strand in the GLV-infected cells. There are less than 1 kb of reaction products. Nonetheless, in gene length, because these crude lysates either contain a large amount of RNAse activity or because under current experimental conditions one of the reaction substrates is reduced. The operation of RNA polymerase seems directly related to viral particles, as it occurs for nearly half the reaction product sediment with VPs in extensively cleansed virus preparations. Unfortunately, several attempts were made to restore RNA polymerase after the centrifuge of crude lysates by saccharose gradient. The cell-lysate polymerase activity can be unstable in saccharose after long periods of time and can inhibit the sucrose role or release by a centrifugation the polymerase factor(s). The GLV virus of Buck KW *et al* 1984, whose killer phenotype is the most well studied, is very similar to mycoviruses. GIA is the best-known *Saccharomyces cerevisiae L-A dsRNA virus*. Both GLV and L-A are viruses of dsRNA that comprise one component of the RNA virus. The particles of the virus consist mainly of one large capsid protein in both cases. No virus seems to have a negative effect on the host's growth. Both viruses produce a ssRNA which is a viral genome strand. And now we have identified the behaviour of RNA polymerase with features similar to the RNA Polymerase of the L-A yeast viruses. Yeast L-A replication virus is identical to mammalian cell replication of reovirus (Silverstein *et al.*, 1976). In host cytoplasm the dsRNA virus persists inside

the viral particles. A RNA-related RNA polymerase conveys a conservative replication of one of the strands of dsRNA (Silverstein *et al.,* 1976), producing a (+) strand released into the cytoplasm. This strand (+) is then mRNA, making the main capsid protein and the RNA polymerase dependent on RNA. Polymerase is created through the frame that transfers the coding sequence of the main capsid protein to a second open measurement frame. Polymerase molecules and large capsid proteins have been assembled and the current viral particle around (+) RNA strand and strand (-) is synthesised with a viral particle. The polymerase activity which synthesises strand (+) is a transcriptase. The synthesised operation (-) is called a replication. It is however clear that both behaviours are caused by the RNA polymerase molecule. The numerous syntheses at that time rely on the components of the viral particle. A similar replication process tends to occur in the GLV. The cytoplasm of GLV cells is overexpressed by a ssRNA. The RNA seems to be a complete copy of a strand of GLV, and the ssRNA synthesised with the kinetics required to replicate the virus intermediately or an mRNA (Furfine *et al.*, 1989). The ssRNA can be the (+) strand synthesised with the transcriptase action of the polymerase RNA using the yeast framework analogy. This paper extends these findings by describing the behaviour of GLV transcriptase that has similar characteristics to the yeast transcriptase. The GLV transcriptase synthesises only one genome strand, the same strand that is released to media by the ssRNA. This result of transcriptase appears to be released at least to some degree from the viral particles into the reaction buffer. The replica activity of polymerase has not been identified since only labelled RNAs are detected that are the same genome strand as intermediate ssRNA. In fact, new viral particles with only (+) strands in large amounts will not be observable in the simple fractions that we have used. Therefore, the replicase activity of this RNA polymerase might require further purification of empty or light-viral particles. GLV and L-A viruses are primarily affected by the fact that GLV is extruded into the culture medium and can invade uninfected *Giardia* cells. The virus and RNA viral behaviour in cells and the culture medium are very similar during the process of infection. The polymerase levels closely correlate with the dsRNA level in infection (Furfine and other sources, 1989). The activity of polymerase does not suit the ssRNA levels in the cell that peak in 24 to 48 hours after infection. In addition, the presence in the culture medium of viral RNA polymerase indicates that mature viruses capable of infection provide all required components for the transcriptase operation. Our principal interest in GLV lies in Giardia's growth potential as a transformation method because successful long-term transfection of parasitic protozoa (Bellafatto V. & Cross GAM 1989) is still not feasible. We need to have a detailed knowledge of the GLV replication process before implementing such a transformation method. An intermediate ssRNA (Furfine *et al.,* 1989) was established and an RNA dependent RNA polymerase will allow to generate full-length virus cDNAs. Using the RNA polymerase activity, these RNAs can after modification be incorporated in the viral particles. Fujimura & Wickner (1988) have demonstrated that low salt viral incubations release endogenous viral genomes and permit the addition and subsequent replication of exogenous templates. The next step towards the development of a GLV transformation method in *Giardia* will be related experiments using GLV.

GLV was one of the first biochemical protozoan viruses to be isolated (Wang & Wang, 1986). Both known GLV viruses have non-segmented dsRNA genomes of 4.5 to 7 kb (Wang & Wang, 1991). Wang *et al.* (1993) obtained a sequence with a contiguous 6100 nt GLV cDNA, which consisted of two large, overlapping, open reading frames (ORFs) in addition to 368 and 123 nucleotides of untranslated, 5' and 3' end areas, respectively, using a combination of cloning methods. cDNA cloning data from the 3' tailed viral RNA prototype indicated that the fragment also contained a 3' terminal dsRNA of GLV. Possibly due to GLV dsRNA's anomalous mobility in agarose gel electrophoresis, a previous size estimate was 7 kb. A multi-strategy virus develops more than one mRNA polypeptide. RNA splice omits GLV*,* as only plus and minus strands of the viral genome comprise virus-infected cell extract. No sub genomic viral RNA has been observed in northern blots. RNA editing has been omitted because if such a happening occurs, RNAs would have been pre-edited and revised.

The dsRNA genome of GLV comprises a 100 kDa polypeptide capsid (p100), ORF1, and a ribosomal frameshifting is synthesised as ORF1-ORF2 fusion protein as the only other viral polypeptide of 190 kDa GLV RNA dependent RNA polymerase (p190). Edman degradation showed that p100 was blocked N-terminally, except that 2 – 5 % showed that N terminal from the amino acid residue 33 was found to be N-terminal. Research using amino acid residue antiserum 6-27 found that the area (NT) does not include viral p100 and p190. Pulse labelling tests showed NT is present in nascent p100 *Giardia lamblia*, synthesised in GLV-infected lamblia, but then excluded. This area, in comparison, was stored in the two *in vitro* synthesised viral proteins and was not extracted when microsomal fractures were prolongedly incubated or included in the in vitro reaction mix. These findings indicate that the endoplasmic reticulum is unable to cleave them or to each other and that p100 and p190 precursors are unable to cleave. This particular cleavage was replicated by the addition of Lysates from GLV *Giardia lamblia*, but not from uninfected cells. While cleaving activity was fairly resistant to phenylmethylsulfonyl fluoride, leupeptin or the E-64, two recognised particular cysteine protease inhibitors, was inhibitable. (Wang *et al.,* 1988).

*Giardia lamblia* is one of the earliest eukaryotic divergences and an intestinal protozoan parasite. The trophozoite multiplies by means of asexual binary fission and lacks all normal lateral gene transmission means. A long-term expression mechanism (Yu *et al.,* 1996) of a foreign gene was developed in this body through the use of recombinant virions from the double-stranded RNA virus *Giardia* (GLV), which infects several *Giardia* isolates. An *in vitro* GLV cloned cDNA transcript, consisting of 5' and 3' fragments of GLV. The GLV positive-strand RNA firefly luciferase-encode region was into GLV Trophozoites. The activity of luciferase in electroporated cells peaked at the level 6 above-ground on day 2. In the absence of selective pressures, expression of this external gene remained 80 % of its peak after 30 days. A double strand of chimeric RNA was replicated and wrapped in virus-like particles. Recombinant virions have been partially isolated by CsCl balance gradient-densities centrifugation from the wild-like aid virus and used to superinfect trophozoites of *Giardia*. These chimeric virions were able to initiate new rounds of luciferase activity in the

superinfected cells at multiplicity of infection from 100 or above. Via the modified virion, a heterologous gene can be successfully inserted into this eukaryotic microorganism and effectively expressed. The RNA transcript of pC670-Luc has been shown to be capable of conducting luciferase syntheses in transfected *Giardia lamblia* trophozoites, which showed more than a millionfold luciferase activity above background and lasting at least 30 days in the absence of selective pressure by serial passing. Obviously, the recombinant RNA was packed into VLP, which are both present in the cytoplasm and in the culture medium of the transfected cells. Such recombinant VLPs are capable of infecting and inducing subsequent rounds of luciferase expression in *Giardia lamblia*. This enables the introduction of foreign genes without electroporation into these trophozoites. It is particularly effective when electroporation is impractical, such as transfecting the *Giardia* trophozoite into a mammalian host under certain circumstances.

In a capsid consisting of an essential 100-kDa protein (p100) and a small 190-kDa protein, *Giardiavirus* encapsidates a 6.2 kb double-stranded (dsRNA). In the current research, two 6.2 kb dsRNA of non-homologous RNAs cohabiting in *Giardia lamblia* trophozoites, one (designated GLV) with capside of p100, and one (designated GLV[p95]), which is made up of a 95 kDa protein and a minor p190, equivalent. Both forms of viruses enrich the membranous fraction of a virus infected lysate *G. lamblia* cells. CsCl gradient centrifugation after osmotic breakdown of the viral particles achieved isolation of these virions. The 6.2 kb ds RNAs of GLV [p100] were extracted with this procedure, while the GLV [p95] remained theoretically unchanged, with differential hybridisation characteristics demonstrated by the 26.2 kb ds of RNAs filtered by this protocol to viral samples of cDNA. Western blotting and peptide mapping experiments show that P100 and P95, though each with distinct amino acid sequences, were closely related proteins. Viral purification and pulse-chase studies have shown that GLV [p100] is selectively secreted in the medium, while GLV [p95] persists in the *G. lamblia* trophozoites in the late cell growth period. Brefeldin A has not inherited the secretion of GLV [p100] which shows that several *Giardiavirus* species are living together in *G. lamblia* (Tai *et al*., 1995).

Gene expression can be controlled in *Giardia lamblia* with electroporation of GLV infected *Giardia lamblia* trophozoites in 50 and 30 non-translated regions (UTRs) of the GLV Genome. The *Giardia lamblia* Genetic Manipulation Model (Davis-Hayman SR 2002, Dan *et al.,* 2000) was used for this transfection procedure.

### *4.1.3 Eimeriavirus*

*Eimeria* (*Eimeriidae*) is a genus of *Apicomplexa* (parasitic alveolates) which infect different types of animals, including several which are farmed, such as cattle, goats, sheep and in particular, poultry; causing a disease known as *coccidiosis*. Symptoms of coccidiosis are malabsorption, diarrhoea and haemorrhage (Chartier & Paraud, 2012).

*Eimeria* has a normally, but not exclusively monoxenous and stenoxenous life cycle i.e. one which is completed in a single host from a narrow range of hosts (Figure 4.1). Transmission of parasites between hosts takes place mainly through ingestion of infested faecal matter. Infections in agricultural settings, where many animals are concentrated in a small area, are common, as an infected host may emit many thousands of oocysts into the environment which have multi-layered walls that are extremely resistant to environmental degradation. Air and moisture cause the oocyst to sporulate, becoming infectious after two to seven days. The oocysts must undergo excystation when swallowed by a host, which releases thousands of sporozoites into the intestinal lumen.

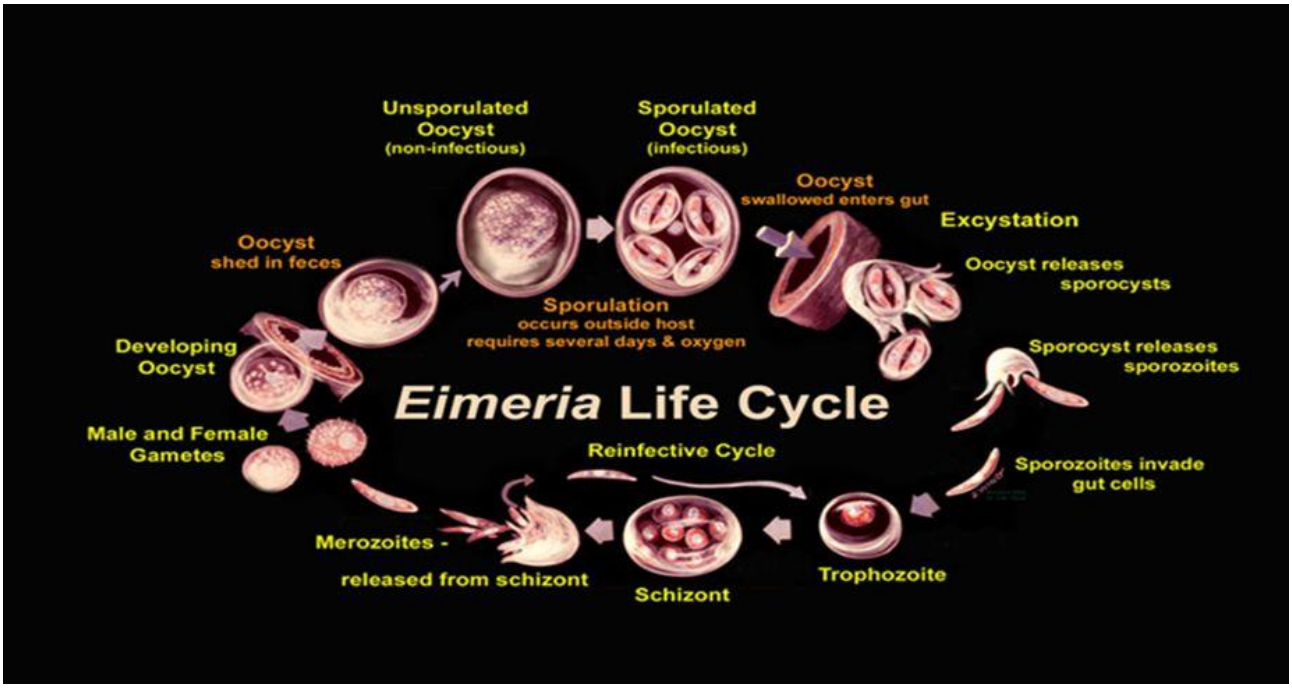



Figure 4.1: Typical Eimeria Species life cycle, from Agricultural Research Service (ARS) website.

Host species include: 30 species of bats, 2 turtles, 130 fish, 2 seals (E*. phocae* and *E. weddelli)*, 5 llamas and alpacas (*E. alpacae, E. ivitaensis, E. lamae, E. macusaniensis* and *E. punonensis),* numerous rodents (*inter alia E. couesii, E. kinsellai, E. palustris, E. ojastii* and *E. oryzomysi)*. Species which infect domesticated animals include *E. maxima*, *E. necatrix* and *E. tenella* of poultry, *E. stiedae* of rabbits and *E. bovis, E. ellipsoidalis* and *E. zuernii* of cattle. *E. bovis, E. zuernii,* and *E. auburnensis*, all causing disease in cattle, are the most common species (Fayer, 1980).

The RNAs found in *Eimeria maxima* have been characterised, cytoplasmic RNA, probably an excess of ribonucleoprotein, has been shown to be resistant to RNAse treatment in sub cell fractionation studies (Ellis & Revets, 1990). Electron microscopy has shown that the RNA found in any *Eimeria maxima* strains to date is double stranded. The protozoan parasite *Eimeria necatrix* has recently been found to host two species of double-banded RNA viruses of ~5.6 kb and ~4.5 kb, however, CsCl centrifuge methods only detected RNA of ~5.6 kb (Lee *et al.*, 1996). It has been known that these viruses have a diameter of ~42 nm and have icosahedral morphology. RNA-dependent RNA

polymerase activities related to RNase-sensitive nucleic acid has also been found in *Eimeria nieschulzi* (Sepp *et al.,* 1991).

Small-molecular bands were detected in gel electrophoresis results when characterising chromosomes of several *Eimeria* species of chickens (*Eimeria acervulina, Eimeria brunetti, Eimeria maxima* and *Eimeria necatrix)*. Host RNAs were degraded significantly in these preparations, meaning they were either small molecules or exceptionally stable sections of RNAs. These bands are characteristically double-stranded RNA, confirming they are viral infections. A putative virus isolated from *Eimeria necatrix* oocytes showed two dsRNA segments (Miller *et al.*, 1989).

Three dsRNA viruses with an unusually small diameter of ~38 nm were isolated from *Eimeria tenella* sporulated oocysts in China (Han *et al.,* 2011) and labelled as VLPs. The VLPs were extra chromosome dsRNA segments of 1.4, 2.4 and 3.6 kb total nucleic acid. RdRp activity was also observed in this study, it was found to be immune to high salt (0.3 M NaCl) digestion of RNase A. The isolates were called *Eimeria tenella virus* (ETV), in accordance with the commonly accepted nomenclature for protozoan viruses and represent the first isolated virus of *Eimeria tenella*. Another recent study of E. *tenella* sporulated oocysts (Bin *et al.,* 2016) found dsRNA and virus particles of ~30 nm diameter. Xin *et al.* (2016) followed a three-step approach for complete genomic sequencing of this novel dsRNA virus. The full genome sequence was 6007 bp, with two open reading frames (ORF) with five-nucleotide overlaps (UGA / UG) (2367 bp with ORF1 and 3216 bp with ORF2). The ORF1 and ORF2 predicted a putative capsid protein of 788 codons (84.922 kDa) with a putative RdRp protein of 1071 codons (118.190 kDa). BLAST analysis showed the *E. tenella* amino acid sequences were identical to *E. brunetti RNA virus,* with an equivalence of 29 % to capsid and 36 % to RdRp proteins. There were 349 bp (50UTR) and 78 bp (30UTR) in the 2 untranslated areas. This isolate was given the name, as prescribed, *Eimeria tenella RNA virus 1.* The full *E. tenella* genome sequence resembled those of *Totiviridae,* suggesting that this virus was a new *Totiviridae* member. Phylogenetic analyses revealed that *Eimeria tenella virus*, and *Eimeria brunetti RNA virus 1* are in the same lineage as *Victorivirus* and a new subgenus, *Eimeriavirus* (Xin *et.al*., 2016).

Revets *et al.* (1989) found virus-like particles (VLPS) in *Eimeria stiedae*. However, the full *E. stiedae virus* genome has not yet been sequenced. A new *E. stiedae virus* has been isolated (Xin *et al.*, 2016), the entire genome sequence from this isolate was 6219 bp in length and contained two tetranucleotide overlapping open reading frames (ORFs). ORF 1 (ORF1) is 2400 bp (86471 kDa), a putative capsid protein, having an amino acid sequence similar in proportional composition to *Eimeria tenella RNA Virus 1* (EtRV1; 43 % NC 026140). ORF " (ORF2) is 3303 bp and is suspected to be RdRp. The sequence data provided enough information to classify the new virus and phylogenetically it is placed in the *Totiviridae* (Xin *et.al*, 2016)

It remains unknown how *Eimeriaviruses* viruses infect *Eimeria* hosts, or how they affect their host's behaviour or phenology. Only *Giardia lamblia virus* amongst totivirus is known to occur freely in

culture media and certain uninfected strains of its host parasite have been known to become infected with this virus (Miller *et al.*, 1989).

### *4.1.4 Prevalence of Trichomonasvirus*

The most common non-viral sexually transmitted infection (STI) in the world is *Trichomonas vaginalis*. Patients with *T. vaginalis* may show specific symptoms, including vaginal fluid and dysuria in women and urethral fluid and male dysuria. Nevertheless, many compromised patients are never symptomatic. Infertility and adverse birth outcomes were associated with untreated or recurrent *T. vaginalis* in women. While less is known in men about *T. vaginalis*, nongonococcal urethritis (NGU), prostatitis and epididymitis have been recorded. Often related to the increased risk of HIV, *T. vaginalis* presents a significant threat to public health. However, due to lack of commitment to public health, *T. vaginalis* is not known. Currently it is not reportable in the U.S., as only three of seven conditions were previously found to satisfy the World Health Organization (WHO). The WHO reported 156 million cases of *T. vaginalis* worldwide in 2016, accounting for approximately half of the global STI incidence that year (Rowley *et al.,* 2019). In this report, the new *T. vaginalis* epidemiology, indications and treatment findings will be updated. A new epidemiological study was released in 2018 (Patel *et al.*, 2018), Local *Trichomonas* in the USA. These results were obtained in 2013 – 2014 using the Hologic Gen Probe Aptima *T. vaginalis* on urine specimens in the National Health & Nutrition Survey (NHANES). The prevalence of *T. vaginalis* was 1.8 % in females and 0.5 % in males 18 – 59 years of age (Krieger *et al.*, 1993 and Schwebke *et al.,* 2011). Prior to this study, *T. vaginalis* had a low national prevalence in US men due to diagnostic difficulties; NHANES did not test men until 2013 – 2014 for *T. vaginalis*. Spontaneous resolution (36 – 69 percent) of *T. vaginalis* in men is common, with a smaller prevalence than in women in the recent NHANES report. Although *T. vaginalis* is less common in men, during penile-vaginal intercourse, as the partner who is infected is asymptomatic, it is easily transmitted between sexual partners. The diagnosis of contaminated people is therefore a significant issue for public health of 14 cases. *T. vaginalis* is rarely of little benefit in men with sex with men (MSM), and urethral or rectal infection is small in asymptotes (Kelley *et al.,* 2012).

In the recent NHENES study, a pronounced racial regression was observed among African American females and men, in comparison with 0.4 % among other groups (Patel *et al.,* 2018) in relation to *T. vaginalis*, with an approximate prevalence of 6.8 % among black people. In line with the 2001 – 2004 NHANES results, *T. vaginalis* was found to be higher among African American women in comparison with women of other races ethnicities (Sutton *et al.,* 2007). This marked race gap is possibly several-faceted, involving discrepancies in the social networks, social risk activity at the individual rates, such as a greater number of sexual partners and systemic disparities (i.e. inadequate access to healthcare resources) (Ford & Browning, 2011, Kraut-Becher *et al.,* 2008, Sorvillo *et al.,* 2001).

In the latest NHANES report, *T. vaginalis* was reported as being associated with having two and more sexual partners in the last year, being substantially correlated with the elderly, a lower

education level and a decreased socio-economic status (Patel *et al.,* 2018). In the United States, *T. vaginalis* prevalence are higher than in other high-income nations, such as the UK (Field *et al.,* 2018). This is likely due to the lack of overt considerations of infections in public health. The only individuals currently approved in the United States for the regular screening of *T. vaginalis* are women with HIV (Workowiski & Bolan, 2015). Even without signs, *Trichomonas vaginalis* was associated with high rates of adverse effects such as PIDs and poor birth outcomes in the same population (Cotch *et al.,* 1997, Moodley *et al.,* 2002). This population is not symptomatic, in some recent studies have reported high prevalence of HIV-infected women with *T. vaginalis* (17,4 – 20 %) and re-infections (up to 22,7 % over a period of 16 months) (Munzy *et al.*, 2016, Sorvillo *et al.,* 1998, Price *et al.,* 2018). *T. vaginalis*, similar to in HIV-uninfected men, is less common in men infecting HIV and uncommon in MSM infected with HIV (Munzy *et al.,* 2016).

Considering that the effect on pregnant women of *T. vaginalis* was correlated with adverse birth effects (Silver BJ. *et al.,* 2014), no guidelines are currently available for the screening of *T. vaginalis* in asymptomatic pregnant women. This is partly because of an ongoing randomised controlled trial (RCT) involving asymptomatic females with *T. vaginalis*. This study showed an increased risk of preterm addiction between 16 and 23 hours and between 24 and 29 weeks of gestation compared to placebo between two doses (2 grams each) of metronidazole (MTZ). This experiment, however, had some restrictions, including atypical MTZ. Furthermore, between 24 and 29 weeks the second cycle of MTZ was administered, although the highest rise in delivery before the duration was in the sample at 35 – 36 weeks. Therefore, it is difficult to make conclusive findings about the relationship between treatment of asymptomatic *T. vaginalis* during pregnancy and pre-term birth. (Klebanoff *et al.,* 2001, Coleman *et al.,* 2018).

Geographic variability in global prevalence of *T. vaginalis* in pregnant women. A 2016 longitudinal survey of 75 pregnant women with STI prevalence studies showed that the prevalence of *T. vaginalis* in low- and middle-income countries (i.e. Latin America and Southern Africa) ranged between three and four.6 % (Joseph Davy *et al.,* 2016). Recent reports have shown 20 % prevalence for *T. vaginalis* in HIV-infected pregnant women in South Africa (Price *et al.,* 2018) and high levels for incident-infected females in South Africa and Zimbabwe (92/100 person-years), respectively (Teasdale *et al.,* 2018). In the case of pregnant women in the southern United States, Lazenby *et al.,* 2019 has been shown an unusually high incidence of recurrent *T. vaginalis* (44 % at least 21 days after treatment) by nucleic acid amplification test (NAAT). It exceeds the pace found in a previous US study by 7 percent of pregnant women (Klebanoff *et al.,* 2001). Based on their results, Lazenby *et al.,* (2019) proposed retested with NAATs around 3 weeks after treatment for any pregnant women with *T. vaginalis*. (Lazenby *et al.,* 2019).

### *4.1.5 Prevalence of Giardiavirus and Eimeriavirus*

Not medically important report so far to assess the prevelance of *Giardiavirus* and *Eimeriavirus* because both of them have transmit the gene horizontal which means infection for each other. So, no need to motion of prevelance for *Giardiavirus* and *Eimeriavirus*.

### *4.1.6 Virulence of Trichomonasvirus*

For 150 years our understanding of its importance for human health have been impeded by the enigmatic existence of *T. vaginalis*, the most dangerous non-viral sexually transmitted pathogen. The combination of epidemiology, molecular cell biology, and more recent genomic and omics and other related factors leads to a new perspective in the identification of a significant human pathogen, which results in a large sequela of the health of the individual, human microbiology, bacterial pathogens and virus because of multifaceted interactions with their human host. The synthesis of these various data lead to improving our understanding of the pathobiology of parasites and of virulent factors. In fact, it is becoming increasingly appropriate to incorporate the widest possible range of human-microbial-parasite-virus interactions in terms of qualitative and quantum variations in human innate and adaptive defence response with regard to rationalising successful prophylactic and therapeutic treatments for people's pathogens. The goal of this short review is to provide a detailed overview of *T. vaginalis* virulent factors by considering the significance of interplay between human-microbiota-parasite-viruses. In the biological and medical literature, it also refers to other cellular features of the parasite that are sometimes ignored.

Since the discovery in 1836 by the French physician and microbiologist Alfred F. Donné, *Trichomonas vaginalis* (TV) has revealed itself gradually and steadily from a relatively insignificant commensal to an important pathogen, leading to substantial health sequelae in male and female patients, as well as adverse pregnancies (HP). Many of the latest advances in our understanding of TV pathogens include epidemiological involvement with HIV and the high incidence of exposures of the population to sexually transmitted infections (STI) (McClelland *et al.*, 2008, Johnston VJ, Mabey DC, 2008). Highlights of the pathetic sense of this common human parasite, which promotes the spread of HIV and is associated with a number of reproductive complications, including sterility, premature birth, and underweight babies, have a significant effect on human health. HIV conditions are resource restricted for hundreds of millions of people worldwide and TV infects a wide range of people, particularly men without any apparent symptoms, making their diagnosis and control more complex (Van der Pol B 2007). However, the elusive existence of the parasite is even less straight forward in the sense of a debate regarding virulent TV causes, as it is emphasised for bacterial pathogens, which are equally elusive (Falkow S 2004). Indeed, it is increasingly clear that without taking into account host immunological status and host associated microbiota (Clemente *et al.,* 2012), the outcome of human – symbiont interactions (including mutualists, parasites and commensal pathogens) cannot be fully understood. In order to understand their various roles and influence on each other and how these have positive and negative impacts on human health, it is

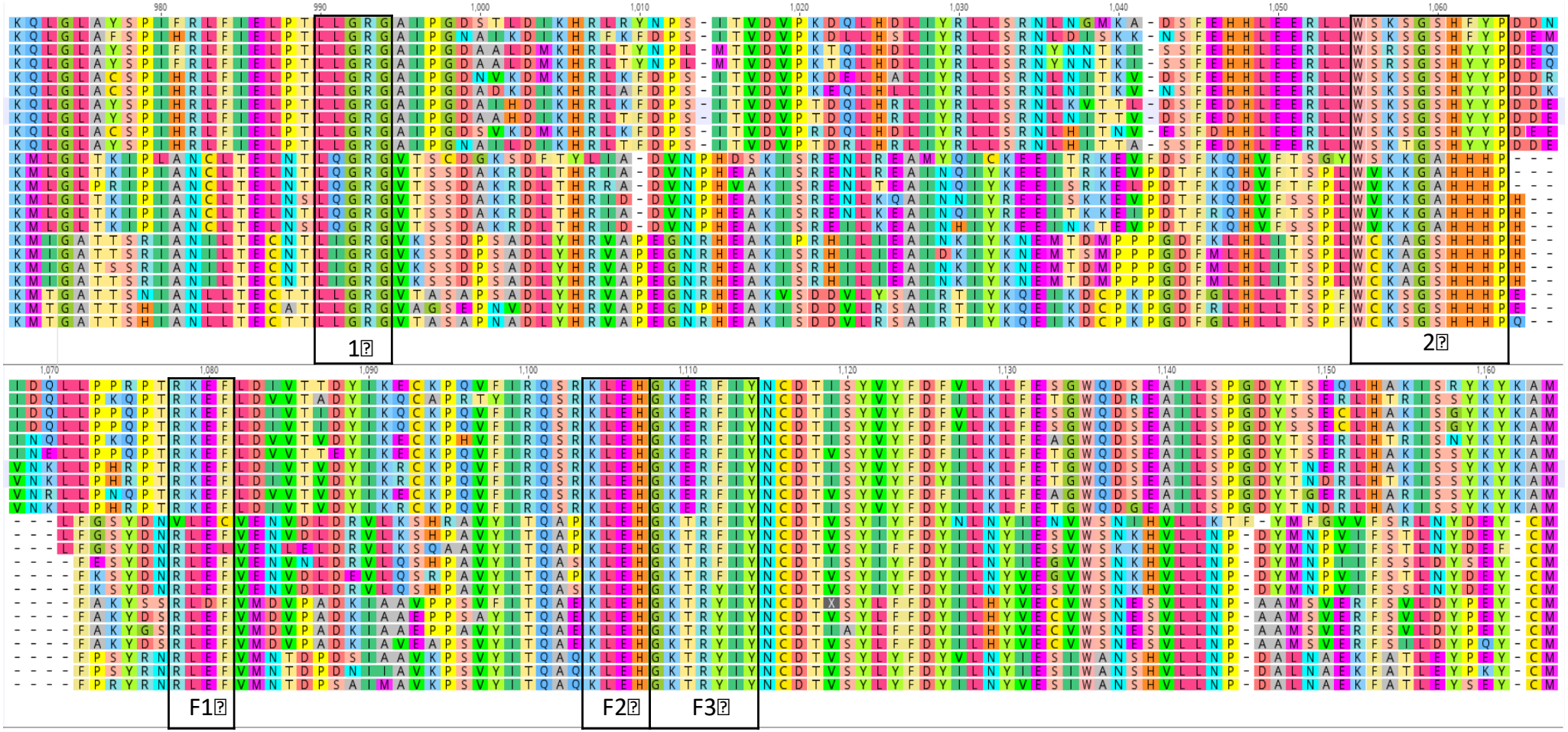
important to incorporate all the human-microbe interactions into all their diversity (Clemente *et al.,* 2012). The restrictive approach suggested by the original four Koch postulates and their molecular children used to classify pathogens and their virulence factors often highlight these aspects (Falkow S 2004). The host reaction to microorganism exposures has now been known to be an integral component of the identification and virulence factors of pathogens (Falkow 2004, Clemente *et al.,* 2012, Casadevall & Pirofiski, 2009). Nonetheless, as seen in a number of recent studies (Sutcliffe *et al.,* 2012, Brotman *et al.* 2012, Conrad *et al.,* 2013, Hirt *et al.,* 2011, Figueroa Angulo *et al.,* 2012), these concerns are increasingly recognised in the TV research community. An integrative summary is therefore provided here to understand the dynamic interplay between the host microbiota parasite-virus and the effects of human-TV interactions for the causes of TV virulence.

### *4.1.7 Virulence of Giardiavirus and Eimeriavirus*

No medical important have been reported for virulence of *Giardiavirus* and *Eimeriavirus* infection because of transition infection of the gene which is horizontal.

## *4.2* Motifs

We have determined the *Leishmaniavirus* motifs that have been discussed in chapter three of this study, and most of the totivirus motifs are identical to each ether. There are also the same motifs for *Trichomonasvirus* as were seen in (Figure 4.2), and *Giardiavirus* in (Figure 4.3), and for *Eimeriavirus* in (Figure 4.4) below. Some of the *Trichomonasviruses* have certain motifs and this is why three separate primers have been created to be deployed for their detection. However, in *Eimeriavirus* motifs, the difficulties of determination of the motifs area lead us to design the accurate primers for each species separately. Each party identified the unique part of totivirus genes.


980
990
1,000
1,010
1,020
1,030
1,040
1,050
1,060
1
2
1,070
1,080
1,090
1,100
1,110
1,120
1,130
1,140
1,150
1,160
F1
F2
F3

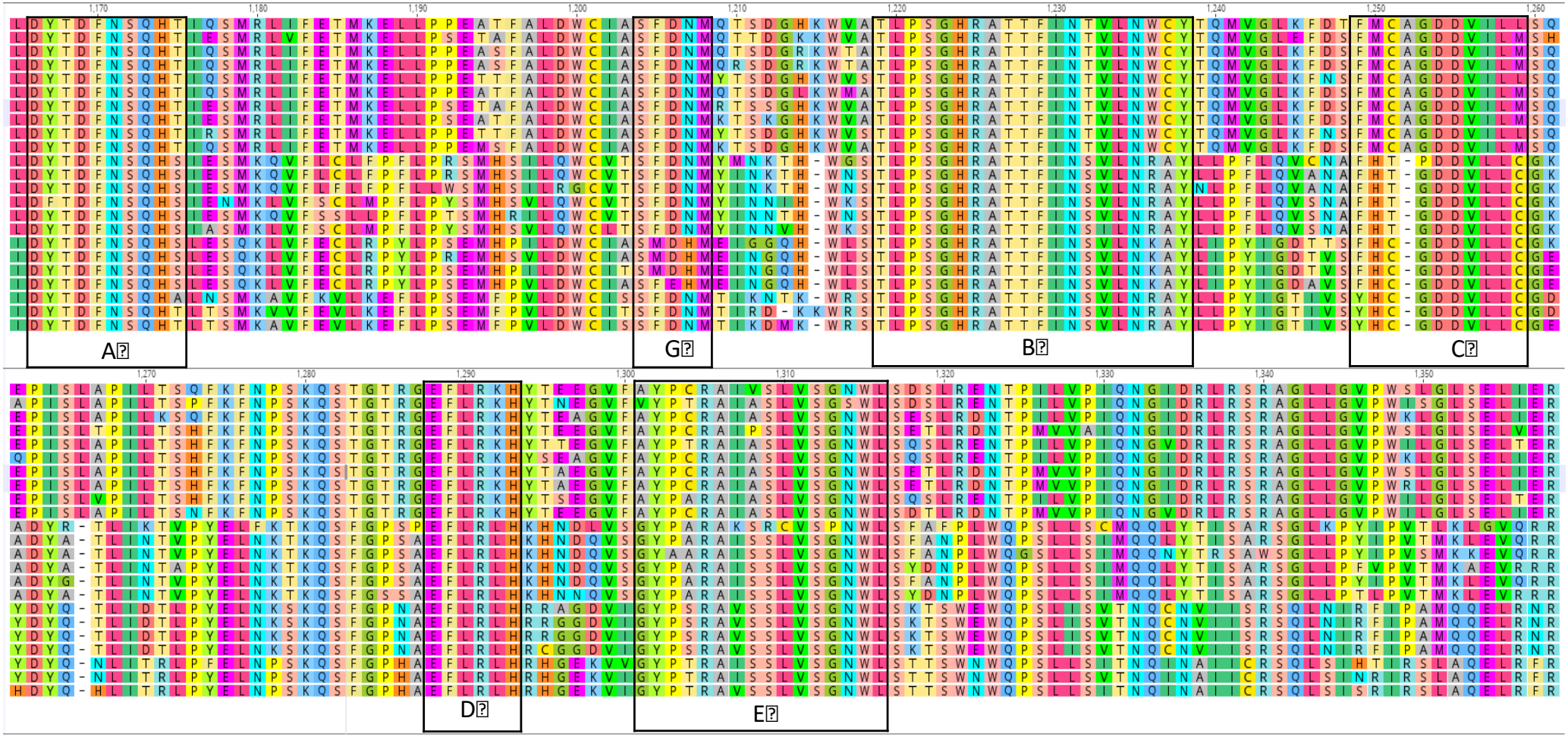



Figure 4.2: Amino acid alignment of RdRp gene from selected *Trichomonasvirus* displaying known motif areas (1,2,F1,F2,F3,A,B,C,D,and E) and highlighting the newly suggested motif area (depicted by letter G), performed by Geneious v10.1 with cost matrix Blosum62.

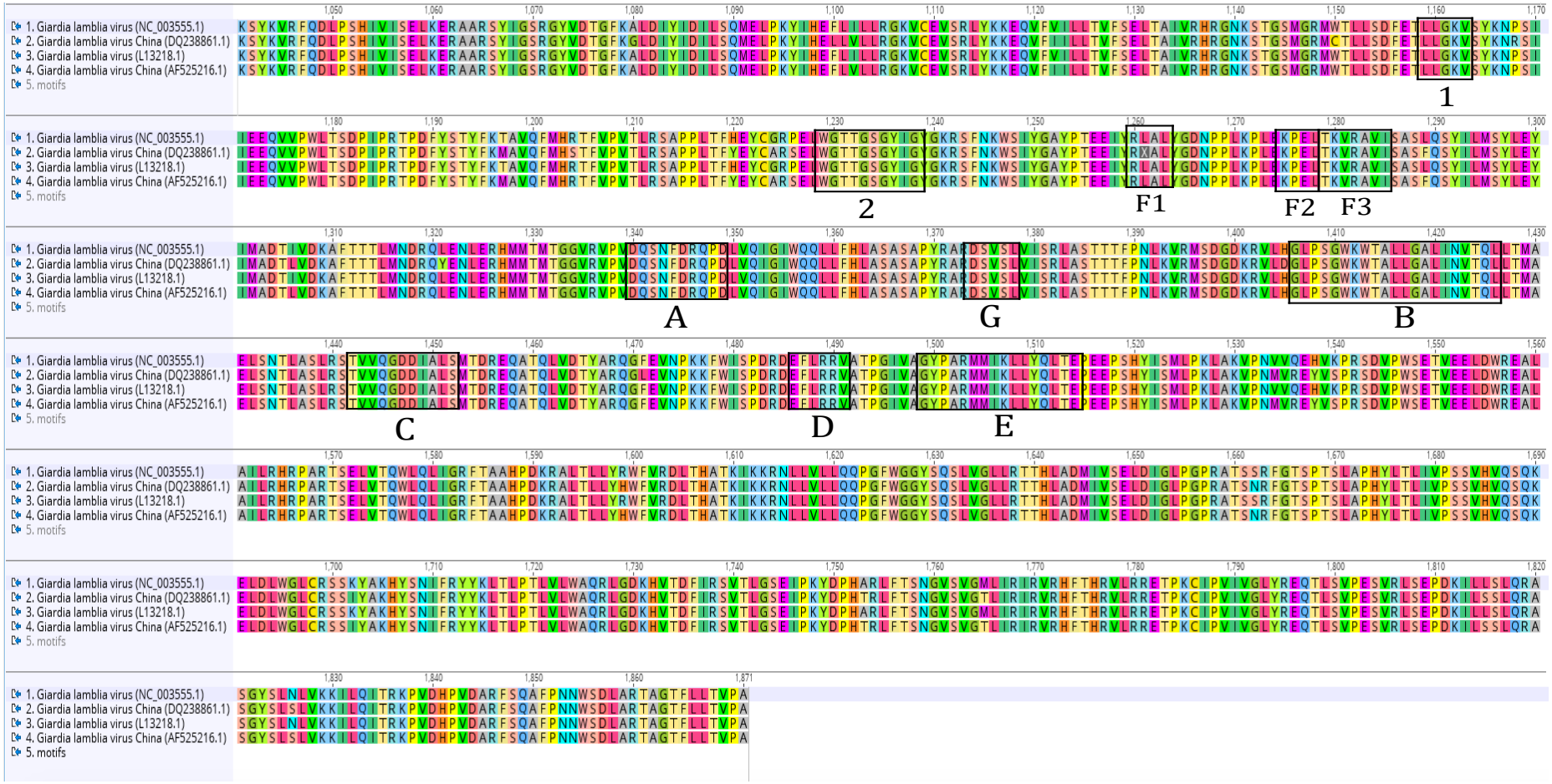



Figure 4.3: Amino acid alignment of RdRp of *Giardia lamblia virus* (GLV) according to identified motifs. Colour scheme is for contrast only. The new suggested motif is called G. Alignment was performed by Geneious v10.1.

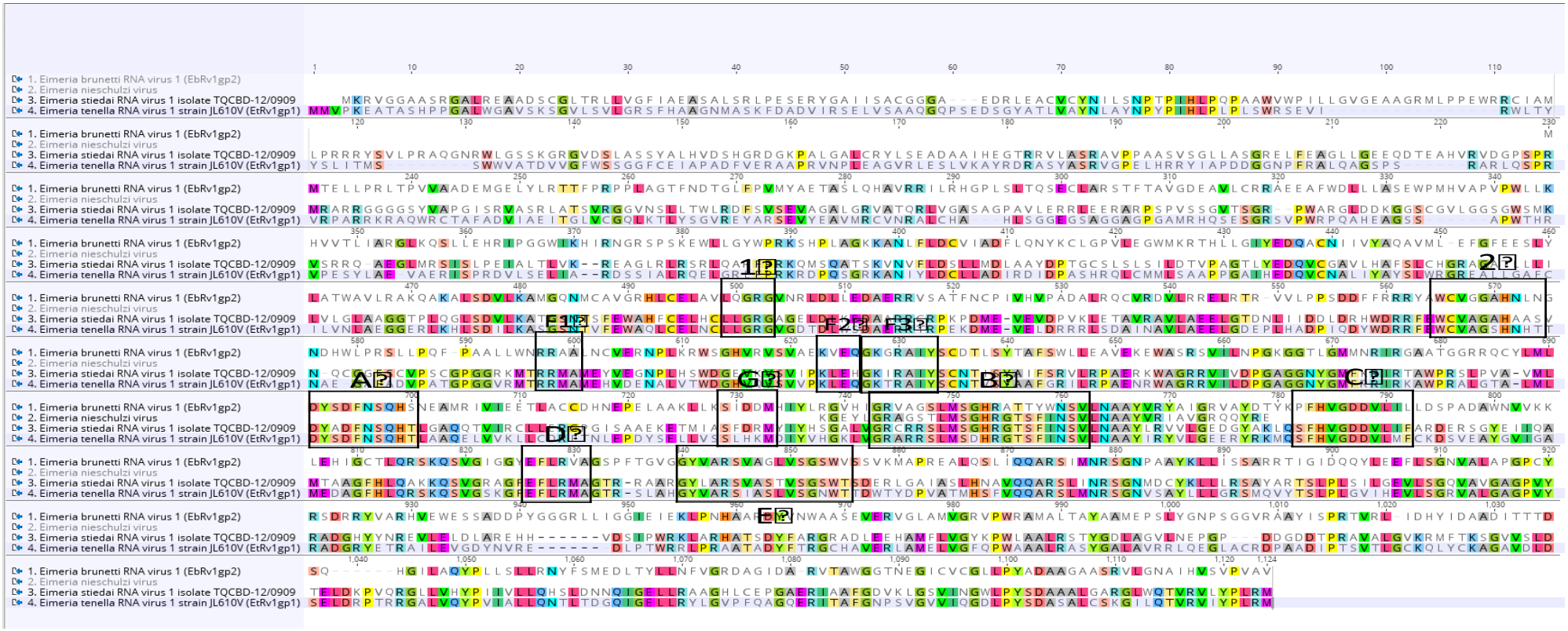



Figure xx protein alignment by Geneious with cost matrix Blosum62 of RdRp *Eimeriavirus* that shows motifs area with new motif area which named G.

Figure 4.4: Amino acid alignment with cost matrix Blosum62 of RdRp *Eimeriavirus* Showing Motifs. A new suggested motif area is labelled G. Alignment was performed by Geneious v10.1.

## *4.3 Aims*

There were few studies in detecting of totivirus in some parasites like *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus.* Here we aim and try to detect the virus in all parasites species that we have got, according to our aims:

- Develop universal, degenerate unique and specific primers, thereby providing totivirus RT-PCR assays for different species of *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus*.
- Sequence the RNA-dependent RNA polymerase of viruses in case of positive samples.
- Align all sequences that belong to *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus,* and determine the most conserve area which is called motif area.
- Try to detect novel totivirus in other species of *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus*.
- Try to detect totiviruses and assess or resolve the severe cases of *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus* species which could associate with totiviruses.
- Try to screen most of different species of *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus* to see how widespread totivirus*.*

## *4.4 Methods and Materials*

The Ethics Review Committee of The Vector Borne and Zoonotic Diseases Department of the Saudi Arabia Ministry of Health authorised and approved this study to collect samples directly from patients such as blood and swab samples, in order to culture them at our laboratory. The health and safety department in Bangor University has approved this experiment, and the ethical approval number is CNS2018AG01.

### *4.4.1 Samples*

In this study many samples were used in several experiments in order to test isolations and PCRs kits and to detect totivirus.

#### *4.3.1.1 Trichomonas Sample*

Samples that have been used in this chapter were provided from University of East Anglia, UK; 3 strains A, C, and M of *Trichomonas gallinae* that infect birds. However, we could not acquire any *Trichomonas virginals* sample from our collaborators.

#### *4.3.1.2 Giardia sample*

The following faecal (unless specified otherwise) samples were obtained from animals that infected with *Giardia* species. All *Giardia* species samples were tested for totivirus.

*a) Canine*

21 from Spain, Carlos III Health Institute, Parasitology Service, Spanish National Centre for Microbiology, 7 from Institute for Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, Slovenia; 1 (as blood) from The European Veterinary Centre, Dubai.

*b) Human*

90 from patients in Uganda, various hospitals; 1 from King Abdullah Hospital, Bisha, Saudi Arabia.

*c) Cattle, Sheep, Goats*

18 cattle, 1 sheep from Axiom Veterinary Laboratories Ltd, Devon, UK; 6 cattle, 3 goats from Slovenia.

*d) Cat*

1 from Slovenia.

#### *4.3.1.3 Eimeria sample*

The following faecal (unless specified otherwise) samples were obtained from animals that infected with *Eimeria* species. All *Eimeria* species samples were tested for totivirus.

*e) Cattle, Sheep, Goats*

3 cattle, 4 sheep from Axiom Veterinary Laboratories Ltd, Devon, UK; 7 cattle, 4 goats from Institute for Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, Slovenia; 5 sheep, 5 goats from Medicina Veterinária Preventiva, Brazil.

### *4.4.2 RNA extractions*

For *Trichomonasvirus* RNA extractions, two kits were used, SV total RNA Isolation System by Promega, and RNeasy Plus Micro kit by QIAGEN. The SV total RNA Isolation System was used in the extraction of RNA from positive a control. The RNeasy Plus Micro kit was used to gain a high concentration of RNA isolation. The extraction was performed according to the following protocol in tables 4.1 and 4.2 below. RNA concentrations for *Trichomonas gallinae* strains were shown in Tables 4.5.

Table 4.1: Extraction protocol according to manufacturer's specifications

| Components | | Volume (µL) |
|---|---|---|
| BL + TG Buffer | | 100 |
| 100 % Isopropanol | | 35 |
| RNA Wash Solution (RWA) | | 500 |
| DNase I incubation mix | Yellow Core Buffer | 24 |
| | $MnCl_2$, 0.09M | 3 |
| | DNase I 3 | 3 |
| Column Wash Solution (DSA) | | 200 |
| RNA Wash Solution (RWA) | | 500 |
| RNA Wash Solution (RWA) | | 300 |
| Nuclease-Free Water | | 15 |

The SV total RNA Isolation System protocol was used to extract positive control samples.

Table 4.2: Extraction protocol according to manufacturer's specifications

| Components | Volume (µl) |
|---|---|
| RLT Buffer | 350 |
| 70 % ethanol | 350 |
| RW1 Buffer | 700 |
| RPE | 500 |
| 80 % ethanol | 500 |
| RNase-free water | 14 |

The RNeasy Plus Micro protocol was used to extract total RNA from tissue or cultured sample.

Two kits were used for the *Giardiavirus* and *Eimeriavirus* RNA extractions, the RNeasy PowerMicrobiome® and RNeasy FFPE®, both from QIAGEN. The first is designed for fast and easy RNA isolation from biosolid samples and was used for the faecal samples; the second for the two samples from Slovenia preserved in formalin. Extractions were performed following the manufacturer's protocols, with parameters as shown in Tables 4.3 and 4.4 below.

RNA concentrations for *Giardiavirus* and *Eimeriavirus* were shown in Tables 4.6 and 4.7 below.

Table 4.3: RNeasy PowerMicrobiome Extraction Reagents

| Components | Volume (µl) |
|---|---|
| PM1–β-ME Buffer | 650 |
| IRS Buffer | 150 |
| PM3 Buffer | 650 |
| PM4 Buffer | 650 |
| PM5 Buffer | 500 |
| DNase I | 50 |
| PM7 Buffer | 400 |
| RNase-free water | 50 |

Table 4.4: RNeasy FFPE Extraction Reagents

| Components | Volume (µl) |
|---|---|
| Deparaffinization Solution | 320 |
| PKD Buffer | 240 |
| Proteinase K | 10 |
| DNase Booster Buffer | 25 |
| DNase I | 10 |
| RBC Buffer | 500 |
| 100 % ethanol | 1200 |
| RBE Buffer | 500 |
| RBE Buffer | 500 |
| RNase-free water | 30 |

Table 4.5: RNA concentrations for *Trichomonas gallinae* strains

| | | |
|---|---|---|
| 16 | *Trichomonas gallinae* A | 39.86 |
| 17 | *Trichomonas gallinae* C | 63.61 |
| 18 | *Trichomonas gallinae* M | 45.47 |

Table 4.6: RNA concentrations for all *Gardai* samples

| No. | Species | RNA (ng/µL) |
|---|---|---|
| **Dog samples from Spain, Slovenia, and Dubai** | | |
| 1 | *Giardia canine* | 87.07 |
| 2 | *Giardia canine* | 97.75 |
| 3 | *Giardia canine* | 90.29 |
| 4 | *Giardia canine* | 48.17 |
| 5 | *Giardia canine* | 90.82 |
| 6 | *Giardia canine* | 35.24 |
| 7 | *Giardia canine* | 49.52 |
| 8 | *Giardia canine* | 76.80 |
| 9 | *Giardia canine* | 70.28 |
| 10 | *Giardia canine* | 65.28 |
| 11 | *Giardia canine* | 120.81 |
| 12 | *Giardia canine* | 109.48 |
| 13 | *Giardia canine* | 97.81 |
| 14 | *Giardia canine* | 145.77 |
| 15 | *Giardia canine* | 156.13 |
| 16 | *Giardia canine* | 95.25 |
| 17 | *Giardia canine* | 55.79 |
| 18 | *Giardia canine* | 77.22 |
| 19 | *Giardia canine* | 57.05 |
| 20 | *Giardia canine* | 80.44 |
| 21 | *Giardia canine* | 98.28 |
| 22 | *Giardia canine* | 90.54 |
| 23 | *Giardia canine* | 65.36 |
| 24 | *Giardia canine* | 91.25 |
| 25 | *Giardia canine* | 83.20 |
| 26 | *Giardia canine* | 32.10 |
| 27 | *Giardia canine* | 52.80 |
| 28 | *Giardia canine* | 32.01 |
| 29 | *Giardia canine* | 110.50 |
| **Human samples from Uganda, and Saudi Arabia** | | |
| 30 | *Giardia lamblia* | 87.07 |
| 31 | *Giardia lamblia* | 97.75 |
| 32 | *Giardia lamblia* | 90.29 |
| 33 | *Giardia lamblia* | 48.17 |
| 34 | *Giardia lamblia* | 90.82 |
| 35 | *Giardia lamblia* | 35.24 |
| 36 | *Giardia lamblia* | 49.52 |
| 37 | *Giardia lamblia* | 76.80 |
| 38 | *Giardia lamblia* | 70.28 |
| 39 | *Giardia lamblia* | 65.28 |
| 40 | *Giardia lamblia* | 102.81 |
| 41 | *Giardia lamblia* | 109.48 |
| 42 | *Giardia lamblia* | 97.81 |
| 43 | *Giardia lamblia* | 105.77 |
| 44 | *Giardia lamblia* | 156.13 |
| 45 | *Giardia lamblia* | 95.25 |
| 46 | *Giardia lamblia* | 55.79 |
| 47 | *Giardia lamblia* | 87.07 |
| 48 | *Giardia lamblia* | 97.75 |
| 49 | *Giardia lamblia* | 90.29 |
| 50 | *Giardia lamblia* | 48.17 |
| 51 | *Giardia lamblia* | 90.82 |
| 52 | *Giardia lamblia* | 35.24 |
| 53 | *Giardia lamblia* | 49.52 |
| 54 | *Giardia lamblia* | 76.80 |
| 55 | *Giardia lamblia* | 70.28 |
| 56 | *Giardia lamblia* | 65.28 |
| 57 | *Giardia lamblia* | 112.81 |
| 58 | *Giardia lamblia* | 139.48 |

| | | |
|---|---|---|
| 59 | *Giardia lamblia* | 97.81 |
| 60 | *Giardia lamblia* | 104.77 |
| 61 | *Giardia lamblia* | 106.13 |
| 62 | *Giardia lamblia* | 95.25 |
| 63 | *Giardia lamblia* | 55.79 |
| 64 | *Giardia lamblia* | 87.07 |
| 65 | *Giardia lamblia* | 97.75 |
| 66 | *Giardia lamblia* | 90.29 |
| 67 | *Giardia lamblia* | 48.17 |
| 68 | *Giardia lamblia* | 90.82 |
| 69 | *Giardia lamblia* | 35.24 |
| 70 | *Giardia lamblia* | 49.52 |
| 71 | *Giardia lamblia* | 76.80 |
| 72 | *Giardia lamblia* | 70.28 |
| 73 | *Giardia lamblia* | 65.28 |
| 74 | *Giardia lamblia* | 92.81 |
| 75 | *Giardia lamblia* | 103.48 |
| 76 | *Giardia lamblia* | 97.81 |
| 77 | *Giardia lamblia* | 185.77 |
| 78 | *Giardia lamblia* | 176.13 |
| 79 | *Giardia lamblia* | 95.25 |
| 80 | *Giardia lamblia* | 91.79 |
| 81 | *Giardia lamblia* | 97.07 |
| 82 | *Giardia lamblia* | 99.75 |
| 83 | *Giardia lamblia* | 80.29 |
| 84 | *Giardia lamblia* | 48.17 |
| 85 | *Giardia lamblia* | 90.82 |
| 86 | *Giardia lamblia* | 95.24 |
| 87 | *Giardia lamblia* | 49.52 |
| 88 | *Giardia lamblia* | 76.80 |
| 89 | *Giardia lamblia* | 70.28 |
| 90 | *Giardia lamblia* | 75.28 |
| 91 | *Giardia lamblia* | 126.81 |
| 92 | *Giardia lamblia* | 109.48 |
| 93 | *Giardia lamblia* | 97.81 |
| 94 | *Giardia lamblia* | 155.77 |
| 95 | *Giardia lamblia* | 125.13 |
| 96 | *Giardia lamblia* | 95.25 |
| 97 | *Giardia lamblia* | 97.79 |
| 98 | *Giardia lamblia* | 98.07 |
| 99 | *Giardia lamblia* | 93.75 |
| 100 | *Giardia lamblia* | 80.29 |
| 101 | *Giardia lamblia* | 48.17 |
| 102 | *Giardia lamblia* | 90.82 |
| 103 | *Giardia lamblia* | 35.24 |
| 104 | *Giardia lamblia* | 49.52 |
| 105 | *Giardia lamblia* | 76.80 |
| 106 | *Giardia lamblia* | 70.28 |
| 107 | *Giardia lamblia* | 65.28 |
| 108 | *Giardia lamblia* | 92.81 |
| 109 | *Giardia lamblia* | 134.48 |
| 110 | *Giardia lamblia* | 99.81 |
| 111 | *Giardia lamblia* | 145.77 |
| 112 | *Giardia lamblia* | 116.13 |
| 113 | *Giardia lamblia* | 95.25 |
| 114 | *Giardia lamblia* | 55.79 |
| 115 | *Giardia lamblia* | 98.07 |
| 116 | *Giardia lamblia* | 97.75 |
| 117 | *Giardia lamblia* | 80.29 |
| 118 | *Giardia lamblia* | 94.17 |
| 119 | *Giardia lamblia* | 90.82 |
| 120 | *Giardia lamblia* | 35.24 |
| 121 | *Giardia lamblia* | 49.52 |
| **Cattle, sheep, and goat samples** | | |
| 122 | *Giardia* | 87.07 |
| 123 | *Giardia* | 97.75 |
| 124 | *Giardia* | 90.29 |

| | | |
|---|---|---|
| 125 | *Giardia* | 98.17 |
| 126 | *Giardia* | 90.82 |
| 127 | *Giardia* | 35.24 |
| 128 | *Giardia* | 49.52 |
| 129 | *Giardia* | 76.80 |
| 130 | *Giardia* | 70.28 |
| 131 | *Giardia* | 65.28 |
| 132 | *Giardia* | 102.42 |
| 133 | *Giardia* | 129.23 |
| 134 | *Giardia* | 97.81 |
| 135 | *Giardia* | 145.77 |
| 136 | *Giardia* | 156.13 |
| 137 | *Giardia* | 95.35 |
| 138 | *Giardia* | 95.59 |
| 139 | *Giardia* | 97.07 |
| 140 | *Giardia* | 99.75 |
| 141 | *Giardia* | 80.29 |
| 142 | *Giardia* | 48.17 |
| 143 | *Giardia* | 90.82 |
| 144 | *Giardia* | 35.24 |
| 145 | *Giardia* | 49.52 |
| 146 | *Giardia* | 96.70 |
| 147 | *Giardia* | 70.28 |
| 148 | *Giardia* | 65.28 |
| **Cat sample** | | |
| 1 | *Giardia* | 94.54 |

Table 4.7: RNA concentrations for all Eimeria samples

| **No.** | **Species** | **RNA (ng/µL)** |
|---|---|---|
| **Cattle, sheep, and goat samples from United Kingdom** | | |
| 1 | *Eimeria* | 27.07 |
| 2 | *Eimeria* | 57.75 |
| 3 | *Eimeria* | 30.29 |
| 4 | *Eimeria* | 38.17 |
| 5 | *Eimeria* | 42.82 |
| 6 | *Eimeria* | 19.24 |
| 7 | *Eimeria* | 49.52 |
| **Cattle, sheep, and goat samples from Slovenia** | | |
| 1 | *Eimeria* | 70.28 |
| 2 | *Eimeria* | 65.28 |
| 3 | *Eimeria* | 12.81 |
| 4 | *Eimeria* | 29.48 |
| 5 | *Eimeria* | 57.81 |
| 6 | *Eimeria* | 45.77 |
| 7 | *Eimeria* | 56.13 |
| 8 | *Eimeria* | 95.25 |
| 9 | *Eimeria* | 55.79 |
| 10 | *Eimeria* | 77.22 |
| 11 | *Eimeria* | 57.05 |
| **Sheep, and goat samples from Brazil** | | |
| 1 | *Eimeria* | 98.28 |
| 2 | *Eimeria* | 90.54 |
| 3 | *Eimeria* | 65.36 |
| 4 | *Eimeria* | 91.25 |
| 5 | *Eimeria* | 83.20 |
| 6 | *Eimeria* | 32.10 |
| 7 | *Eimeria* | 52.80 |
| 8 | *Eimeria* | 32.01 |
| 9 | *Eimeria* | 20.50 |
| 10 | *Eimeria* | 87.07 |

### *4.4.3 Designing Primers*

The primers were designed using Geneious V10.1 for all species of *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus* according to amino acids alignment, we have constructed the primers according to the generous arrangement of nucleotides. Three separate primers groups were designed to detect totivirus in the species of *Trichomonas* shown below in (Table 4.8) and (Figure 4.5).

Table 4.8: *Trichomonasvirus* Primers

| Set | Name | Sequence | Size fragment | Temp | PCR type |
|---|---|---|---|---|---|
| Outer primers | | | | | |
| 1 | *Trichomonasvirus* RdRp 2220F | RTTACCTTAYGAYGATGATCTTTT | 338 bp | 56 ˚C | Nested |
| | *Trichomonasvirus* RdRp 2558R | AGCGGYGTTTGTGATGCATT | | | |
| 2 | *Trichomonasvirus* RdRp 799F | CTCATCGATGGYSCTCTCGC ACC | 350 bp | 60 ˚C | Nested |
| | *Trichomonasvirus* RdRp 1148R | RATGTTCAAYGGWGAYCGCR | | | |
| 3 | *Trichomonasvirus* RdRp 2196F | CGAGTTAGCTGCGCTTTTCG | 253 bp | 58 ˚C | Nested |
| | *Trichomonasvirus* RdRp 2448R | AGTGAATTGTTACGCGCGAA | | | |
| 4 | *Trichomonasvirus* RdRp 2328F | WGAAACAGAAYTACAACTYTTYCCA | 250 bp | 56 ˚C | Nested |
| | *Trichomonasvirus* RdRp 2577R | GAGTGAAGTCCTDTCGGTTAATGC | | | |
| Inner primers | | | | | |
| 5 | *Trichomonasvirus* RdRp 2331F | AACAGAAYTACAGCTYTTYCC | 157 bp | 59 ˚C | Nested |
| | *Trichomonasvirus* RdRp 2488R | CAATGTGCTYTVATGTGGTC | | | |
| 6 | *Trichomonasvirus* RdRp 940F | KCTYGTCGATGCCGCYTTCT | 165 bp | 60 ˚C | Nested |
| | *Trichomonasvirus* RdRp 1104R | AGTTTCGAYCAAGCKCTCGG | | | |
| 7 | *Trichomonasvirus RdRp* 2215F | GCARTTCTCGCCTAACTCGC | 198 bp | 59 ˚C | Nested |
| | *Trichomonasvirus RdRp* 2412R | TTTCCCACTGAAGAAGGCCC | | | |
| 9 | *Trichomonasvirus* RdRp 2535F | WATTAGCGGYGTTTGTGATGCA | 80 bp | 59 ˚C | Nested |
| | *Trichomonasvirus* RdRp 2614R | GTTGTTTRAACCAATCTGAYATACGT | | | |

*Giardiavirus* primers were designed in Geneious V10.1 by aligning amino acid sequences of *Giardiavirus* RdRp downloaded from the NCBI online database, then nucleotides separately downloaded (Figure 4.6) below. Four primer pairs were designed, each with >50 % G and C content and ideal lengths between ~18 - ~24 bp (Table 4.9) below. As the concentrations of RNA were high in all isolates, primers were optimised for normal PCR.

Table 4.9: *Giardiavirus* Primers

| **Set** | **Name** | **Sequence** | **Frag. Size (bp)** | **Temp. °C** | **PCR type** |
|---|---|---|---|---|---|
| 1 | *Giardiavirus* RdRp 2930 F1 | TGGAATTCGTATGCGCTCCA | 488 | 59 | Normal |
| | *Giardiavirus* RdRp 3417 R | CCCCGTGGACTTGTTACCTC | | | |
| 2 | *Giardiavirus* RdRp 2989 F2 | GCTCCGATTCAGCTCCAAGAACC | 429 | 59 | Normal |
| | *Giardiavirus* RdRp 3417 R | CCCCGTGGACTTGTTACCTC | | | |
| 3 | *Giardiavirus* RdRp 2934 F3 | ATTCGTATGCGCTCCACTGT | 484 | 59 | Normal |

| | | | | | |
|---|---|---|---|---|---|
| | *Giardiavirus* RdRp 3417 R | CCCCGTGGACTTGTTACCTC | | | |
| 4 | *Giardiavirus* RdRp 2991 F4 | TCCGATTCAGCTCCAAGACG | 427 | 59 | Normal |
| | *Giardiavirus* RdRp 3417 R | CCCCGTGGACTTGTTACCTC | | | |

*Eimeriavirus* conserved areas of the proposed primer regions revealed differences in the RdRp amino acid sequences downloaded from the National Center for Biotechnology Information (NCBI) databank. For this reason, primers were designed separately for each *Eimeriavirus* species.

Three species were identified as having sequences deposited in GenBank:

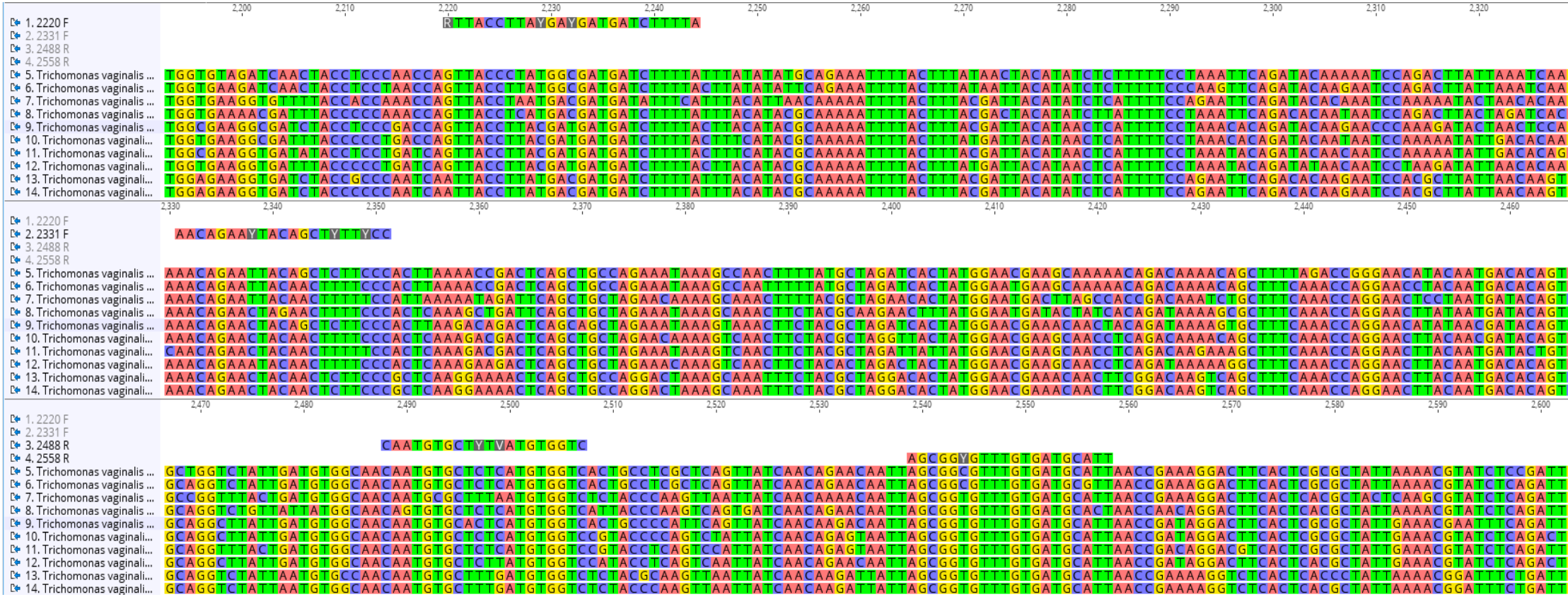
- *Eimeria stiedai RNA virus 1*,
- *Eimeria tenella RNA virus 1*, and
- *Eimeria brunetti RNA virus 1*

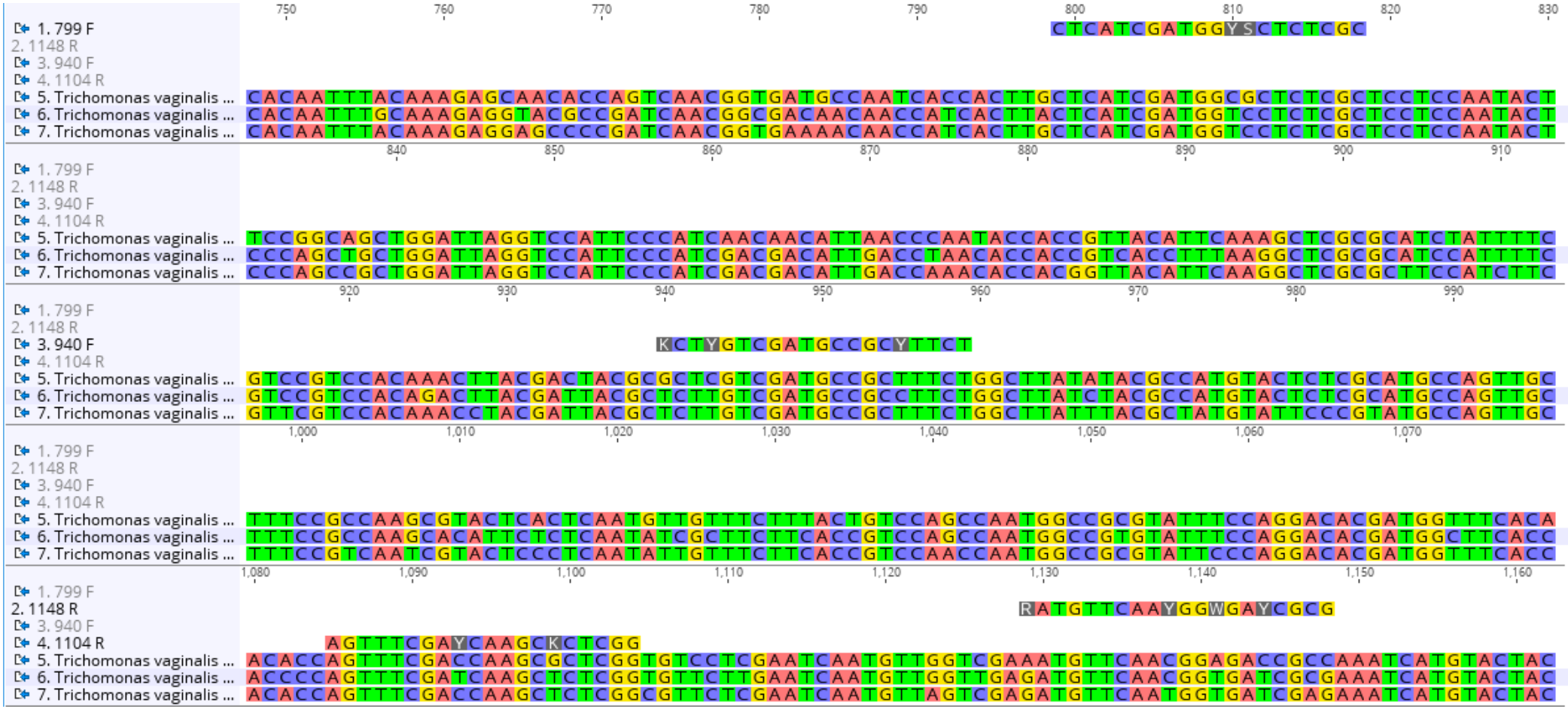
It is recognised that to be fully effective, primers should neither be too short nor too long, typically between ~18 - ~22 bp. Furthermore, G and C content should be 55 % in the sequence or higher, compared to A and T. The nucleotide sequences and alignments of the Geneious v10.1 designed primers per species are shown in (Table 4.10) and (Figure 4.7, 4.8, and 4.9) below.

Table 4.10: *Eimeriavirus* Primer

| Set | Name | Sequence | Frag. Size (bp) | Temp. (°C) | PCR type |
|---|---|---|---|---|---|
| *Eimeria stiedae RNA virus 1* | | | | | |
| 1 | *Eimeriavirus stiedai* RdRp 292F-759R | ATGTTGCCCCCTGAATGGAG<br>TCCCCGTACAGATGTGGCTA | 468 | 58 | Normal |
| *Eimeria tenella RNA virus 1* | | | | | |
| 2 | *Eimeriavirus brunetti* RdRp 1183F-2465R | CATCTATGTGCACGGCAAGCACC<br>TACCGGGTCGTATGTCCAGT | 385 | 58 | Normal |
| *Eimeria brunetti RNA virus 1* | | | | | |
| 3 | *Eimeriavirus brunetti* RdRp 1183F- 1569R | GGGAAGGGTCGTGCCATTTA<br>GGCACGGTGTCCACTCATTA | 387 | 58 | Normal |

Primers were tested by a BLAST search of the NCBI online database.


1. 2220 F
2. 2331 F
3. 2488 R
4. 2558 R
5. Trichomonas vaginalis ...
6. Trichomonas vaginalis ...
7. Trichomonas vaginalis ...
8. Trichomonas vaginalis ...
9. Trichomonas vaginalis ...
10. Trichomonas vaginali...
11. Trichomonas vaginali...
12. Trichomonas vaginali...
13. Trichomonas vaginali...
14. Trichomonas vaginali...


1. 799 F
2. 1148 R
3. 940 F
4. 1104 R
5. Trichomonas vaginalis ...
6. Trichomonas vaginalis ...
7. Trichomonas vaginalis ...
750
760
770
780
790
800
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950
960
970
980
990
1,000
1,010
1,020
1,030
1,040
1,050
1,060
1,070
1,080
1,090
1,100
1,110
1,120
1,130
1,140
1,150
1,160

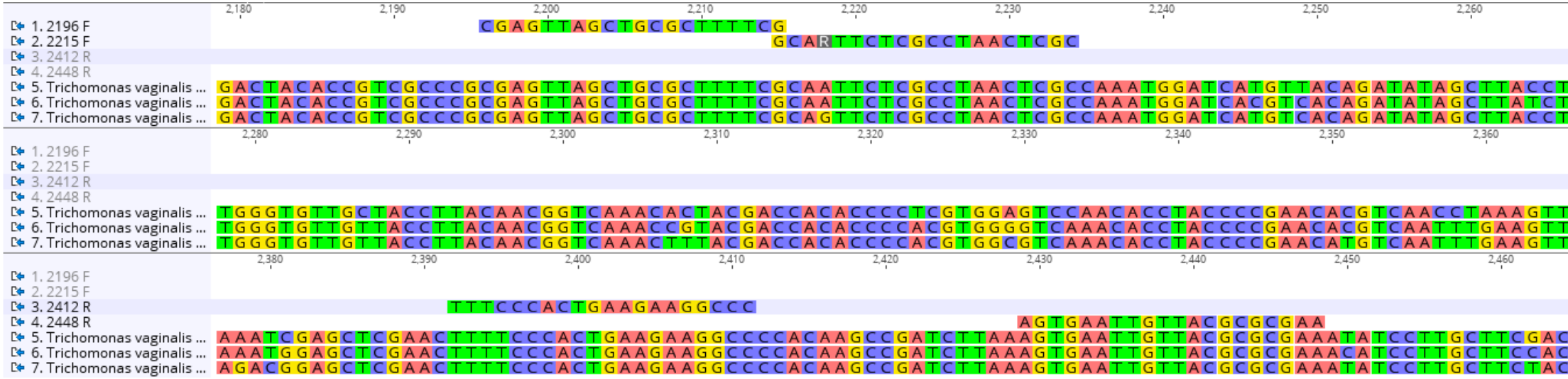



Figure 4.5: Nucleotide alignment of RdRp of *Trichomonas vaginalis virus* (TVV). All sets of position of primers for TVV nested and TVV semi-nested PCR.

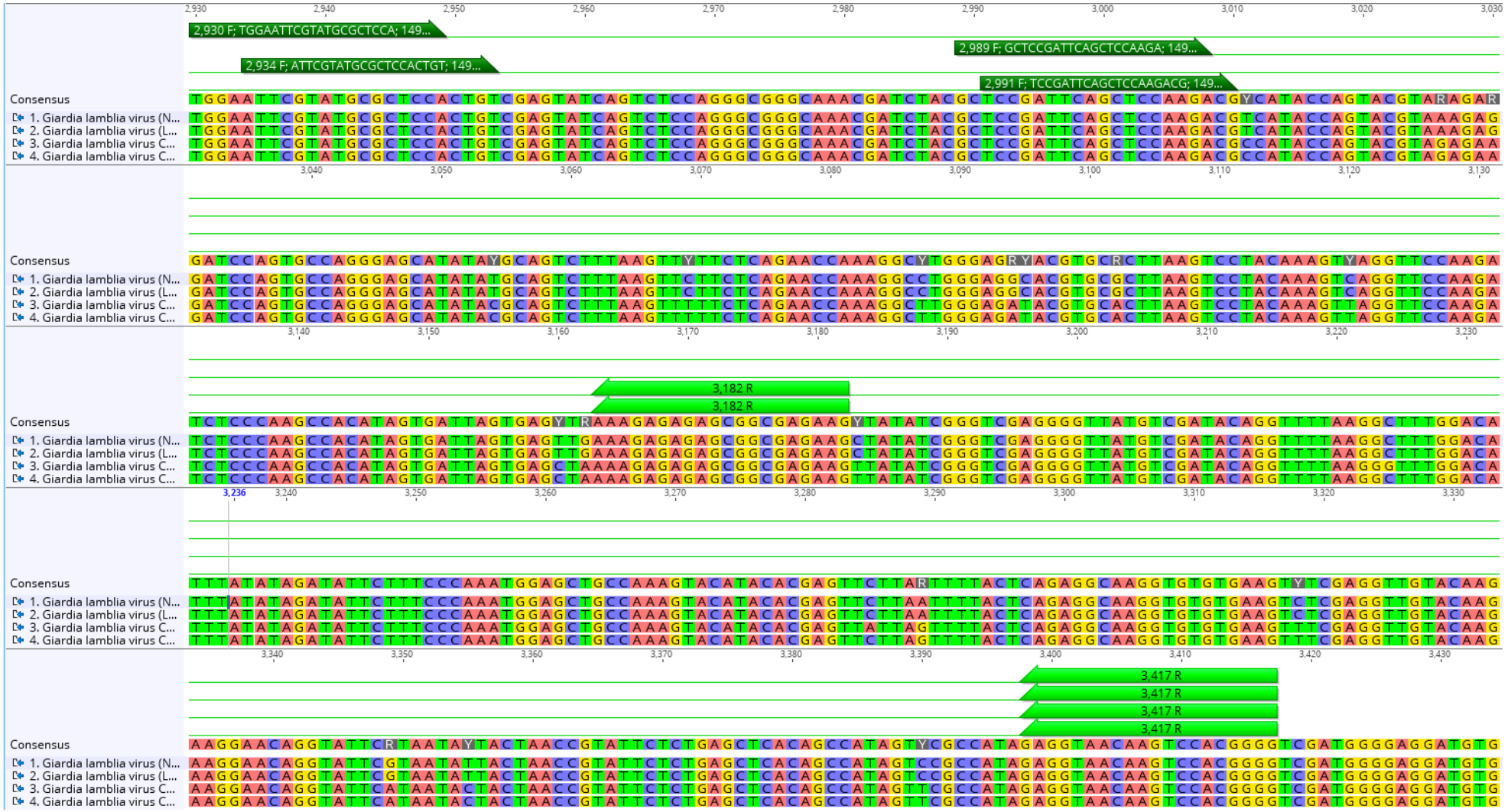



Figure 4.6: Nucleotide alignment of RdRp of *Giardia lamblia virus* (GLV), and primers position for normal PCR

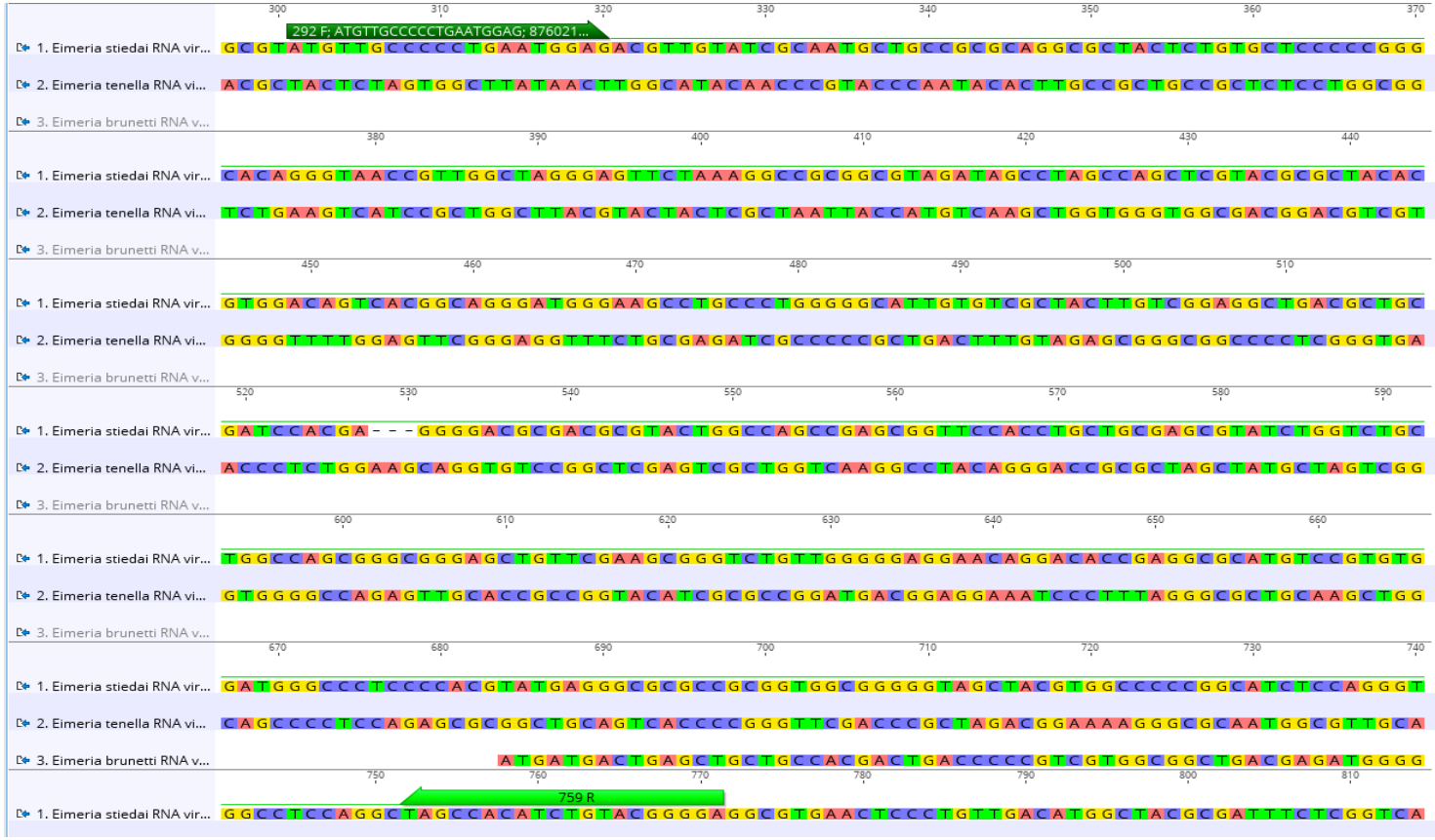



Figure 4.7: Nucleotide alignment of RdRp of *Eimeria stiedai RNA virus 1*, and Primer Positions for normal PCR.

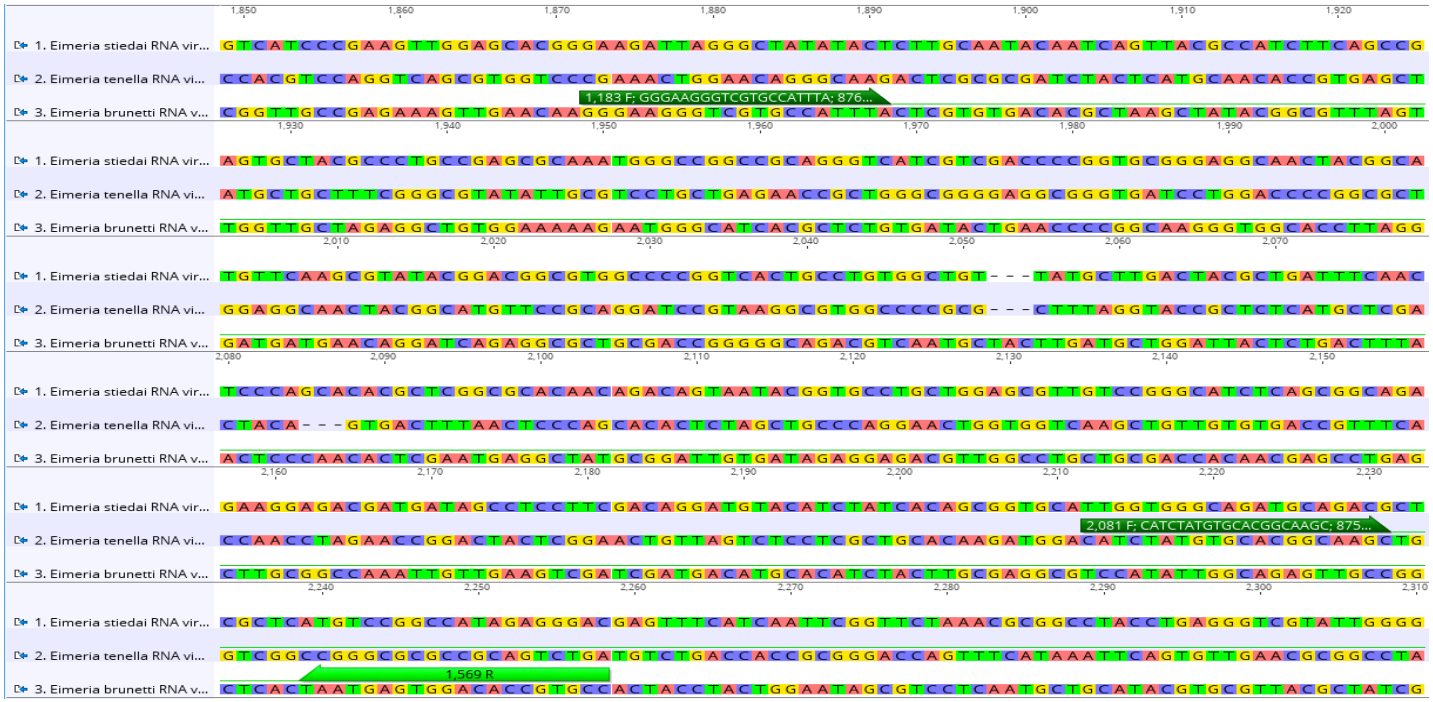



Figure 4.8: Nucleotide alignment of RdRp of *Eimeria tenella RNA virus 1*, and Primer Positions for normal PCR.

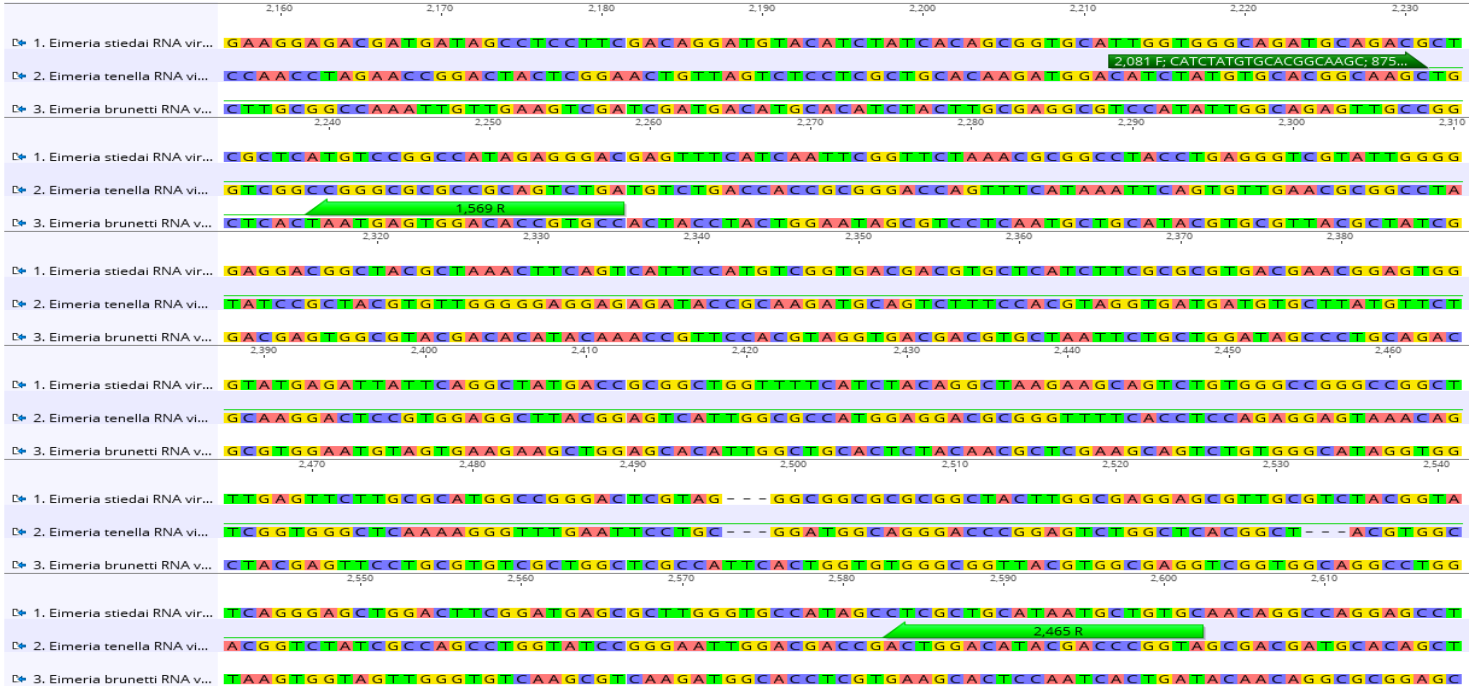



Figure 4.9: Nucleotide alignment of RdRp of *Eimeria brunetti RNA virus 1*, and primer positions for normal PCR.

## 4.5 Results

### 4.5.1 Trichomonasvirus

We were unsuccessful in obtaining any samples of *Trichomonas vaginalis* from our suppliers, so we were then unable to test for totivirus. Also, we could not find totivirus in *Trichomonas gallinae* species.

### 4.5.2 *Giardiavirus* and *Eimeriavirus*

Reverse transcription polymerase chain reaction (RT-PCR) was used to screen the protozoan *Giardia duodenalis* species and *Eimeria* species from a human and diverse animal sources for *Giardiavirus* and *Eimeriavirus*. However, *Giardiavirus* and *Eimeriavirus* were not detected in any sample. Extractions were successful, as demonstrated by the presence of RNA, as was cDNA synthesis and RT-PCRs, which were repeated with a range of annealing temperatures.

## 4.6 Discussion

### 4.6.1 Trichomonasvirus

An early investigation (Alice *et al.,* 1986) has provided strong evidence that virus-like particles (VLPs) of *Trichomonas vaginalis virus* (*TVV*) containing the 1.5 µm linear ds RNA, and a major protein of 85 kDa is present in *T. vaginalis*. Since the dsRNA has been detected in some forty independent isolates and strains of *T. vaginalis*, the virus must have a prevalent presence in the parasite. The presence of virus-like particles before and after long-term implantation was examined using Electron Microscopy and data were thoroughly analysed (Wang *et al.,* 1987). The data indicate strongly that the loss of viral dsRNA correlates to the development of the parasite after long- *in vitro* growth and phenotypic changes in parasites of the isolates concerned. *T. vaginalis* was thought to acquire human viruses based on observations of amoebic behaviour and macrophage activity of the parasite. To test this hypothesis a study was designed (Pindak *et al.,* 1989), where results have been shown to by phagocytosis of infected cells or viruses whose remnants get entry to vaginal. In the first step of the acquisition, *Trichomonads* maintain close contact with the virus that contains cells and cell fragments before ingestion. Considering the current evidence that the P270 mRNA is more abundant during TVV infection than the *Trichomonas* thus corresponding to observations of increased amounts of P270 in type I parasites, the relationship between the dsRNA virus and phenotypic variation of the P270 protein was examined (Khoshnan and Alderete, 1994). Results showed the loss of the dsRNA virus, which occurs during the in vitro cultivation of some isolates, resulted in the absence of a detected P270 mRNA. However, the presence of the P270 protein

in V-trichomonads indicates that transcription and translation of the P270 gene occurs in all *T. vaginal* organisms. Viral infection of *T. vaginalis* has three main differential properties, cellular pathology associated with viral growth, the nuclear site of the virus, and the release of free virus into the culture medium. An irregular cell groups grow after the organism has decomposed and the viral molecules have been released. Such morphological modifications were not documented in other Trichomonas vaginalis virus infected strains or other RNA-containing protozoan parasites (Champney *et al.,* 1995). TVV has four separate viral genotypes to date (Goodman *et al.,* 2011a, b). DNA and amino acid are expected to have properties along with meta-analysis according to the RT-PCR assay. TVV-type 1 (one strain) and 2 (two strains) of the TVV type match the three chains strain. It is an approach to characterize the types of TVV in circulation in the *T. vaginalis* strains in Cuba (Fraga *et al.,* 2011). Also, in Iran (Bandehpour *et al.,* 2013, and Khanaliha *et al.,* 2017), in Philippian (Rivera *et al.,* 2015), in south Brazil (Becker *et al.,* 2015), and Egypt (El-Gayar *et al.,* 2016). In recent decades, interest in the *T. vaginalis* virus has increased from the stranded RNA viruses, as they are considered to be able to influence the severity and cause of the hairy (Wendel *et al.,* 2002). Numerous studies have identified four types of viruses in different groups in the same coating cell in various parts of the world. (Benchimol *et al.,* 2002; Fraga *et al.,* 2012; Goodman *et al.,* 2011; Rivera *et al.,* 2017).

### *4.6.2 Giardiavirus*

Positive control samples were not available at the time of this study, therefore the ability to test primer efficiency was limited. Therefore, while the newly designed primers were tested by matching to sequences available online, they could not be tested with template cDNA from host species known to be infected. A number of characteristics of primers can lead to their failure, despite working in online tests. Length and GC content have been identified as such (Beasley *et al.*,1999), so that while we attempted to design within known, successful parameters of >20 bp and GC content >50 % (as specified by Beasley *et al.*), 7 of 8 primer sequences of exactly 20 bp and only one longer and all with GC > 55 %. It is also important in developing primers, that they are conserved across potential variations in the virus sequence, false-negative results can occur when this is not the case and there are at least 3 strains of *Giardiavirus*. No identical sequences and significant diversity between *Giardiavirus* sequences generated with their own primers, albeit for the capsid protein and those of previous authors were found in isolations from domestic animals by Miska *et al.* (2009). Dultana *et al.* (2009) state that existing primer design tools such as Primer3 used by Geneious are not efficient for designing primers when there is significant heterozygosity in the target virus, since they specifically target a single known sequence. Primers developed by Weinberger *et al.* (2000) matched < 50 % of tested strains of hepatitis B virus. Sequence

variants can be common; in hepatitis C virus, 27 samples were tested by Morris *et al.* (1996) but forward, reverse primer, and probe sequences, respectively matched 24, 23, and 22 sequences respectively. Both studies highlight the need for primer testing with virus-positive template RNA. Therefore, failure, to design effective primers cannot be ruled out as a cause of the PCR amplification failures observed here, in the absence of suitable testing and appropriate positive controls.

Another PCR methodology issue is reported in a study by De Jonckheere & Gordts, (1987). RNA consistent with a double-stranded RNA virus was found to be denatured by RNase A, but not by RNase T1, in about 50 % of *Giardia*-isolates. RNA expression by country of origin was found to range from nil to 100 % depending on the strain tested. White and Wang (1989) found that raw cell lysates and cell culture media can show RNA polymerase activity and that its efficacy depends on how long and how warm the reaction mix is. The RNA product tested by White and Wang was a single strand RNA of a cell infected with the *Giardia lamblia virus* (GLV). As we did not test for the presence of this protein, nor for influence on its activity of our trial conditions, it is possible that some or all of the viruses, if indeed present in our samples, were denatured this way.

Some strains of *Giardia* species appear to lack a virus receptor on the surface membrane of the trophozoites and some research which uses cloning as a step after RT-PCR to further increase the amount of virus available for sequencing has shown success, for example Wang *et al.* (1993).

Despite the increasing number of published reports indicating the prevalence of the *Giardia* protozoan host in a variety of animal hosts, there is evidence that recovery of GLV is not inevitable and may be less common than appreciated. For example, while GLV was found in *Giardia* spp. from a range of domesticated animals, it was not detected in different individuals of the same species nor in other species, despite all of them supporting *Giardia* protozoa (Miller *et al.*, 1988). Miska *et al.* (2009) found numerous sheep and cattle faecal samples that were positive for *Giardia* protozoa, but negative for GLV; furthermore, although the human reference stain of *Giardiavirus* Portland-1-CCW has been identified and persisted in several dozen host-cell subcultures, recent trials have failed to detect dual stranded RNAs, despite artificial GLV infection (Wang & Wang, 1986). It is, therefore, the case that absence of GLV in some or all of our test samples is a likely cause of our inability to detect GLV.

### *4.6.3 Eimeriavirus*

In this exploratory study, our samples of *Eimeria* all came from cattle, sheep and goats, none of which have previously been tested for Eimeriaviruses. It is likely, therefore, that some or all

were negative for this virus. All previous studies of *Eimeriavirus* are derived from infected domestic fowl or chickens (Lee & Fernando, 1999; Lee *et al.*, 1996a and b; Roditi *et al.*, 1994; Sepp, *et al.*, 1991) or the livers of domestic rabbits (Wang & Wang, 1986a and b; Tarr *et al.* 1988; Revets *et al.* 1989), known hosts of *Eimeriavirus* positive *Eimeria* protozoa. Further, many previous studies have tested the Guelph strain of *Eimeria maxima,* which has been confirmed positive for *Eimeriavirus* in every case used. Attempts to isolate a related, small RNA virus, infectious bursal disease virus (IBDV), from 151 samples of the Bursa of Fabricius from chickens, using RT-PCR, found only 48 contained the virus signature and that PCR amplifications were poor in some cases preventing sequencing. Diversity among virus strains was also common in these samples (Jackwood & Nielsen, 1997); both issues are discussed in the *Giardiavirus* part in this chapter as likely to have influenced virus negative results in our case. No previous studies have used PCRs to detect *Eimeriavirus*, except Marugan-Hernandez *et al.*, (2016) who used a cloning stage after the PCR and who, therefore, were successful with this method, illuminating perhaps a different molecular biological strategy to future, more successful trials with *Eimeriavirus*.

## *4.7 Conclusion*

Totiviruses genes have been reported in several studies on *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus* since the mid 1980s*.* Here, despite an extensive bioinformatic search and primer design exercise focusing on *Trichomonasvirus*, *Giardiavirus*, and *Eimeriavirus*, the chapter presented null results. The apparent absence of totiviruses in our samples could mean that our samples were not infected with totivirus and the lack of *Trichomonas virginals* samples has meant that we were unable to screen effectively for *Trichomonasvirus*. Also, it could be that there were only small amounts of totivirus present, especially in the case of *Trichomonasvirus,* meaning that if present, the concentrations of the virus were beyond the limit of amplification. We did screen the sister group of *Trichomonas*, *Trichomonas gallinae*, but yet again, no detection was apparent. Furthermore, have did not have the exact samples that have detected totivirus in previous studies. For example, in *Giardia sp.*, and *Eimeria sp.* cases, our samples were from dogs, cat, cattle, sheep, and goats which have not yielded positive detections for totivirus before. Clearly, having access to known positive controls would have demonstrated the efficacy of the designed primers, yet such samples were elusive, despite our best efforts of acquisition.

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# *Chapter 5:* PHYLOGENY OF *TOTIVIRIDAE*

## *5.1 Introduction*

The *Totiviridae* family is a group of dsRNA viruses that have been increasingly discovered in a variety of different hosts, such as fungi, parasites, Arthropods, fish, animal, plants, fruit, algae, and grassland soil. Viruses in this family contain a monopartite double stranded RNA (dsRNA) genome and comprise non-enveloped icosahedral virions of ~40nm. Their genome size ranges from 4.6 to 7.0 kbp. The majority of these totiviruses are organised in two Open Reading Frames (ORF1 and ORF2). ORF1 is capsid protein with range of sizes between 70–100 kDa and ORF2 is an RNA-dependent RNA polymerase (RdRp) which is important for replication (Wickner R, *et al*., 2012). The *Totiviridae* family consists of five genera: *Leishmaniavirus*, *Trichomonasvirus*, and *Giardiavirus* comprising viruses that infect protozoa; and *Totivirus* and *Victorivirus* which exclusively infect fungi according to the latest report by the Taxonomy Committee of Viruses (ICTV) (King et al., 2011).

The number of studies identifying new species belonging to the *Totiviridae* family has increased considerably in recent years and most of them have not yet been officially listed in ICTV. In the last five years, at least 37 new *Totiviridae* genome sequences have been added to the National Center for Biotechnology Information (NCBI) databank. Notably, some of these totiviruses had an unexpected distribution of host species, including numerous insects, plants, and even fish. (Koyama et al., 2015; Martinez et al., 2016; Chen et al., 2016; Mor and Phelps, 2016a, b). As a result of this wide host heterogeneity, the *Totiviridae* family has increasingly become an excellent model for studies that attempt to clarify the relationships between phylogeny and structural genome features (de Lima et al. 2019). More recently, the unassigned totiviruses have reached total 187 in the course of this study (ICTV website, January 2020).

A new genus, called *Artivirus,* was proposed by Zhai et al. (2010), which comprises arthropod *Totiviridae* species based on IMNV, DTV and AsV genome characteristics, phylogenetic relationships and host type. Also, Dantas and his team (2016) conducted phylogenetic and genomic analysis, which is supports the presence of typical characteristics observed in IMNV, DTV, AsV, OMRV and ToV.

In this study, I attempted to construct the first phylogenetic tree for all available *Totiviridae* family RdRp gene sequences, including all newly described species - 319 sequences in total. Also, I have updated the phylogenetic relations of *Totiviridae* family and compare it with de Lima, 2019 tree that included only 90 sequences of *Totiviridae*. We also, added our novel results of detecting 16 novel RdRp sequences that we have generated in *Leishmaniavirus* in chapter 3 which is not mentioned in de Lima, 2019 tree.

## *5.2 Methods*

The methods closely follow de Lima *et al.* (2019) which are as following:

- De Lima *et al.* used 90 protein sequences which are amino acids for reconstruction of the RdRp-based phylogenetic tree which downloaded from NCBI Taxonomy database. We have used all the totiviruses sequences until January 2020 which are 319 protein sequences.
- De Lima *et al.* used *Amalgaviridae* as outgroup and we have used it too, due to the close relationship between *Amalgaviridae* and *Totiviridae* in phylogenetic tree.
- De Lima *et al.* used methods that using ProtTest program (Darriba et al., 2011) in order to estimate the best amino acid substitution model, followed by a Bayesian phylogenetic analysis using the BEAST v2.4 program (Bouckaert et al., 2014). The phylogenetic inference quality was analysed in the TreeAnnotator v1.10.4 program (Drummond et al., 2012) and the tree topology was generated and edited using the FigTree v1.4.4 program (Drummond et al., 2012). We have done exactly same methods.
- Alignment was performed using the online version of MAFFT (Yamada et al., 2016). However, we have performed the alignment with ClustalW by Geneious v10.1.

### *5.2.1 Sequences*

*Totiviridae* RdRp sequences database for amino acid and nucleotide were downloaded from GenBank which is available online at NCBI website using the following criteria:

l) sequences marked as "*Totiviridae*" or "unclassified *Totiviridae*" deposited before 30 January 2020; de Lima until October 2017.

m) in instances where the same sequence had more than one ancestor, all the sequences were included for all related ancestors to the totivirus. However, de Lima chose only the largest one from totiviruses ancestors sequencing.

n) Sequences of over 130 amino acids and sequences with the highly conserved core domain (GDD) motif were selected; and

o) three viruses of *Amalgaviridae* were selected as the outgroup and sequences also downloaded. Because of phylogenetic analysis clearly shows a close link between the *Amalgaviridae* and *Totiviridae* members, even though *Amalgaviridae* genome organization has a closer relationship to *Totiviridae* members (Martin R, *et al.,* 2011) specially with plant virus (Sabanadzovic S, *et a.l,* 2009).

p) In addition, 16 novel sequences generated in Chapter 3, *Leishmaniavirus*, were included.

q) Moreover, we have also downloaded all totiviruses sequences and aligned for nucleotide. However, de Lima did not use nucleotide sequences.

Amino acid and nucleotide were aligned as described below. Downloaded amino acid and nucleotide sequences, including those selected as outgroups, are listed in Appendix 5.1, novel sequences are in Appendix 3.1.

### *5.2.2 Bayesian Analysis*

Bayesian analysis has been done for all totiviruses sequences in amino acid and nucleotide according to phylogenetic trees made easy book fifth edition (Hall G., 2018).

*a) Amino acid*

Geneious v10.1 was used to download *Totiviridae* RNA dependant RNA polymerase (RdRp) sequences using its implementation of BLAST, according to the criteria specified above, and the amino acid sequences aligned using known motifs. Sequences were separately aligned in MEGA v10.1.7 (Stecher *et al.*, 2020). BEAUti (included in BEAST v2.7.4) was used to add information needed to compile a tree and to convert the data to a BEAST compatible file format. Alignments were passed to ProtTest v3 (Abascal *et al.*, 2005) to estimate the most appropriate model of amino acid substitution for tree building analyses from 108 candidate models (Appendix 6.2). ProtTest 3 results for the amino acid sequences show that best fit model was VT+I+G+F (Appendix 5.2). However, BEAST analysis could not be performed with this model, so instead the next best fit model, WAG+I+G+F, was used.

Using the best-fit models predicted by ProtTest v3, separate Bayesian phylogenetic analyses was conducted using BEAST v2.7.4 (Heled & Drummond, 2009; Bouckaert *et al.*, 2014) and rooted, maximum likelihood trees of aligned amino acid sequences constructed. The Bayesian inference was performed from two independent runs using the best fit substitution models, a strict type molecular clock with a discrete trait informing which group each of the viruses could belong to in a constant population coalescing model. The Markov chain Monte Carlo (MCMC) was performed with $10^7$ generations, with log and tree samplings per 1000 generations. The consensus trees were obtained from the combination of both independent runs, discarding a 10 % burn-in of the initial states. The phylogenetic inference quality was analysed in Tracer v1.6 (Rambaut *et al.*, 2014) and the tree topology was generated and edited using FigTree v1.4.4 (Rambaut, 2009).

*b) Nucleotide*

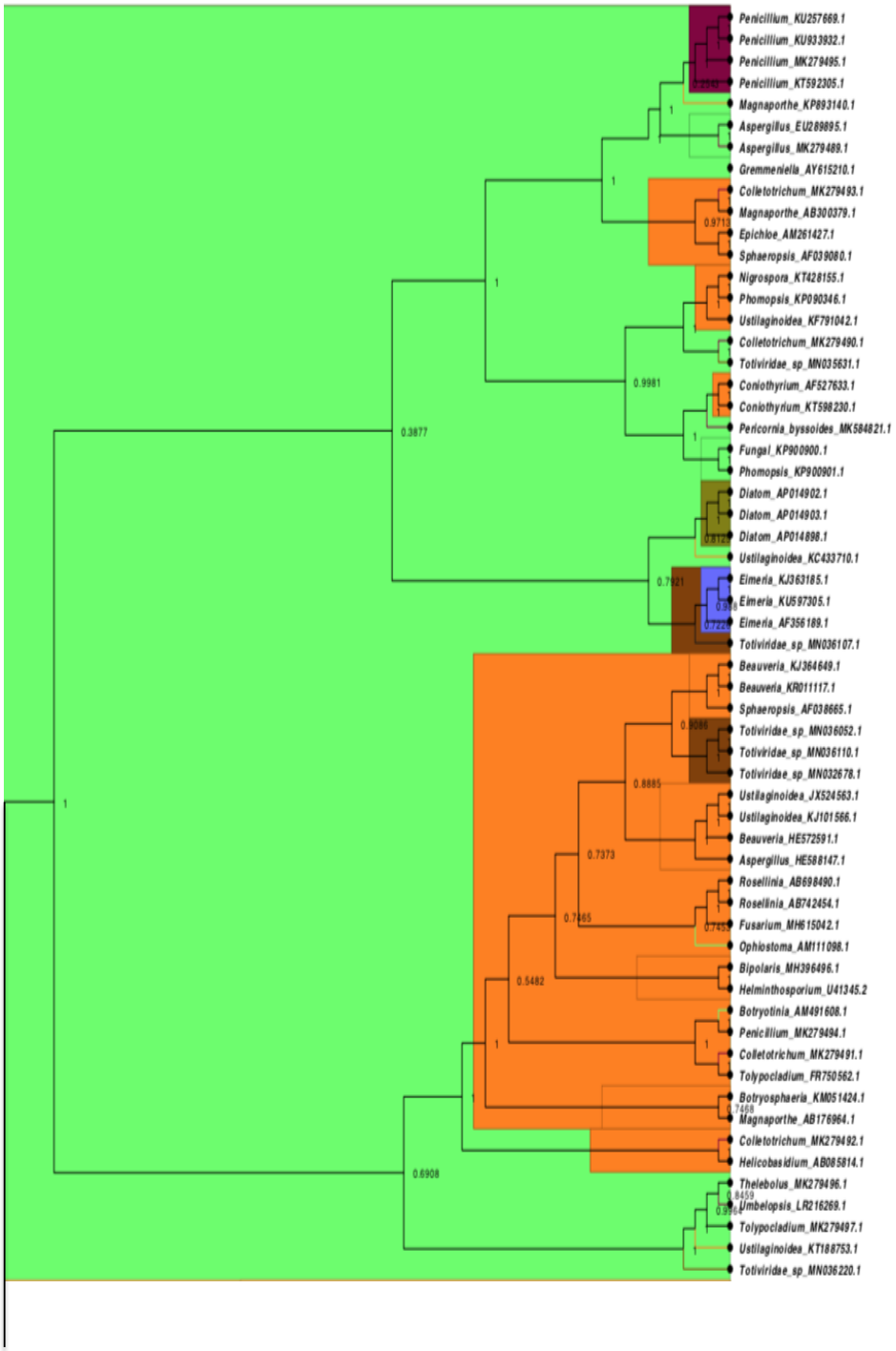
Totiviruses sequences were downloaded as described in Amino acid part. However, MEGAX program have been used to convert the nucleotide sequence to amino acids sequence to made it easier to determine the exact motifs area in the nucleotide sequence, which is easier in amino acid sequence alignment. So, I've used MEGAX program to help me to find the right position of the motifs as it was for amino acid. Then all steps were followed as described above in amino acid analysis using BEAST2 programme for most appropriate substitution model for tree building analyses. After that the outcome result that came for model was GTR +I +G and selected as best fit for the nucleotide tree.

## *5.3 Results*

Results are shown for the amino acid alignments only, since the trees generated from them and the nucleotide alignments were similar. So, the data for the nucleotide tree not showed here because there was no significant difference between them.

### *5.3.1 Totiviridae Phylogeny*

The maximum likelihood phylogenetic tree resulting from BEAST Bayesian analysis, Figure 5.1, was constructed with conserved amino acid domains in the RNA dependent RNA polymerase (RdRp) region of each sequence. A numbered hierarchy is included for each clade, with higher numbers representing progressively larger clades. Branch lengths represent the extent of changes that occurred between separation (speciation events).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Penicillium_MK279495.1
Penicillium_KT592305.1
Magnaporthe_KP893140.1
Aspergillus_EU289895.1
Aspergillus_MK279489.1
Gremmeniella_AY615210.1
Colletotrichum_MK279493.1
Magnaporthe_AB300379.1
Epichloe_AM261427.1
Sphaeropsis_AF039080.1
Nigrospora_KT428155.1
Phomopsis_KP090346.1
Ustilaginoidea_KF791042.1
Colletotrichum_MK279490.1
Totiviridae_sp_MN035631.1
Coniothyrium_AF527633.1
Coniothyrium_KT598230.1
Pericornia_byssoides_MK584821.1
Fungal_KP900900.1
Phomopsis_KP900901.1
Diatom_AP014902.1
Diatom_AP014903.1
Diatom_AP014898.1
Ustilaginoidea_KC433710.1
Eimeria_KJ363185.1
Eimeria_KU597305.1
Eimeria_AF356189.1
Totiviridae_sp_MN036107.1
Beauveria_KJ364649.1
Beauveria_KR011117.1
Sphaeropsis_AF038665.1
Totiviridae_sp_MN036052.1
Totiviridae_sp_MN036110.1
Totiviridae_sp_MN032678.1
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Ustilaginoidea_KJ101566.1
Beauveria_HE572591.1
Aspergillus_HE588147.1
Rosellinia_AB698490.1
Rosellinia_AB742454.1
Fusarium_MH615042.1
Ophiostoma_AM111098.1
Bipolaris_MH396496.1
Helminthosporium_U41345.2
Botryotinia_AM491608.1
Penicillium_MK279494.1
Colletotrichum_MK279491.1
Tolypocladium_FR750562.1
Botryosphaeria_KM051424.1
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Colletotrichum_MK279492.1
Helicobasidium_AB085814.1
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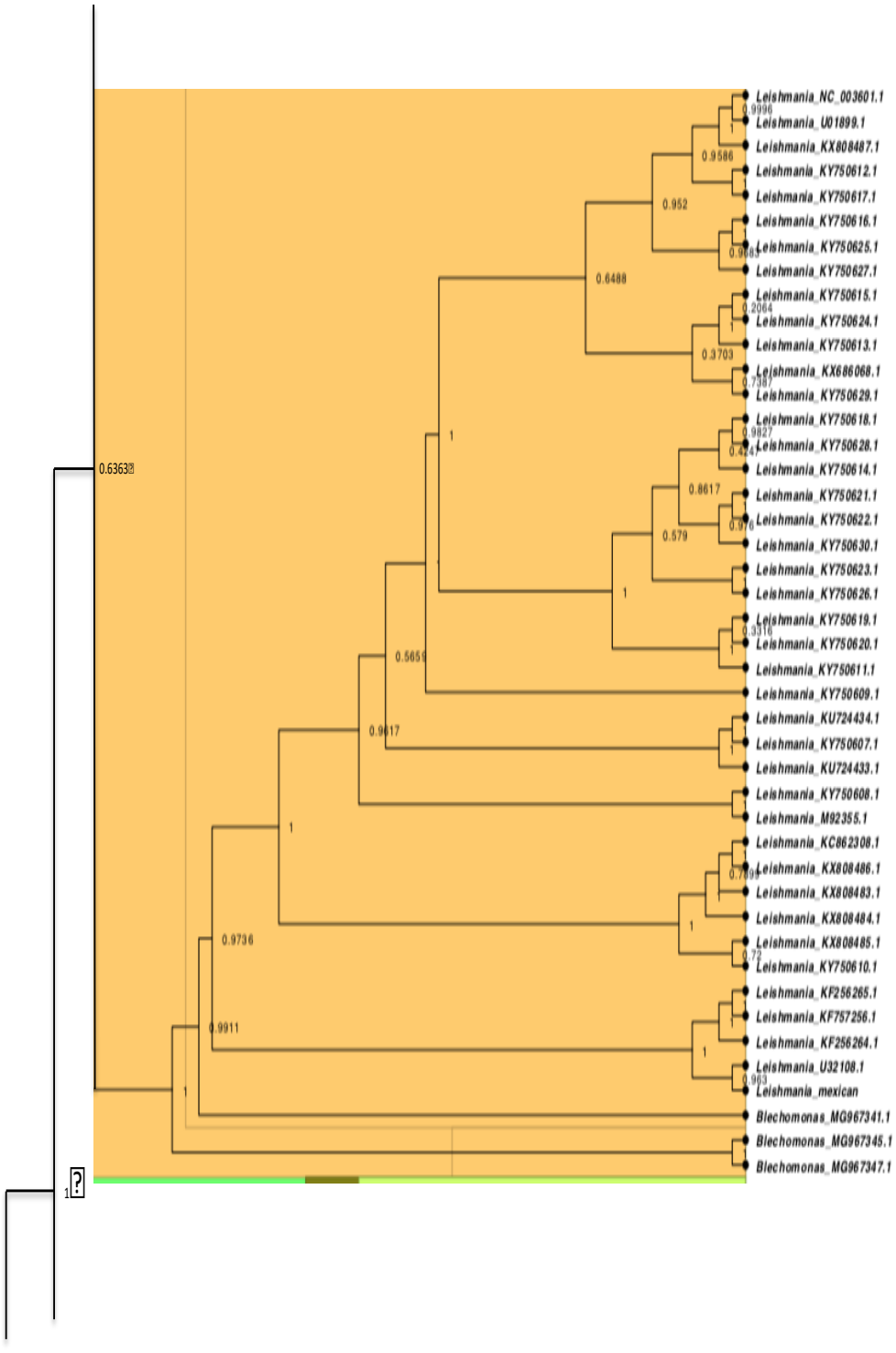
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Leishmania_KY750617.1
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Leishmania_KY750625.1
Leishmania_KY750627.1
Leishmania_KY750615.1
Leishmania_KY750624.1
Leishmania_KY750613.1
Leishmania_KX686068.1
Leishmania_KY750629.1
Leishmania_KY750618.1
Leishmania_KY750628.1
Leishmania_KY750614.1
Leishmania_KY750621.1
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Leishmania_KY750623.1
Leishmania_KY750626.1
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Leishmania_KY750611.1
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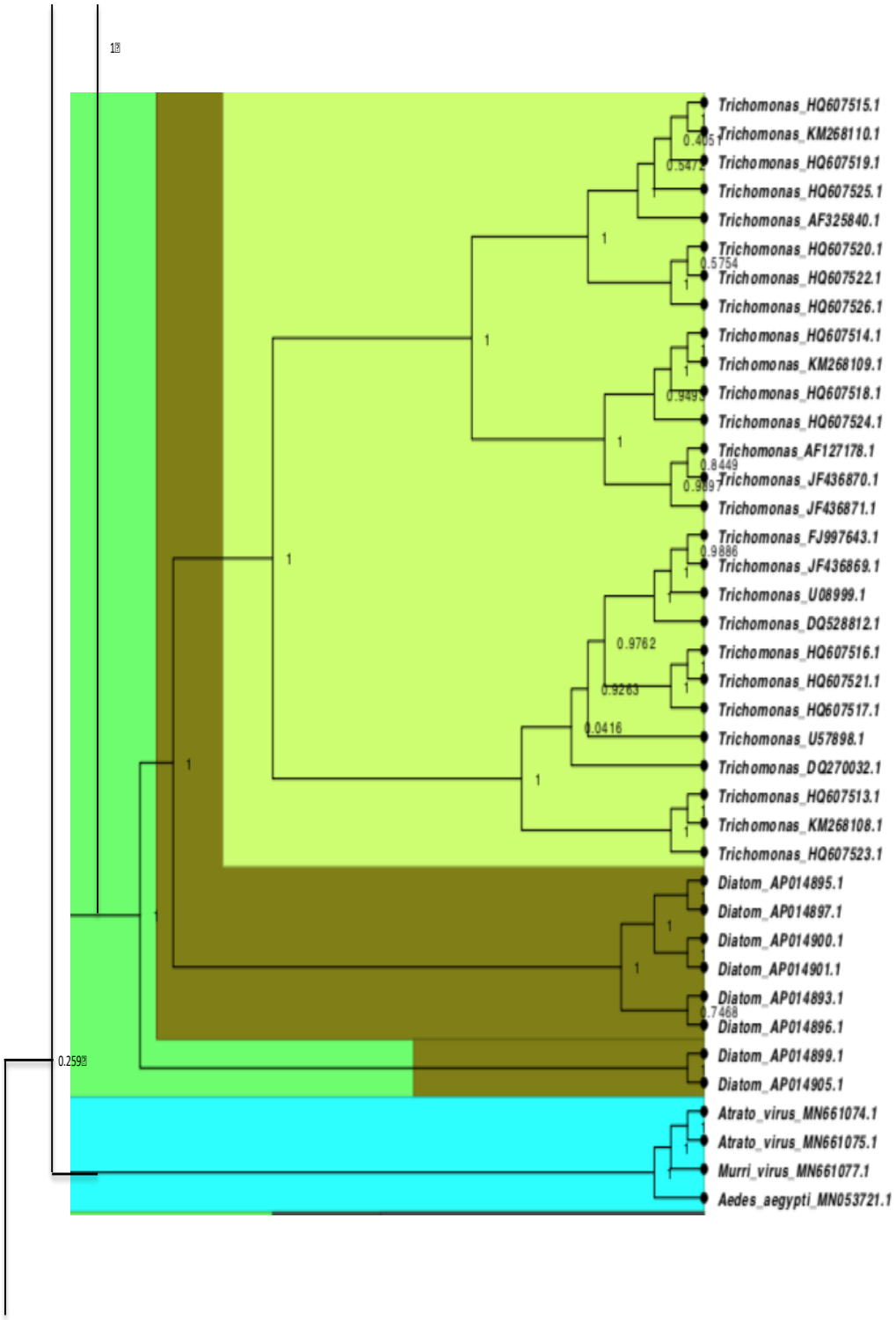
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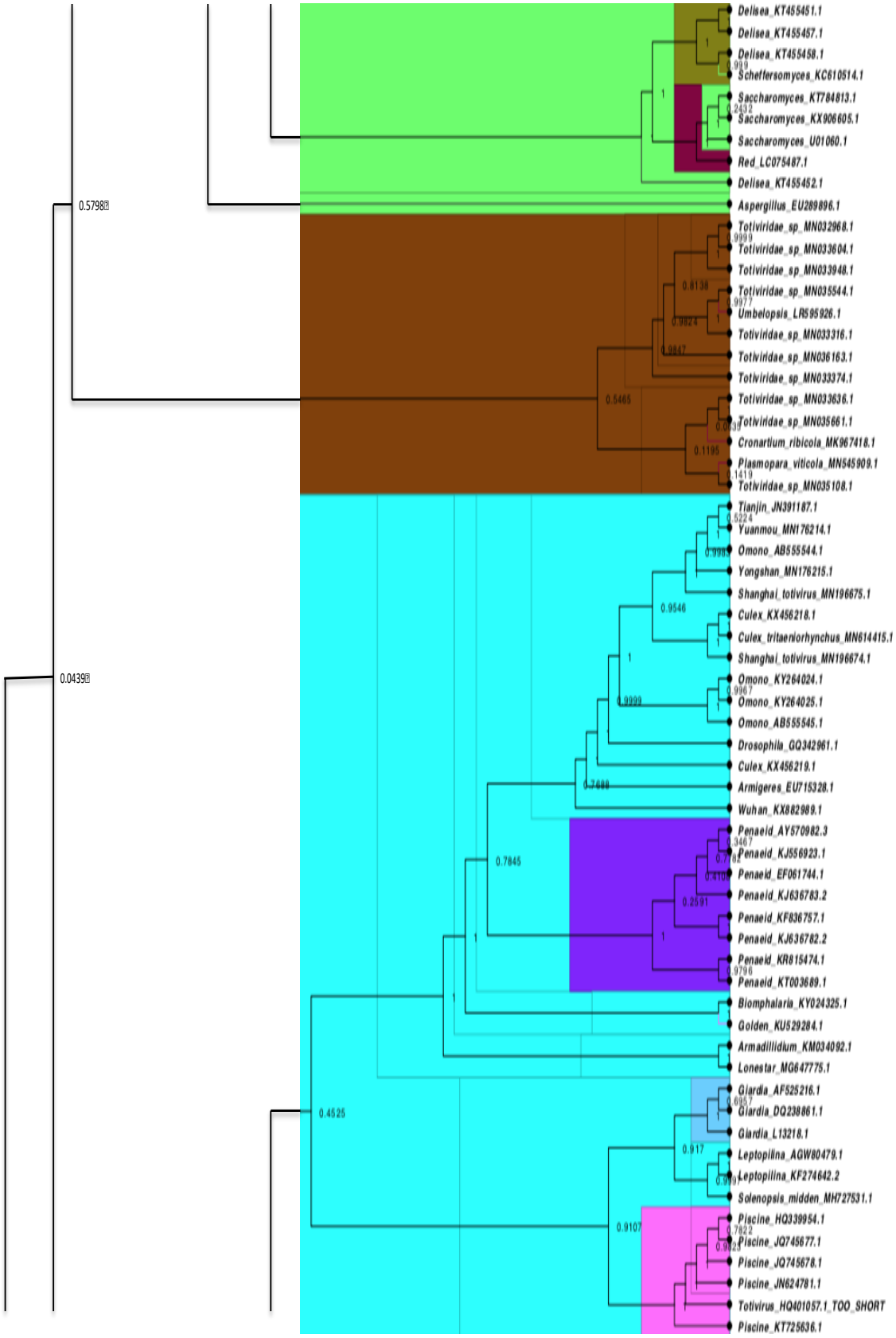
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Culex_KX456218.1
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Wuhan_KX882989.1
Penaeid_AY570982.3
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Penaeid_EF061744.1
Penaeid_KJ636783.2
Penaeid_KF836757.1
Penaeid_KJ636782.2
Penaeid_KR815474.1
Penaeid_KT003689.1
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Golden_KU529284.1
Armadillidium_KM034092.1
Lonestar_MG647775.1
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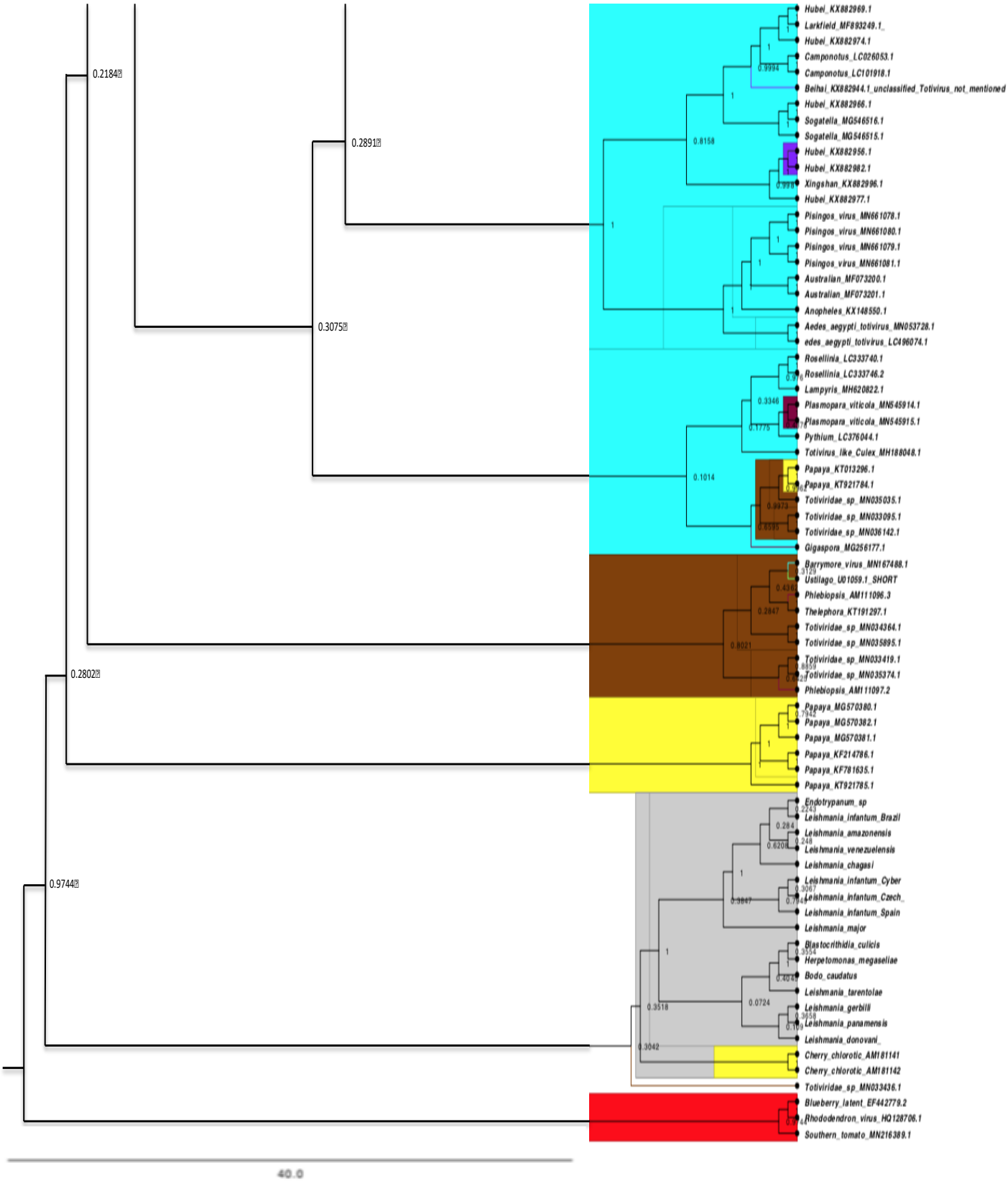


Figure 5.1: *Totiviridae* Phylogenetic Tree Genome organisation of 16 novel of totiviruses found in this Phylogenetic tree of 319 sequences of *Totiviridae* family. . Maximum likelihood tree of totivirus constructed with conserved amino acid domains in the RNA dependent RNA polymerase (RdRp) extracted using Geneious 10.1 version and BEAST2 2.7.4 version. Genome sequences with Genbank accession numbers see appendix 5.1. Node values are the posterior probability.

The phylogenetic tree (Figure 5.1) of the Bayesian inference relationships in the *Totiviridae* family of viruses contains all 319 sequences of *Totiviridae*, held by the NCBI database up to the download date. Branch tips represent downloaded sequences, some species are represented by multiple accessioned sequences (see Appendix 5.1). The analysis recognises 11 distinct clades of *Totiviridae*, as described below, including an out-group of three sequences.

Coloured regions indicate ancestral groups which means a colour have been given to each genus group, detailed in Table 5.1. The 5 genera currently recognised by the Committee on Taxonomy of Viruses (ICTV) are *Totivirus, Trichomonasvirus, Giardiavirus, Victorivirus* (including *Eimeriavirus*) and *Leishmaniavirus*: all 5 are recognised as monophyletic. A further 186 sequences, currently unclassified by the ICTV, are placed in the clades with one of these 5 genera or as distinct clades. The highest number of unclassified viruses is clustered in the clades of the *Totivirus* genus, next in diversity is the *Giardiavirus* (arthropod, Artivirus) clade.

Table 5.1: Highlighted Groups on Figure 5.1 by Colour

| **Group** | **Colour on Figure 5.6** |
|---|---|
| *Totivirus* | Bright green |
| *Victorivirus* | Tangerine orange |
| *Leishmaniavirus* | Cantaloupe / Silver |
| *Trichomonasvirus* | Honeydew |
| *Giardiavirus* | Ice |
| *Eimeriavirus* | Orchid |
| Unclassified *Totiviridae* Hosts | |
| Arthropods | Turquoise |
| Fish | Bubblegum |
| Animal | Grape |
| Plants | Iron |
| Fruit | Yellow |
| Fungi | Maroon |
| Algae | Asparagus |
| Grassland Soil | Mocha |
| Novel Viruses | Silver |
| Outgroup. | Red |

The major clades identified in Figure 5.1 are summarised in the phylogenetic tree Figure 5.2 below (branch lengths are not significant). The phylogeny shows that recognising the ICTV genera as separate clades, leads to 2 clades of *Leishmaniavirus*.

The 16 novel sequences of virus from the newly recognised "New World" *Leishmania* species form the clade *Leishmaniavirus* (2) and are an outgroup to all the recognised genera of *Totiviridae* and are a sister group to the outgroup which is absent from the remaining genera

and, therefore, is the most distantly group related to the other genera. This includes sequences for *L. infantum virus* (including those in hosts from Cyprus, Czech Republic and Spain as well as Brazil), *L. major virus*, *L. panamensis virus*, *L. amazonensis virus*, *L. venezuelensis virus*, *L. chagasi virus*, *L. donovani virus*, *L. gerbilli virus,* and *L. tarentolae virus. L. hertigi virus* was not included in the phylogeny. All of the related viral sequences tested with positive results for *Leishmaniavirus* in Chapter 3, *Endotrypanum* sp., *Herpetomonas megaseliae*, *Blastocrithidia culicis* and *Bodo caudatus* are also in this clade. This monophyletic group was not included in de Lima *et al.* (2019).

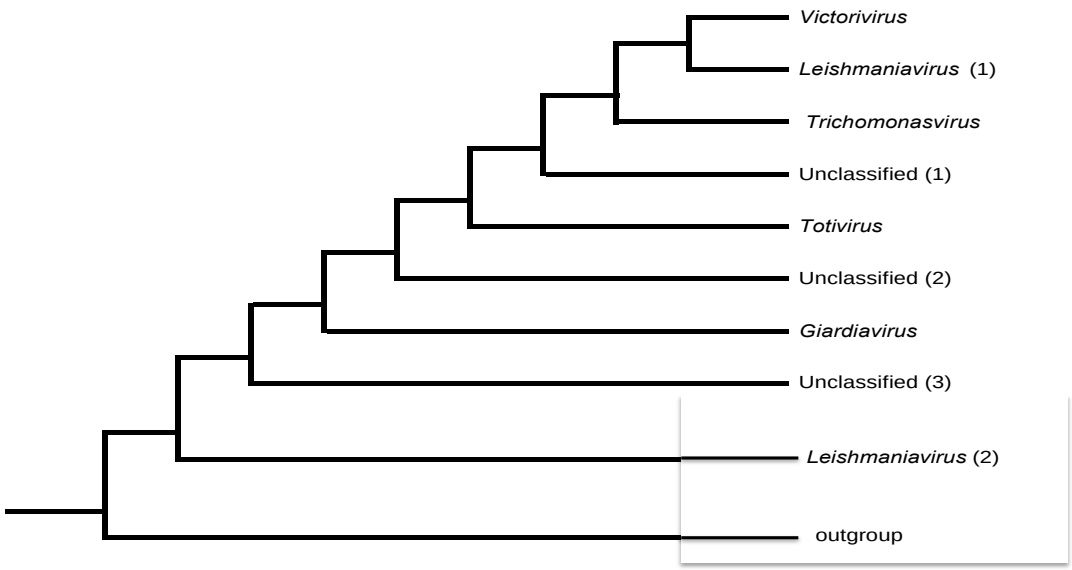


Figure 5.2: Major Clades of the *Totiviridae* cladogram in Figure 5.1

The *Leishmaniavirus* (1) clade contains sequences from the Old World Leishmania species and also one of *L. mexicana* virus and 3 unclassified viruses. It is sister to the recognised genus *Victorivirus*, which in this phylogenetic tree contains all 3 *Eimeriavirus* sequences as expected, along with numerous unclassified viruses predominantly, but not exclusively, from fungal hosts. A small number of sequences are from grassland soil samples, which may also be fungi, and a few are Diatoms (algae). This monophyly and sister relationship is similar to that recovered by de Lima *et al.* (2019)

The major clades of the *Totiviridae* cladogram in figure 5.2 shows that *Totivirus* is an outgroup to the *Victorivirus, Leishmaniavirus* (1) and *Trichomonasvirus* groups, a similar finding to de Lima *et al.* (2019), however, viruses of fungal hosts, typical of *Totivirus*, occur throughout the phylogeny. A monophyletic *Totivirus*, which did not recognise the newly recognised *Trichomonasvirus* sequences would include all of the New World *Leishmaniavirus* and *Victorivirus* sequences and would have the further disadvantage of including Unclassified virus clade (1) which comprises viruses of entirely Arthropod hosts. The *Trichomonasvirus* group includes all the protozoan born viruses, *Trichomonasvirus* sequences from *Trichomonas*, and a small number of Diatoms. The *Totivirus* group is more diverse, containing plant, fungal and algal host sourced sequences.

The *Giardiavirus* clade, includes all three sequences of *Giardia*, and numerous unclassified sequences from arthropods, animals (shrimp) and fish hosts, as well as some from grassland soil and 2 fungi. It contains two monophyletic groups which conform largely to those recovered by de Lima *et al.* (2019), one containing *Giardia lamblia virus* along with viruses from the wasp (*Leptopilina*) and a fish (*Salmo salar*) which de Lima *et al.* refers to as *Giardia lamblia virus* (GLV), the other contains more arthropod hosts, including *inter alia* fruit flies (Drosophila), Mosquitos (Armigeres) and true flies (Culex). However, two more sister clades were recovered in the much enlarged *Giardiavirus* clade, one comprised entirely of viruses of diverse arthropod hosts, for example the mosquito *Aedes aegypti*, the other with mixed sequences from arthropods, fungi such as *Plasmopara viticola*, a plant, *Papaya meleira*, and from environmental samples.

There are three additional clades, Unclassified (1), (2) and (3) which are not monophyletic with any of the 4 recognised genera, (2) and (3) comprise mainly but not entirely environmental samples from garden soil, and (1) a small number of arthropod hosted viruses.

## *5.4 Discussion*

Species of hosts with vertical transmission, for example *Leishmaniavirus* (New World), *Blechomonasvirus* and *Trichomonasvirus,* frequently present adjacently, in respect of genetic distance and distantly from the species transmitted horizontally, which also cluster, they include *Giardiavirus*, *Artivirus*, and *Insevirus* species.

The division of *Giardiavirus* into two monophyletic clades, as recognised by de Lima *et al.* (2019) (hereafter de Lima *et al.*), was initiated following isolation of infectious myonecrosis virus (IMNV) in Penaeid shrimps by Tang *et al.* (2005) and picked up by Poulos *et al.* (2006) (and others) who used a neighbour-joining method of phylogenetic analysis with RdRp sequences to isolate two monophyletic clusters around *Giardia lamblia*[1] *virus* (GLV) hosted by a protozoan and the IMNV hosted by shrimps. Poulos *et al.* go on to suggest that at least IMNV might be distinct from the *Totiviridae* and potentially a new dsRNA family of viruses, however, our findings suggest this is not the case and that, just as de Lima *et al.* found, both *Giardia* clades are nested in a monophyletic *Totiviridae*. *Giardiavirus* IMNV differs in hosts (Shrimps), but also in other characteristics from GLV, notably, GLV replicates in the nucleus of its host and they often have a fusion protein consisting of the major capsid protein and the RNA polymerase, whereas IMNV replicates in shrimp muscle cell cytoplasm and does not appear to have a fusion protein (Wang & Wang, 1986; Wang *et al.*, 1993). There is, therefore accumulating evidence that these groups should be considered for recognition at generic level.

Host specificity has been fundamental to the delineation of the *Totiviridae*, both internally and between related families (Ghabrial, 2008) since its first proposal. Our results challenge this hypothesis, and are supported by both de Lima *et al.*'s results and their estimation. The evidence for reconsidering this taxonomic theory is largely the diminished host specificity in the broadly recovered *Giardiavirus* clades: IMNV we confirmed includes *Bomphalariavirus* (mollusc) and Golden Shiner virus (Fish) as well as arthropods ranging from insects (mosquito) to crustaceans (shrimp); and GLV includes arthropods from insects such as *Leptopilina* (wasp) and *Solenopsis invicta* (ant, though the sequence came from a midden sample (Valles & Rivers, 2019), so is not necessarily of arthropod origin), Salmo salar (fish), as well as the *Giardia lamblia* protozoan. However, the relatively recent inclusion of *Eimeriavirus* whose host is a protozoan in the genus *Victorivirus*, which prior to this comprised

[1] The name *Giardia lamblia* is used in these results as it is the name under which the sequences of this species are deposited in GenBank and also the name preferred by de Lima *et al*. (2019) however, as discussed in Chapter 4, the currently accepted name is *G. duodenalis*.

viruses of fungal hosts only, (for example *Helminthosporium victoriae*, a fungal disease of oats (*Avena* spp., Poaceae), also supports this reappraisal of *Totiviridae*, a placement that is fully supported in our phylogeny, as it was in de Lima *et al.*'s. de Lima *et al.*'s suggestion is that host specificity may have been artefactual based on the limited scope of research into these viruses. Improvements in the availability, and therefore more widespread application of, PCR and next generation sequencing, means that the number of *Totiviridae* sequences available is proliferating with a general trend towards a more diverse range of hosts being positively sampled. It is likely that as this research expands, so will the range of viruses and hosts that meet the morphological definition of *Totiviridae* proposed by Ghabrial (2008) and others. The theory that Artivirus, is both a new genus of *Totiviridae* from *Armigeres* mosquitos and contains just viruses of arthropod hosts as proposed by Zhai *et al.*, (2010) is similarly challenged by de Lima *et al.* and our own phylogeny, since it is embedded within the *Giardiavirus* IMNV clade and, as discussed, also infects vertebrates and molluscs.

De Lima *et al.* did not include viruses from diatoms, from environmental samples or unpublished sequences and only used the longer sequence where more than one was available for a species. They also removed, post assessment, those sequences which adversely affected posterior probability confidence values of their clades. This is the reason for some of the detailed differences in our results, for example the Unclassified (2) clade comprises mainly sequences from environmental samples (grassland soil) and diatom viruses comprise the entirety of a sister group to the *Trichomonasvirus* sequences within the *Trichomonasvirus* clade, which would otherwise be monophyletic for the three known *Trichomonas vaginalis* viruses, as well as comprising a significant proportion of the sequences of the *Totivirus* clade. The two additional *Giardiavirus* clades also largely comprise sequences avoided or unavailable to de Lima *et al.*

The structure of the *Totiviridae* proposed by de Lima *et al.* is very similar to that recovered by the similar Bayesian analysis in this study. So that the outline of the major clades shown in Figure 5.2 is largely reflected in de Lima's phylogeny. We found *Trichomonasvirus* outgroup to *Victorivirus* and *Leishmaniavirus* which are sisters, totivirus sister to this group, *Giardiavirus* sister to the three and including monophyletic GLV and IMNV clades and the *Amalgaviridae* outgroup to them all. This is reflected exactly in de Lima *et al.*'s proposed phylogeny. Key differences between the two studies are the inclusion of a monophyletic clade of New World viruses, *Leishmania* (2) which is sister to the *Amalgaviridae* and, therefore, is an outgroup to the 5 recognised *Totiviridae* genera, likely, therefore, to be worthy of consideration as a new genus. Other key differences include the presence of 2 additional mixed host clades within *Giardiavirus* and the three clades of unclassified viruses, largely from Diatoms and

environmental field samples, none of which sequences were included in the de Lima *et al.* study.

## *5.5 Conclusion*

The results report here strengthen a significant structural host and genome diversity within the *Totiviridae* family, particularly with regard to the expanded clade of *Giardiavirus*, Artivirus, and Insevirus and the rest of unclassified species, which are transmitted horizontally. Species of hosts with vertical transmission, for example *Leishmaniavirus* (New World), *Blechomonasvirus* and *Trichomonasvirus,* frequently present adjacently, in respect of genetic distance and distantly from the species transmitted horizontally. The phylogenetic analysis showed that GLV and IMNV groups have close evolutionary relationships and are therefore shared by the same common forefather, in spite of their considerable differences, in particular in comparison to ORF1. *Leishmaniavirus* (Old World) become near clade of *Amalgaviridae* family which was outgroup of the *Totivirdae* tree.

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# *Chapter 6: HOST ORGANISMS ASSOCIATED WITH TOTIVIRUS*

New results were gathered which were expected to be bioinformatically analysed. Unfortunately, due to the COVID 19 pandemic situation of the past 18 months made it extremely difficult if not impossible to perform the analysis. This was entirely due to the fact that all such facilities which were to be used to sequence RNA template samples produced in this study were diverted in full to support UK Governmental priority requirements for Cov-19 antigen testing. Also, the closure of the University due to COVID19 prevented me from finishing the practical work of Chapter 6.

# *Chapter 7:* FINAL CONCLUSION

*Totiviridae* is a family of double-stranded RNA viruses (dsRNA) of five recognised genera and many unclassified species of totivirus that are being discovered in new host species. Numerous host species have so far been found infected with totivirus

In laboratory research, several dsRNA detection techniques are available, and mostly used is the Dot blot technique. Although it is considered a highly sensitive method and very widely used for its easy and quick methodology, yet in this work it was found to be not accurate enough to detect specific species of *Totiviridae* or dsRNA viruses using the standard J2 antibody due to the fact that the latter is not sensitive enough and the operational window where the antibody is highly selective, is too narrow to be useful for exploratory dot blot assays. Unfortunately, there are no other rapid tests or even ELISA tests available for totivirus detection, especially at low RNA concentrations such as in *Leishmaniavirus* (Zangger et al. 2013). However, this technique can be used with high concertation of RNA virus such as in plant and fungal (Okada et al., 2015). Also, with using highly sensitive antibody like 9D5 (Cheng et al. 2015).

The absence of any simpler or quicker test necessities the use of an alternative approach, the polymerase chain reaction, PCR commonly applied for amino acid and nucleotide amplification prior to sequencing. Specifically, reverse transcriptase PCR (RT-PCR) has already been used successfully to detect some totivirus species. It proved to be accurate and more sensitive compared to Dot blot technique and consequently was adopted for host samples and virus sequences some of which have barely if ever been tested until now. The current method for detecting totiviruses in *Leishmania* samples is by PCR (Margarita *et al.*, 2019; Abtahi *et al.*, 2020; Kariyawasam *et al.*, 2020; Parra-Muñoz *et al.*, 2021). Much research has been published describing its use to detect totivirus species and its sister group *Leishmania*, for example, *Leishmania RNA virus 1* and *Leishmania RNA virus 2* were found this way. However, little is published for *Eimeriavirus* or *Giardiavirus*, being so new, so degenerate primers are needed to be designed prior to its evaluation with samples of the hosts of these viruses. Another widely and successfully used modern method for detecting viruses in general is Illumina whole genome sequencing. However, this technique remains relatively financially expensive, time consuming and complex. Also. Furthermore, considerable effort is required to analyse the resulting data preceded by sample preparation and library building ready for this process.

In our analysis of *Giardiavirus* and *Eimeriavirus* the inclusion of positive-control, virus-positive samples have clearly enabled more precise conclusions to be drawn for the absence of isolated viruses. A systematic testing of the efficacy of the newly designed primers *in vitro*, given that BLAST testing in isolation is recognised not to be sufficient for this purpose and of the PCR conditions, using positive controls, would enable host sample genomes to be accurately evaluated for virus infection and the prevalence of virus infection to be determined. Available research and published data demonstrate the non-uniform distribution of the virus among it's hot and in some cases, even when present, cannot be detected due to primer specificity issues in the presence of divergent strains. Where our RT-PCR was successful, in 16 samples of *Leishmania* and sister group, we discovered that of 43 samples were analysed, 42 were positive isolates by our several primers that we have designed for Old and New world Leishmania, reinforcing the view that in *Leishmaniavirus* at least, infection is very common. In both Old and New World Leishmania, it is worthwhile mentioning that this particular finding is different to what is reported by Hartley et al. (2012), Hajjaran et al. (2016) hwrer they reported only 2 sample positive out 50 tested. Also, Sukla et al. (2017) could not isolate it at all L. donovani, a species successfully isolated in this study. Many virus groups are not so widespread as *Leishmaniavirus*, or at least regularly not isolated, so that clearly there is much to be learnt about both the biological and methodological reasons for negative results.

The absence of *Leishmaniavirus* in some studies that showed negative result can be attributed to the following factors:

1. The low concentration and prevalence of the totivirus because genetic exchange that occurs then the virus transmits (Hartley et al. 2012).

2. These viruses might be lost in laboratory culture (Ronet et al., 2011).

3. Inaccurate non sensitive primer design that detect the totiviruses

So, more research is needed to investigate the absence of totiviruses in all *Leishmania* species and when virulence is associated with prevelance of the totiviruses especially in Old World Leishmania.

RT-PCR technique can be highly successful as demonstrated when discovering important, novel viruses of New World host species such as *Leishmania infantum virus*, *L. major virus, L. panamensis virus, L. hertigi virus, L. mexicana virus, L. amazonensis virus, L. venezuelensis virus, L. chagasi virus, L. donovani virus, L. gerbilli virus,* and *L. tarentolae* virus. When included in our overarching phylogenetic analysis of the *Totiviridae*, these strains

were placed in a monophyletic clade, distinct from previously discovered Leishmaniaviruses, an outgroup to all recognised genera and sister to the *Amalgaviridae.*

Totivirus, a relatively new detected virus discovered in the genus *Endotrypanum*, in *Herpetomonas megaseliae*, and in *Blastocrithidia culicis* of the *Trypanosomatidae*, is currently outside taxonomic groupings of parasites. In addition, the first totivirus was discovered in *Bodo caudatus* (*Bodonida*: *Kinetoplastida),* widely expanding the host range of vertically transmitted totiviruses and the probable time when these viruses entered their host lineage. Further research is needed to establish the following:

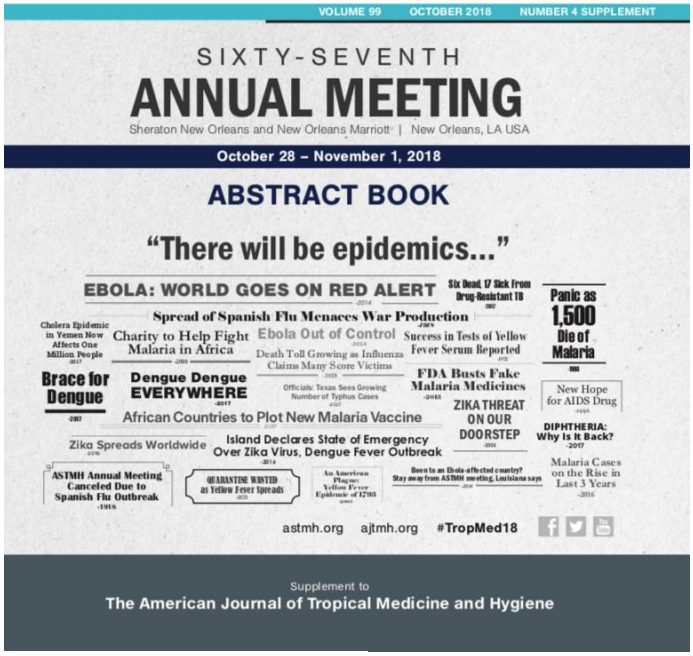
1. How widespread this group of viruses is among species related to *Leishmania*.

2. Determine what detrimental or beneficial effects they may have on their host's efficacy and perhaps compare it to more highly pathogenic species.

This applies particularly to their relationship to the globally important, disease causing *Leishmania* affecting vertebrate hosts. While considerable work on their occurrence in some *Leishmania* species is available, a broad characterisation of the diversity of strains is yet to emerge. The phylogeny built using Bayesian inference showed great affinity to an earlier one by de Lima *et al*., (2019), confirming the effectiveness of the analysis. The major clades recovered were largely equivalent to de Lima *et al*., save for the considerable number of additional sequences included in our dendrogram. Based on these results we can strongly suggest the need to move away from a host-based classification of the *Totiviridae*, too many genera were shown to be far more host-diverse than has previously been widely recognised. Furthermore, it is clear that there are strong lineages within the family which would benefit from broader, deeper analysis with a view to future taxonomic recognition.

# *Chapter 8:* APPENDICES

## Appendix 1.1

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### TOTIVIRUSES, PARASITES, AND EVERYTHING ELSE

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Totiviridae are unsegmented, icosahedral dsRNA viruses which display a fascinating diversity of hosts, a disparity of host effects, and a divergence of transmission strategies. Hosts include human parasites like Giardia, plant parasitic oomycetes, fungi and yeasts, red macroalgae (seaweed), terrestrial crustaceans like woodlice, insects like flies, mosquitoes, ants and wasps, marine crustaceans like shrimp, but also fish, fresh water snails that are intermediate hosts to parasites, and plants like papaya, notoginseng, maize, and wild petunias. In Leishmania and Trichomonas, the viruses increase the virulence of the parasites (hypervirulence), while in Victoria blight of oats it reduces the virulence of the fungus (hypovirulence). In salmon, smelt, and shrimp, it causes myocarditis and myonecrosis, in golden shiners it is asymptomatic. In Leishmania and many fungi and some plants, it is non-infectious and vertically transmitted, while in Giardia, fish, shrimps, and papaya, it is horizontally transmitted. Using PCR with degenerate primer sets, we are trying to explore the taxonomic boundaries of the vertically transmitted viruses in parasites to estimate the evolutionary age of first infection, the virulence in Giardia, and the evolutionary origin of dsRNA viruses in arthropods, especially sand flies, which are vectors of Leishmania. In a new development, Leishmania species not infected with totiviruses show an infection with Narnaviruses. Both viruses are vertically transmitted. The original concept that the Leishmania clade has only been infected once and subsequently lost the infection in several lineages has been challenged with these new discoveries.

Figure 8.1: 1 Appendix 1.1: Abstract from the 67th Annual Meeting of the American Society of Tropical Medicine and Hygiene (ASTMH) conference, 2018

**Abstract, describing this study, published at the 67th Annual Meeting of the American Society of Tropical Medicine and Hygiene (ASTMH) conference, which was held in New Orleans, LA USA, from October 28 to November 1, 2018**

## *Appendix 3.1*

## *Leishmaniavirus RNA virus 1* & *2* sequences

*Leishmania major*

GGCTGAGTTATTTACTAGACAGGCGCGAAGCACTGACAAGTGCTATGTCATGTGTCAGAACGATTATCTTAGC
TTGACTGGGCCATGATATCAGCTATGTCAGCACCGTTTTGTCGCAGCTCACACTCTCAGCGTATGANTGNATG
GTNTCGTAGATANTCCGCTGTGGCAAACATTTCATATTTGAATGTCCGTGTCTTGTCATAATCCATATTGACAT
TTATATCAACTTTCGTCGCACTGCTGCACAGCTTCCCGTAGATTACGCCTTCGCCCAAACAAGCTTTACCGTA
CAGTAATTTGCGTATGTGTTTTTCTTCGACACAACATCGCTTCGCAACGCTTAGTGCTACAACACGACCTATTG
GATTGTCCTCATCAGATTGTGTACGATNCATAATCGTYSTNACACRWGWYMTAGCATTTGTCAATGCTTCTCG
CCGGTCCCAGATGTGATCAGACACCCAATTACCGCTGACACAGCTTGCAATAGCACGTGCGACATACCCAGT
ACTATAGTGTTTCATGTGAGCAACGCGTAAGAACTCACCACTATATTCACTTGCACACTGTTTATGTGGGTAAT
ATGTCGAACGTGCTACTGCAAGCTGTGTCACCGGAATTGGGTGTCTGATTTTTTCTCGCACCGCCCAGAAGC
GTGCATAACGCTATATAAAAGTAGGGGGACGTTTTTT

*Endotrypanum* sp.

TTTTTGTCGCAGCATCACACTCTCRGCGNAYSAYGKNTGRTATNGTWGATAMTCCGCTGTGGNAAACATTNTC
ATATTTGAATGTCCGTGTCTTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTT
CCCGTAGATTACGCCTTCGCCCAAACAAGCTTTACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAAC
ATCGCTTCGCAACGCTTAGTGCTACAACACGACCTATTGGATTGTCCTCATCNAGAYNGTGTMCKNAKTCNAY
AAYMGTWMTRACWMAYSNTAYRSCAYNNGTCAAAGCTTCTCGCCGGTCCCA

*Leishmania amazonensis*

TTAGGACCGGCGAGAAGCTTTGACAAAWGYTNAKMTCRKGTGNTCMGAACGATTATGANTCGTACACAATCT
GATGAGGACAATCCAATAGGTCGTGTTGTAGCACTAAGCGTTGCGAAGCGATGTTGTGTCGAAGAAAAACAC
ATACGCAAATTACTGTACGGTAAAGCTTGTTTGGGCGAAGGCGTAATCTACGGGAAGCTGTGCAGCAGTGCG
ACGAAAGTTGATATAAATGTCAATATGGATTATGACAAGACACGGACATTCAAATATGAAATGTTNGCCACAGC
GGATTATCTACGATNACMATWNNCAYCRTACGCTGAGAGTGTGATGCTGCGACAAAAA

*Leishmania venezuelensis*

TGGGACCGGCGAGAAGCTTTGACARATSCTANNYCRKGKGTNASWACGATTATGANTCGTACACAATCTGAT
GAGGACAATCCAATAGGTCGTGTTGTAGCACTAAGCGTTGCGAAGCGATGTTGTGTCGAAGAAAAWCACATA
CGCAAATTACTGTACGGTAAAGCTTGTTTGGGCGAAGGCGTAATCTACGGGAAGCTGTGCAGCAGTGCGACG
AAAGTTGATATAAATGTCAATATGGATTATGACAAGACACGGACATTCAAATATGAAATGTTTGCCACAGCGGA
TNATCTACGATMCCATMCWKCMTMCGCTGAGAGTGTGATGCTGCGACAAAACATTTCATATTTGATTGTCCG
TGTCTTGTGTTAATCCATATTGACATTTATATCAACTTTCGTCAGCTGCTAACTCCGTATATTACGCTCAGCACA
AAAAGCTTTTCCCCTAAGAAATTTAGCTATGGTTTTTTTTTTCAACACTTCCTTGAACCGTTAAGTGAACCACCC
ACCCAAGGGAATGGCCCTAGAAATTTGGGAAAGAA

*Leishmania chagasi*

TTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTTCCCGTAGATTACGCCTTCGCCCAAACAAGCTTT
ACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAACATCGCTTCGCAACGCTTAGTGCTACAACACGAC
CTATTGGATTGTCCTCATCAGATTGTGTACGATNCATAATCGTYSTNACACMWGNTAKWSCATTT

*Leishmania major*

AGCAAGCTCTAGAGTCAAGCCATCAAGNATGCGCGTNACGTTGTCNCGTAGTGATTTAGGTGTATTCACGATG
CCAGTATGTGATGTTGAACGTTTCCCAGTCCGTAAAGGCGTGTTTGAAAAATATCCATTATTGATGATGATCAA
AGAACGAATACCATTACGCGAGGCACTAGATCTGGCACAGGGAATAGGATACCATTGCCCACATGGTTGTGC
GGAGGATCTTNGGGNCGGAACTATGCGTWKGNNWSTGMAMYWKACGGTGTATTACCCTACACAGATGCTTG
TCAAAAATTT

*Leishmania gerbilli*

TTAGACCGGCGAGAAGCTTTGACAWAKGCTANANCRKKTGTNASRACGATTATGANTCGTACACAATCTGATG
AGGACAATCCAATAGGTCGTGTTGTAGCACTAAGCGTTGCGAAGCGATGTTGTGTCGAAGAAAAACACATAC
GCAAATTACTGTACGGTAAAGCTTGTTTGGGCGAAGGCGTAATCTACGGGAAGCTGTGCAGCAGTGCGACGA
AAGTTGATATAAATGTCAATATGGATTATGACAAGACACGGACATTCAAATATGAAATGTTNGCCACAGCGGAT
NATCTACGATACCATACATCATACGCTGAGAGTGTGATGCTGCGACAAAAAAATTTCATATTTGAATGTCCGTG

TCTTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCATTTCCCGTAAATTACGCCTTCGC
CAAAACAAGCTTTACCGTACAGTATTTTGGGTTATGGGTTTTTTCTTCAACAACATCCCTCCCCACCGCTAAGT
GCTTCAACACGCCCCTATGGGATTGCCCTCATCAAATTGGGGGTAGAATTAAAAATCTTTTGGACCATGAATA
AAAACAATTTGTCAAAGCTTTCCCCCCGGCTCAAAAA

*Leishmania tarentolae*

TTTTTGACCGGCGAGAAGCTTTGACAAATGMTNTWTCATGNTGTCNGANCGATTATGANTCGTACACAATCTG
ATGAGGACAATCCAATAGGTCGTGTTGTAGCACTAAGCGTTGCGAAGCGATGTTGTGTCGAAGAAAAACACAT
ACGCAAATTACTGTACGGTAAAGCTTGTTTGGGCGAAGGCGTAATCTACGGGAAGCTGTGCAGCAGTGCGAC
GAAAGTTGATATAAATGTCAATATGGATTATGACAAGACACGGACATTCAAATATGAAATGTTNGCCACAGCG
GATTATCTACGATMCCATMCMYCMTMCGCTGAGAGTGTGATGCTGCGACAAAAAAA

*Herpetomonas megaseliae*

TTTGTCGCAGCATCACACTCTCAGCRWAYKAYGYRTGSNATCGYAGMTMMWCCGCTNGTGGCAAACATTTCA
TATTTGAATGTCCGTGTCTTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTTC
CCGTAGATTACGCCTTCGCCCAAACAAGCTTTACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAACA
TCGCTTCGCAACGCTTAGTGCTACAACACGACCTATTGGATTGTCCTCATCNAGATTSTGTMCKMWTCNATAR
TCGYYCYKMCWMATSRTMYMSCATTTGTCAAAGCTTCTCGCCGGTCCCAGCTATGTGCCATACTGGGTGATT
AATACATTTATTTATAAACTGTCTGCTCTTAATCTTGCTGGCTGCTTGTTCACCCCCGCGAGCTGTCATCAGTG
GGTATAACTAAGCGGTCCCCACCCCT

*Blastocrithidia culicis*

TCTCAGCRWAYKAYGYRTGSNATCGYAGMTMMWCCGCTNGTGGCAAACATTTCATATTTGAATGTCCGTGTC
TTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTTCCCGTAGATTACGCCTTC
GCCCAAACAAGCTTTACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAACATCGCTTCGCAACGCTTA
GTGCTACAACACGACCTATTGGATTGTCCTCATCNAGATTSTGTMCKMWTCNATARTCGYYCYKMCWMATSR
TMYMSCATTTGTCAAAGCTTCTCGCCGGTCCCAGCTATGTGCCATACTGGGTGATTAATACATTTATTTATAAA
CTGTCTGCTCTTAATCTTGCTGGCTGCTTGTTCACCCCCGCGAGCTGTCATCAGTGGGTATAACTAAGCGGTC
CCCACCCCT

*Bodo caudatus*

GGACCGGCGAGAAGCTTTGACAAATGCYAYANCANGTGTCNGWRCKAYTRTNGAMTNMGTACACARTCNTN
GATNGAGGACAATCCAATAGGTCGTGTTGTAGCACTAAGCGTTGCGAAGCGATGTTGTGTCGAAGAAAAACA
CATACGCAAATTACTGTACGGTAAAGCTTGTTTGGGCGAAGGCGTAATCTACGGGAAGCTGTGCAGCAGTGC
GACGAAAGTTGATATAAATGTCAATATGGATTATGACAAGACACGGACATTCAAATATGANANATGTTTGCCAC
AGCNRGANYATMTACGMTNACCWYASMYCATACGCTGAGAGTGTGATGCTGCGACAAA

*Leishmania infantum* from Brazil

GGACCGGCGAGAAGCTTTGACAAATGCKRYRKCMKGTGTYMGANCGATTATGANTCGTACACAATCTGATGA
GGACAATCCAATAGGTCGTGTTGTAGCACTAAGCGTTGCGAAGCGATGTTGTGTCGAAGAAAAACACATACG
CAAATTACTGTACGGTAAAGCTTGTTTGGGCGAAGGCGTAATCTACGGGAAGCTGTGCAGCAGTGCGACGAA
AGTTGATATAAATGTCAATATGGATTATGACAAGACACGGACATTCAAATATGAAATGTTTGCCACAGCGGATN
ATCTACGATACCATMMAKCMTACGCTGAGAGTGTGATGCTGCGACAAA

*Leishmania infantum* from Czech

TTTGTCGCAGCATCACACTCTCAGCGKAYSMKGTWTGGWAWCGTAGATANTCCGCTGTGGCAANCATTTCAT
ATTTGAATGTCCGTGTCTTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTTC
CCGTAGATTACGCCTTCGCCCAAACAAGCTTTACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAACA
TCGCTTCGCAACGCTTAGTGCTACAACACGACCTATTGGATTGTCCTCATCAGATTGTGTACGATNCATAATC
GTNCKRACANCAWKAWRKMSCATTTGTCAAAGCTTCTCGCCGGTCCAA

*Leishmania infantum* from Cyprus

TTTGTCGCAGCATCACACTCTCAGMGKMYGRNGWAKGGKANTCGTAGATAATCCGCTGTGGCAANCATTTCA
TATTTGAATGTCCGTGTCTTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTTC
CCGTAGATTACGCCTTCGCCCAAACAAGCTTTACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAACA
TCGCTTCGCAACGCTTAGTGCTACAACACGACCTATTGGATTGTCCTCATCAGATTGTGTACGATNCATAATC
GTYSTNACACAKGNTANAGCMYTTGTCAAAGCTTCTCGCCGGTCC

*Leishmania infantum* from Brazil

TTTGTCGCAGCATCACACTCTCAGMKTAWNATGTRTGNTATCGTAGATANTCCGCTGTGGCAAACATTTCATA
TTTGAATGTCCGTGTCTTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTTCC
CGTAGATTACGCCTTCGCCCAAACAAGCTTTACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAACAT
CGCTTCGCAACGCTTAGTGCTACAACACGACCTATTGGATTGTCCTCATCAGATTGTGTACGATNCATAATCG
TNCNGACACMTSMTWTMGCMYYTGTCAAAGCTTCTCGCCGGTCCAA

*Leishmania infantum* from Spain

TTTTGTCGCAAGCACACACTCTCAGCGTAWRMTGNATGGTATCGTAGATANTCCGCTGTGGCAANCATTTCAT
ATTTGAATGTCCGTGTCTTGTCATAATCCATATTGACATTTATATCAACTTTCGTCGCACTGCTGCACAGCTTC
CCGTAGATTACGCCTTCGCCCAAACAAGCTTTACCGTACAGTAATTTGCGTATGTGTTTTTCTTCGACACAACA
TCGCTTCGCAACGCTTAGTGCTACAACACGACCTATTGGATTGTCCTCATCAGATTGTGTACGATNCATAATC
GTNCNGACACMWGWYATAGCATTTGTCAAAGCTTTCGCCGGTCCCAAGGGGGGTGTAGGAAGAAAGACAGA
AAGAAAACCGGGGAAGAAAAAGCAGGGGAAAAAAACGCCTTCGGACAAGAAACTATGGCTTAAAAAAAGCAA
CCCGGCTCAAAATAAAAAAAAAACAAGAGAAACAATACAATAAAAGGAGGAGTAAGGAAAAAAAATTATTGAAA
GAGAAGGTAAAAAAATTAACGATGTAAAAAAAAAACTAAAGAAATAACTGAGCATAAACAAAACTAAATAACCA
AAAAATTCTTCACTATAAATGATAAATGCACACGGAAACCAACCAAATTATAAAGACAATATAATTAAAAAAATC
ATCTTGATGTAGAAAACGAGACAATGAGCTACGTAGAATGCATTTGTTTATGAACATATGAAAGAAATATGTAT
ACTATCCTTATAAACTTTGTGATGTATCTTAGCAAGGCTTATATGCCGAATAGATAAGGATATACACTTAAAGAT
TAAGTCTCTCGGCGACGGCGCTATGATCCCACCTCGCATTTACATCTCCATCGAAGGACGCGAGCCAGCTGA
TCACAGCTGGTAAAAAAAATATGTCCCGGAAGACGTAAATTAGATATCTTAC

*Leishmania donovani*

TACACAATCTGATGAGGACAATCCAATAGGTCGTGTTGTAGCACTAAGCGTTGCGAAGCGATGTTGTGTCGAA
GAAAAACACATACGCAAATTACTGTACGGTAAAGCTTGTTTGGGCGAAGGCGTAATCTACGGGAAGCTGTGC
AGCAGTGCGACGAAAGTTGATATAAATGTCAATATGGATTATGACAAGACACGGACATTCAAATATGAAATGTT
TGCCACAGCGGATNATCTACGATACCATMMAKCMTACGCTGAGAGTGTGATGC

***Appendix 5.1***

All sample sequence of species of totiviruses downloaded from GenBank and used to build the amino acid and nucleotide phylogenetic trees.

Red – sequences of species used as outgroups to root the phylogenetic trees.

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| 1 | *Aedes aegypti toti-like virus* | AATLV | Unclassified | arthropods | MN053721.1 |
| 2 | *Aedes aegypti Totivirus* | AATV | Unclassified | arthropods | MN053728.1 |
| 3 | *Anopheles Totivirus* | AToV | Unclassified | arthropods | KX148550.1 |
| 4 | *Armadillidium vulgare endogenous virus Totivirus* | AVEVT | Unclassified | arthropods | KM034092.1 |
| 5 | *Armigeres subalbatus virus* | SaX06-AK20 | Unclassified | arthropods | EU715328.1 |
| 6 | *Aspergillus mycovirus 178* | AsV-178 | *Totivirus* | fungi | EU289895.1 |
| 7 | *Aspergillus mycovirus 1816* | AsV-1816 | *Totivirus* | fungi | EU289896.1 |
| 8 | *Aspergillus foetidus slow virus 1* | AsFSV1 | *Victorivirus* | fungi | HE588147.1 |
| 9 | *Aspergillus homomorphus Totivirus 1* | AsHTv1 | Unclassified | fungi | MK279489.1 |
| 10 | *Atrato virus strain Mati 1738-6* | ATV-1738-6 | Unclassified | arthropods | MN661074.1 |
| 11 | *Atrato virus strain Mati 1756-3* | ATV-1756-3 | Unclassified | arthropods | MN661075.1 |
| 12 | *Australian Anopheles Totivirus* | AATV | Unclassified | arthropods | MF073200.1 |
| 13 | *Australian Anopheles Totivirus* | AATV | Unclassified | arthropods | MF073201.1 |
| 14 | *Barrymore virus* | BAV | Unclassified | arthropods | MN167488.1 |
| 15 | *Beauveria bassiana victorivirus 1* | BeBV1 | *Victorivirus* | fungi | HE572591.1 |
| 16 | *Beauveria bassiana victorivirus NZL/1980* | BeBV1980 | *Victorivirus* | fungi | KJ364649.1 |
| 17 | *Beauveria bassiana victorivirus 1* | BeBV1 | *Victorivirus* | fungi | KR011117.1 |
| 18 | *Beihai toti-like virus 2* | BHTV2 | Unclassified *Riboviria* | animal | KX882944.1 |
| 19 | *Biomphalaria virus 5* | BiPHV5 | Unclassified | animal | KY024325.1 |
| 20 | *Bipolaris maydis victorivirus 1* | BiMaV1 | *Victorivirus* | fungi | MH396496.1 |
| 21 | *Black raspberry virus F* | BRVF | *Totivirus* | fungi | EU082131.1 |
| 22 | *Blechomonas juanalfonzi Leishmaniavirus-like RNA virus 1* | BJLRV1 | Unclassified | protozoa | MG967341.1 |
| 23 | *Blechomonas maslovi Leishmaniavirus-like RNA virus 1* | BMLRV1 | Unclassified | protozoa | MG967345.1 |
| 24 | *Blechomonas wendygibsoni Leishmaniavirus-like RNA virus 1* | MWLRV1 | Unclassified | protozoa | MG967347.1 |
| 25 | *Blueberry latent virus* | BBLV | *Amalgavirus* | plant | EF442779.2 |
| 26 | *Botryosphaeria dothidea victorivirus 1* | BTDV1 | *Victorivirus* | fungi | KM051424.1 |
| 27 | *Botryotinia fuckeliana Totivirus 1* | BTFTV1 | *Totivirus* | fungi | AM491608.1 |
| 28 | *Cronartium ribicola Totivirus 1* | CrTV3 | Unclassified | fungi | MK967418.1 |
| 29 | *Cronartium ribicola Totivirus 2* | CrTV3 | Unclassified | fungi | MK967419.1 |
| 30 | *Cronartium ribicola Totivirus 3* | CrTV3 | Unclassified | fungi | MK967420.1 |
| 31 | *Cronartium ribicola Totivirus 4* | CrTV4 | Unclassified | fungi | MK967421.1 |
| 32 | *Cronartium ribicola Totivirus 5* | CrTV5 | Unclassified | fungi | MK967422.1 |
| 33 | *Camponotus yamaokai virus* | CaYaV | Unclassified | arthropods | LC026053.1 |
| 34 | *Camponotus nipponicus virus* | CaNiV | Unclassified | arthropods | LC101918.1 |
| 35 | *Cherry chlorotic rusty spot associated totiviral-like dsRNA 3* | ChCTV3 | Unclassified | plant | AM181141.1 |
| 36 | *Cherry chlorotic rusty spot associated totiviral-like dsRNA 4* | ChCTV4 | Unclassified | plant | AM181142.1 |
| 37 | *Colletotrichum caudatum Totivirus 1* | CoCTV1 | Unclassified | fungi | MK279490.1 |

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| 38 | *Colletotrichum eremochloae Totivirus 1* | CoETV1 | Unclassified | fungi | MK279491.1 |
| 39 | *Colletotrichum navitas Totivirus 1* | CoNTV1 | Unclassified | fungi | MK279492.1 |
| 40 | *Colletotrichum zoysiae Totivirus 1* | CoZTV1 | Unclassified | fungi | MK279493.1 |
| 41 | *Coniothyrium minitans RNA virus* | CoMRV | *Victorivirus* | fungi | AF527633.1 |
| 42 | *Coniothyrium minitans RNA virus* | CoMRV | *Victorivirus* | fungi | KT598230.1 |
| 43 | *Culex tritaeniorhynchus Totivirus* | CtTV | Unclassified | arthropods | KX456218.1 |
| 44 | *Culex tritaeniorhynchus Totivirus* | CtTV | Unclassified | arthropods | KX456219.1 |
| 45 | *Culex tritaeniorhynchus Totivirus* | CtTV | Unclassified | arthropods | MN614415.1 |
| 46 | *Delisea pulchra Totivirus IndA_1* | DePTV_IndA1 | Unclassified | eukaryotic algae | KT455449.1 |
| 47 | *Delisea pulchra Totivirus IndA_2* | DePTV_IndA2 | Unclassified | eukaryotic algae | KT455450.1 |
| 48 | *Delisea pulchra Totivirus IndA_3* | DePTV_IndA3 | Unclassified | eukaryotic algae | KT455451.1 |
| 49 | *Delisea pulchra Totivirus IndA_4* | DePTV_IndA4 | Unclassified | eukaryotic algae | KT455452.1 |
| 50 | *Delisea pulchra Totivirus IndA_5* | DePTV_IndA5 | Unclassified | eukaryotic algae | KT455453.1 |
| 51 | *Delisea pulchra Totivirus IndA_8* | DePTV_IndA8 | Unclassified | eukaryotic algae | KT455456.1 |
| 52 | *Delisea pulchra Totivirus IndA_11* | DePTV_IndA11 | Unclassified | eukaryotic algae | KT455457.1 |
| 53 | *Delisea pulchra Totivirus IndA_12* | DePTV_IndA12 | Unclassified | eukaryotic algae | KT455458.1 |
| 54 | *Diatom colony associated dsRNA virus 3* | DiRV3 | Unclassified | algae | AP014893.1 |
| 55 | *Diatom colony associated dsRNA virus 4B* | DiRV4B | Unclassified | algae | AP014895.1 |
| 56 | *Diatom colony associated dsRNA virus 5* | DiRV5 | Unclassified | algae | AP014896.1 |
| 57 | *Diatom colony associated dsRNA virus 6* | DiRV6 | Unclassified | algae | AP014897.1 |
| 58 | *Diatom colony associated dsRNA virus 7* | DiRV7 | Unclassified | algae | AP014898.1 |
| 59 | *Diatom colony associated dsRNA virus 8* | DiRV8 | Unclassified | algae | AP014899.1 |
| 60 | *Diatom colony associated dsRNA virus 9* | DiRV9 | Unclassified | algae | AP014900.1 |
| 61 | *Diatom colony associated dsRNA virus 9B* | DiRV9B | Unclassified | algae | AP014901.1 |
| 62 | *Diatom colony associated dsRNA virus 10* | DiRV10 | Unclassified | algae | AP014902.1 |
| 63 | *Diatom colony associated dsRNA virus 11* | DiRV11 | Unclassified | algae | AP014903.1 |
| 64 | *Diatom colony associated dsRNA virus 12* | DiRV12 | Unclassified | algae | AP014904.1 |
| 65 | *Diatom colony associated dsRNA virus 13* | DiRV13 | Unclassified | algae | AP014905.1 |
| 66 | *Drosophila melanogaster Totivirus SW-2009a* | DTV | Unclassified | arthropods | GQ342961.1 |
| 67 | *Aedes aegypti Totivirus GH115* | AATV GH115 | Unclassified | arthropods | LC496074.1 |
| 68 | *Eimeria brunetti RNA virus 1* | EbRV 1 | Unclassified | protozoa | AF356189.1 |
| 69 | *Eimeria tenella RNA virus 1* | EtRV 1 | Unclassified | protozoa | KJ363185.1 |
| 70 | *Eimeria stiedai RNA virus 1* | EsRV 1 | Unclassified | protozoa | KU597305.1 |
| 71 | *Eimeria nieschulzi virus* | EnRV | Unclassified | protozoa | L25869.1 |
| 72 | *Epichloe festucae virus 1* | EfV 1 | *Victorivirus* | fungi | AM261427.1 |
| 73 | *Fungal Totivirus MpPl* | FuTV MpPl | *Totivirus* | fungi | KP900900.1 |
| 74 | *Fusarium asiaticum victorivirus 1* | FAVV 1 | *Victorivirus* | fungi | MH615042.1 |
| 75 | *Giardia lamblia virus* | GLV | *Giardiavirus* | protozoa | AF525216.1 |

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| 76 | *Giardia canis virus* | GCV | *Giardiavirus* | protozoa | DQ238861.1 |
| 77 | *Giardia lamblia virus* | GLV | *Giardiavirus* | protozoa | L13218.1 |
| 78 | *Gigaspora margarita giardia-like virus 1* | GMGLV 1 | Unclassified | fungi | MG256177.1 |
| 79 | *Golden shiner Totivirus* | GSTV | Unclassified | fish | KU529284.1 |
| 80 | *Gremmeniella abietina RNA virus L2* | GARV L2 | *Totivirus* | fungi | AY615210.1 |
| 81 | *Helicobasidium mompa Totivirus 1-17* | HMTV 1-17 | *Victorivirus* | fungi | AB085814.1 |
| 82 | *Helminthosporium victoriae virus 190S* | HeVV 190S | *Victorivirus* | fungi | U41345.2 |
| 83 | *Hortaea werneckii Totivirus 1* | HoWTV 1 | Unclassified | fungi | MK279498.1 |
| 84 | *Hubei toti-like virus 12* | HuTLV 12 | Unclassified | animal roundworm | KX882956.1 |
| 85 | *Hubei toti-like virus 13* | HuTLV 13 | Unclassified | arthropod | KX882966.1 |
| 86 | *Hubei toti-like virus 14* | HuTLV 14 | Unclassified | arthropod | KX882969.1 |
| 87 | *Hubei toti-like virus 15* | HuTLV 15 | Unclassified | arthropod | KX882974.1 |
| 88 | *Hubei toti-like virus 24* | HuTLV 24 | Unclassified | arthropod | KX882977.1 |
| 89 | *Hubei toti-like virus 12* | HuTLV 12 | Unclassified | animal roundworm | KX882982.1 |
| 90 | *Lampyris noctiluca toti-like virus 1* | LNTV 1 | Unclassified | arthropod | MH620822.1 |
| 91 | *Larkfield virus* | LaV | Unclassified | arthropod | MF893249.1 |
| 92 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KC862308.1 |
| 93 | *Leishmania RNA virus 2* | LRV 2 | *Leishmaniavirus* | protozoa | KF256264.1 |
| 94 | *Leishmania RNA virus 2* | LRV 2 | *Leishmaniavirus* | protozoa | KF256265.1 |
| 95 | *Leishmania RNA virus 2* | LRV 2 | *Leishmaniavirus* | protozoa | KF757256.1 |
| 96 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KU724433.1 |
| 97 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KU724434.1 |
| 98 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KX686068.1 |
| 99 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KX808483.1 |
| 100 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KX808484.1 |
| 101 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KX808485.1 |
| 102 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KX808486.1 |
| 103 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KX808487.1 |
| 104 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750607.1 |
| 105 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750608.1 |
| 106 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750609.1 |
| 107 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750610.1 |
| 108 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750611.1 |
| 109 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750612.1 |
| 110 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750613.1 |
| 111 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750614.1 |
| 112 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750615.1 |
| 113 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750616.1 |
| 114 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750617.1 |
| 115 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750618.1 |
| 116 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750619.1 |
| 117 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750620.1 |
| 118 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750621.1 |
| 119 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750622.1 |
| 120 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750623.1 |
| 121 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750624.1 |
| 122 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750625.1 |
| 123 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750626.1 |
| 124 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750627.1 |
| 125 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750628.1 |
| 126 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750629.1 |
| 127 | *Leishmania RNA virus 1* | LRV 1 | *Leishmaniavirus* | protozoa | KY750630.1 |
| 128 | *Leishmania RNA virus 1 - 1* | LRV 1-1 | *Leishmaniavirus* | protozoa | M92355.1 |
| 129 | *Leishmania RNA virus 1 - 4* | LRV 1-4 | *Leishmaniavirus* | protozoa | NC_003601.1 |
| 130 | *Leishmania RNA virus 1 - 4* | LRV 1-4 | *Leishmaniavirus* | protozoa | U01899.1 |

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| 131 | *Leishmania RNA virus 2 - 1* | LRV 2-1 | *Leishmaniavirus* | protozoa | U32108.1 |
| 132 | *Leptopilina boulardi Toti-like virus* | LPTLV | Unclassified | arthropod | AGW80479.1 |
| 133 | *Leptopilina boulardi Toti-like virus* | LPTLV | Unclassified | arthropod | KF274642.2 |
| 134 | *Lonestar tick Totivirus* | LoTTV | Unclassified | arthropod | MG647775.1 |
| 135 | *Magnaporthe oryzae virus 1* | MaOV 1 | *Victorivirus* | fungi | AB176964.1 |
| 136 | *Magnaporthe oryzae virus 2* | MaOV 2 | *Victorivirus* | fungi | AB300379.1 |
| 137 | *Magnaporthe oryzae virus 3* | MaOV 3 | *Victorivirus* | fungi | KP893140.1 |
| 138 | *Maize-associated Totivirus 1* | MATV1 | *Totivirus* | plant | KP984504.1 |
| 139 | *Maize-associated Totivirus 2* | MATV2 | *Totivirus* | plant | KT722800.3 |
| 140 | *Maize associated Totivirus 1* | MATV1 | *Totivirus* | plant | MF372914.1 |
| 141 | *Maize associated Totivirus 1* | MATV1 | *Totivirus* | plant | MF372916.1 |
| 142 | *Maize associated Totivirus 1* | MATV1 | *Totivirus* | plant | MF372917.1 |
| 143 | *Maize-associated Totivirus 3* | MATV3 | *Totivirus* | plant | MF425844.1 |
| 144 | *Maize-associated Totivirus 3* | MATV3 | *Totivirus* | plant | MF425845.1 |
| 145 | *Maize-associated Totivirus 3* | MATV3 | *Totivirus* | plant | MF425846.1 |
| 146 | *Maize-associated Totivirus 3* | MATV3 | *Totivirus* | plant | MF425847.1 |
| 147 | *Maize-associated Totivirus 3* | MATV3 | *Totivirus* | plant | MF425848.1 |
| 148 | *Maize-associated Totivirus 3* | MATV3 | *Totivirus* | plant | MF425849.1 |
| 149 | *Maize-associated Totivirus Anhui* | MATVA | *Totivirus* | plant | MH055436.1 |
| 150 | *Maize associated Totivirus* | MATV | *Totivirus* | plant | MK037419.1 |
| 151 | *Maize associated Totivirus* | MATV | *Totivirus* | plant | MK037420.1 |
| 152 | *Maize associated Totivirus* | MATV | *Totivirus* | plant | MK066242.1 |
| 153 | *Maize associated Totivirus* | MATV | *Totivirus* | plant | MK066243.1 |
| 154 | *Murri virus* | MuV | Unclassified | arthropod | MN661077.1 |
| 155 | *Nigrospora oryzae victorivirus 1* | NOVTV | *Victorivirus* | fungi | KT428155.1 |
| 156 | *Omono River virus* | ORV | Unclassified | arthropod | AB555544.1 |
| 157 | *Omono River virus* | ORV | Unclassified | arthropod | AB555544.1 |
| 158 | *Omono River virus* | ORV | Unclassified | arthropod | KY264024.1 |
| 159 | *Omono River virus* | ORV | Unclassified | arthropod | KY264025.1 |
| 160 | *Ophiostoma minus Totivirus* | OMTV | *Totivirus* | fungi | AM111098.1 |
| 161 | *Panax notoginseng virus A* | PNV A | Unclassified | plant | KT388111.1 |
| 162 | *Panax notoginseng virus B* | PNV B | Unclassified | plant | MF614101.1 |
| 163 | *Panax notoginseng virus B* | PNV B | Unclassified | plant | MF614102.1 |
| 164 | *Papaya meleira virus* | PMV | Unclassified | plant | KF214786.1 |
| 165 | *Papaya meleira virus* | PMV | Unclassified | plant | KF781635.1 |
| 166 | *Papaya meleira virus* | PMV | Unclassified | plant | KT013296.1 |
| 167 | *Papaya meleira virus* | PMV | Unclassified | plant | KT921784.1 |
| 168 | *Papaya meleira virus 2* | PMV 2 | Unclassified | plant | KT921785.1 |
| 169 | *Papaya meleira virus 2* | PMV 2 | Unclassified | plant | MG570380.1 |
| 170 | *Papaya meleira virus 2* | PMV 2 | Unclassified | plant | MG570381.1 |
| 171 | *Papaya meleira virus 2* | PMV 2 | Unclassified | plant | MG570382.1 |
| 172 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | AY570982.3 |
| 173 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | EF061744.1 |
| 174 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | KF836757.1 |
| 175 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | KJ556923.1 |
| 176 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | KJ636782.2 |
| 178 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | KJ636783.2 |
| 179 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | KR815474.1 |
| 180 | *Penaeid shrimp infectious myonecrosis virus* | IMNV | Unclassified | animal | KT003689.1 |
| 181 | *Penicillium aurantiogriseum Totivirus 1* | PATV 1 | Unclassified | fungi | KT592305.1 |
| 182 | *Penicillium digitatum virus 1* | PDV 1 | Unclassified | fungi | KU257669.1 |
| 183 | *Penicillium digitatum virus 1* | PDV 1 | Unclassified | fungi | KU933932.1 |

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| 184 | *Penicillium digitatum virus 2* | PDV 2 | Unclassified | fungi | MK279494.1 |
| 185 | *Penicillium digitatum virus 1* | PDV 1 | Unclassified | fungi | MK279495.1 |
| 186 | *Pericornia byssoides Totivirus 1* | PBTV 1 | Unclassified | fungi | MK584821.1 |
| 187 | *Phlebiopsis gigantea mycovirus dsRNA 1* | PGM_ dsRNA 1 | Unclassified | fungi | AM111096.3 |
| 188 | *Phlebiopsis gigantea mycovirus dsRNA 2* | PGM_dsRNA 2 | Unclassified | fungi | AM111097.2 |
| 189 | *Phomopsis vexans RNA virus* | PVRV | *Victorivirus* | fungi | KP090346.1 |
| 190 | *Phomopsis longicolla Totivirus 1* | PLTV 1 | *Totivirus* | fungi | KP900901.1 |
| 191 | *Piscine myocarditis virus AL V-708* | PMCV AL V-708 | Unclassified | fish | HQ339954.1 |
| 192 | *Piscine myocarditis virus TT-2012* | PMCV TT-2012 | Unclassified | fish | JN624781.1 |
| 193 | *Piscine myocarditis virus AL V-708* | PMCV AL V-708 | Unclassified | fish | JQ745677.1 |
| 194 | *Piscine myocarditis virus AL V-708* | PMCV AL V-708 | Unclassified | fish | JQ745678.1 |
| 195 | *Piscine myocarditis-like virus* | PMCLV | Unclassified | fish | KT725636.1 |
| 196 | *Pisingos virus* | PIV | Unclassified | arthropod | MN661078.1 |
| 197 | *Pisingos virus* | PIV | Unclassified | arthropod | MN661079.1 |
| 198 | *Pisingos virus* | PIV | Unclassified | arthropod | MN661080.1 |
| 199 | *Pisingos virus* | PIV | Unclassified | arthropod | MN661081.1 |
| 200 | *Plasmopara viticola associated Totivirus-like 5* | PVATVL 5 | Unclassified | fungi | MN545909.1 |
| 201 | *Plasmopara viticola associated Totivirus 1* | PVATV 1 | Unclassified | fungi | MN545910.1 |
| 202 | *Plasmopara viticola associated Totivirus* | PVATV 2 | Unclassified | fungi | MN545911.1 |
| 203 | *Plasmopara viticola associated Totivirus 3* | PVATV 3 | Unclassified | fungi | MN545912.1 |
| 204 | *Plasmopara viticola associated Totivirus-like 1* | PVATVL 1 | Unclassified | fungi | MN545914.1 |
| 205 | *Plasmopara viticola associated Totivirus-like 2* | PVATVL 2 | Unclassified | fungi | MN545915.1 |
| 206 | *Pterostylis sanguinea Totivirus A* | PSTV A | Unclassified | plant | KU291926.1 |
| 207 | *Pterostylis sanguinea Totivirus A* | PSTV A | Unclassified | plant | KU291927.1 |
| 208 | *Pterostylis Totivirus-like* | PTVL | Unclassified | plant | KU291971.1 |
| 209 | *Puccinia striiformis Totivirus 1* | PSTV 1 | Unclassified | fungi | KY207361.1 |
| 210 | *Puccinia striiformis Totivirus 2* | PSTV 2 | Unclassified | fungi | KY207362.1 |
| 211 | *Puccinia striiformis Totivirus 3* | PSTV 3 | Unclassified | fungi | KY207363.1 |
| 212 | *Puccinia striiformis Totivirus 4* | PSTV 4 | Unclassified | fungi | KY207364.1 |
| 213 | *Puccinia striiformis Totivirus 5* | PSTV 5 | Unclassified | fungi | KY207365.1 |
| 214 | *Pythium polare RNA virus 1* | PPRV 1 | Unclassified | fungi | LC376044.1 |
| 215 | *Red clover powdery mildew-associated Totivirus 1* | RPaTV 1a | Unclassified | fungi | LC075485.1 |
| 216 | *Red clover powdery mildew-associated Totivirus 1* | RPaTV 1b | Unclassified | fungi | LC075486.1 |
| 217 | *Red clover powdery mildew-associated Totivirus 2* | RPaTV 2 | Unclassified | fungi | LC075487.1 |
| 218 | *Red clover powdery mildew-associated Totivirus 3* | RPaTV 3 | Unclassified | fungi | LC075488.1 |
| 219 | *Red clover powdery mildew-associated Totivirus 4* | RPaTV 4 | Unclassified | fungi | LC075489.1 |
| 220 | *Red clover powdery mildew-associated Totivirus 5* | RPaTV 5 | Unclassified | fungi | LC075490.1 |
| 221 | *Red clover powdery mildew-associated Totivirus 6* | RPaTV 6 | Unclassified | fungi | LC075491.1 |
| 222 | *Red clover powdery mildew-associated Totivirus 7* | RPaTV 7 | Unclassified | fungi | LC075492.1 |
| 223 | *Red clover powdery mildew-associated Totivirus 8* | RPaTV 8 | Unclassified | fungi | LC075493.1 |
| 224 | *Rhododendron virus A* | RhV A | *Amalgavirus* | plant | HQ128706.1 |
| 225 | *Ribes virus F* | RiV F | Unclassified | plant | EU495331.1 |
| 226 | *Rosellinia necatrix victorivirus 1* | RNVV 1 | *Victorivirus* | fungi | AB698490.1 |

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| 227 | *Rosellinia necatrix victorivirus 1* | RNVV 1 | *Victorivirus* | fungi | AB742454.1 |
| 228 | *Rosellinia necatrix mega Totivirus 1* | RNMTV 1 | Unclassified | fungi | LC333740.1 |
| 229 | *Rosellinia necatrix mega Totivirus 1* | RNMTV 1 | Unclassified | fungi | LC333746.2 |
| 230 | *Saccharomyces cerevisiae virus L-A* | SCV L-A | *Totivirus* | fungi | J04692.1 |
| 231 | *Saccharomyces cerevisiae virus L-BC-lus* | SCV L-BC-lus | *Totivirus* | fungi | KT784813.1 |
| 232 | *Saccharomyces cerevisiae virus L-BC-lus* | SCV L-BC-lus | *Totivirus* | fungi | KU845301.2 |
| 233 | *Saccharomyces cerevisiae virus L-BC-2* | SCV L-BC-2 | *Totivirus* | fungi | KX906605.1 |
| 234 | *Saccharomyces cerevisiae virus L-A* | SCV L-A L1 | *Totivirus* | fungi | M28353.1 |
| 235 | *Saccharomyces cerevisiae virus L-BC* | SCV L-BC | *Totivirus* | fungi | U01060.1 |
| 236 | *Scheffersomyces segobiensis virus L* | SMSV L | *Totivirus* | fungi | KC610514.1 |
| 237 | *Shanghai Totivirus* | SHTV | Unclassified | arthropod | MN196674.1 |
| 238 | *Shanghai Totivirus* | SHTV | Unclassified | arthropod | MN196675.1 |
| 239 | *Snodland virus* | SNV | Unclassified | arthropod | MF893257.1 |
| 240 | *Sogatella furcifera Totivirus 1* | SFTV 1 | Unclassified | arthropod | MG546515.1 |
| 241 | *Sogatella furcifera Totivirus 2* | SFTV 2 | Unclassified | arthropod | MG546516.1 |
| 242 | *Solenopsis midden virus* | SMV | Unclassified | arthropod | MH727531.1 |
| 243 | *Southern tomato virus* | STV | *Amalgavirus* | plant | MN216389.1 |
| 244 | *Sphaeropsis sapinea RNA virus 1* | SSRV 1 | *Victorivirus* | fungi | AF038665.1 |
| 245 | *Sphaeropsis sapinea RNA virus 2* | SSRV 2 | *Victorivirus* | fungi | AF039080.1 |
| 246 | *Taro-associated Totivirus L* | TATV L | Unclassified | plant | MN119621.1 |
| 247 | *Thelebolus microsporus Totivirus 1* | TMTV 1 | Unclassified | fungi | MK279496.1 |
| 248 | *Thelephora terrestris virus 1* | TTV 1 | Unclassified | fungi | KT191297.1 |
| 249 | *Tianjin Totivirus* | ToV-TJ | Unclassified | arthropod | JN391187.1 |
| 250 | *Tolypocladium cylindrosporum virus 1* | ToCV 1 | *Victorivirus* | fungi | FR750562.1 |
| 251 | *Tolypocladium ophioglossoides Totivirus 1* | ToTV 1 | Unclassified | fungi | MK279497.1 |
| 252 | *Totiviridae* sp. | Toti H4 | Unclassified | grassland soil | MN032678.1 |
| 253 | *Totiviridae* sp. | Toti H1 | Unclassified | grassland soil | MN032968.1 |
| 254 | *Totiviridae* sp. | Toti H2 | Unclassified | grassland soil | MN033095.1 |
| 255 | *Totiviridae* sp. | Toti H4 | Unclassified | grassland soil | MN033316.1 |
| 256 | *Totiviridae* sp. | Toti H2 | Unclassified | grassland soil | MN033374.1 |
| 257 | *Totiviridae* sp. | Toti H2 | Unclassified | grassland soil | MN033419.1 |
| 258 | *Totiviridae* sp. | Toti H4 | Unclassified | grassland soil | MN033436.1 |
| 259 | *Totiviridae* sp. | Toti H1 | Unclassified | grassland soil | MN033604.1 |
| 260 | *Totiviridae* sp. | Toti H2 | Unclassified | grassland soil | MN033636.1 |
| 261 | *Totiviridae* sp. | Toti H2 | Unclassified | grassland soil | MN033948.1 |
| 262 | *Totiviridae* sp. | Toti H4 | Unclassified | grassland soil | MN034364.1 |
| 263 | *Totiviridae* sp. | Toti H2 | Unclassified | grassland soil | MN035035.1 |
| 264 | *Totiviridae* sp. | Toti H2 | Unclassified | grassland | MN035108.1 |

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| 265 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN035374.1 |
| 266 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN035544.1 |
| 267 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN035631.1 |
| 268 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN035661.1 |
| 269 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN035895.1 |
| 270 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN036052.1 |
| 271 | *Totiviridae* sp. | Toti H2 | Unclassified | soil grassland | MN036107.1 |
| 272 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN036110.1 |
| 273 | *Totiviridae* sp. | Toti H3 | Unclassified | soil grassland | MN036142.1 |
| 274 | *Totiviridae* sp. | Toti H1 | Unclassified | soil grassland | MN036163.1 |
| 275 | *Totiviridae* sp. | Toti H4 | Unclassified | soil grassland | MN036220.1 |
| 276 | *Totivirus* Atlantic salmon/CMS/Norway | TAS CMS N | Unclassified | soil fish | HQ401057.1 |
| 277 | *Totivirus*-like Culex mosquito virus 1 | TLCMV 1 | Unclassified | arthropod | MH188048.1 |
| 278 | *Trichoderma koningiopsis Totivirus 1* | TKTV 1 | Unclassified | fungi | MK993478.1 |
| 279 | *Trichomonas vaginalis virus 2* | TVV 2 | *Trichomonasvirus* | protozoa | AF127178.1 |
| 280 | *Trichomonas vaginalis virus 3* | TVV 3 | *Trichomonasvirus* | protozoa | AF325840.1 |
| 281 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | DQ270032.1 |
| 282 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | DQ528812.1 |
| 283 | *Trichomonas vaginalis virus* | TVV | *Trichomonasvirus* | protozoa | FJ997643.1 |
| 284 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | HQ607513.1 |
| 285 | *Trichomonas vaginalis virus 2* | TVV 2 | *Trichomonasvirus* | protozoa | HQ607514.1 |
| 286 | *Trichomonas vaginalis virus 3* | TVV 3 | *Trichomonasvirus* | protozoa | HQ607515.1 |
| 287 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | HQ607516.1 |
| 288 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | HQ607517.1 |
| 289 | *Trichomonas vaginalis virus 2* | TVV 2 | *Trichomonasvirus* | protozoa | HQ607518.1 |
| 290 | *Trichomonas vaginalis virus 3* | TVV 3 | *Trichomonasvirus* | protozoa | HQ607519.1 |
| 291 | *Trichomonas vaginalis virus 4* | TVV 4 | *Trichomonasvirus* | protozoa | HQ607520.1 |
| 292 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | HQ607521.1 |
| 293 | *Trichomonas vaginalis virus 4* | TVV 4 | *Trichomonasvirus* | protozoa | HQ607522.1 |
| 294 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | HQ607523.1 |
| 295 | *Trichomonas vaginalis virus 2* | TVV 2 | *Trichomonasvirus* | protozoa | HQ607524.1 |
| 296 | *Trichomonas vaginalis virus 3* | TVV 3 | *Trichomonasvirus* | protozoa | HQ607525.1 |
| 297 | *Trichomonas vaginalis virus 4* | TVV 4 | *Trichomonasvirus* | protozoa | HQ607526.1 |
| 298 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | JF436869.1 |
| 299 | *Trichomonas vaginalis virus 2* | TVV 2 | *Trichomonasvirus* | protozoa | JF436870.1 |
| 300 | *Trichomonas vaginalis virus 2* | TVV 2 | *Trichomonasvirus* | protozoa | JF436871.1 |
| 301 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | KM268108.1 |
| 302 | *Trichomonas vaginalis virus 2* | TVV 2 | *Trichomonasvirus* | protozoa | KM268109.1 |
| 303 | *Trichomonas vaginalis virus 3* | TVV 3 | *Trichomonasvirus* | protozoa | KM268110.1 |
| 304 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | U08999.1 |
| 305 | *Trichomonas vaginalis virus 1* | TVV 1 | *Trichomonasvirus* | protozoa | U57898.1 |
| 306 | *Tuber aestivum virus 1* | TAV 1 | *Totivirus* | fungi | HQ158596.1 |
| 307 | *Umbelopsis ramanniana virus 1* | URV 1 | Unclassified | fungi | LR216268.1 |
| 308 | *Umbelopsis ramanniana virus 2* | URV 2 | Unclassified | fungi | LR216269.1 |
| 309 | *Umbelopsis ramanniana virus 3* | URV 3 | Unclassified | fungi | LR595926.1 |
| 310 | *Umbelopsis ramanniana virus 4* | URV 4 | Unclassified | fungi | LR595928.1 |
| 311 | *Ustilaginoidea virens RNA virus 1* | UVRV 1 | *Victorivirus* | fungi | JX524563.1 |
| 312 | *Ustilaginoidea virens RNA virus* | UVRV 1 | *Victorivirus* | fungi | KC433710.1 |

| No. | Virus Species / Group | Taxon Abbreviation | Genus / Group | Host | GenBank Accession No. |
|---|---|---|---|---|---|
| | *1* | | | | |
| 313 | *Ustilaginoidea virens RNA virus 3* | UVRV 3 | *Victorivirus* | fungi | KF791042.1 |
| 314 | *Ustilaginoidea virens RNA virus L* | UVRV L | *Victorivirus* | fungi | KJ101566.1 |
| 315 | *Ustilaginoidea virens RNA virus 5* | UVRV 5 | *Victorivirus* | fungi | KT188753.1 |
| 316 | *Ustilago maydis virus H1* | UMV H1 | *Totivirus* | fungi | U01059.1 |
| 317 | *Wuhan insect virus 31* | WIV 31 | Unclassified | arthropod | KX882989.1 |
| 318 | *Xanthophyllomyces dendrorhous virus L1A* | XdV-L1A | *Totivirus* | fungi | JN997472.1 |
| 319 | *Xanthophyllomyces dendrorhous virus L1B* | XdV-L1B | *Totivirus* | fungi | JN997473.1 |
| 320 | *Xanthophyllomyces dendrorhous virus L2* | XdV-L2 | *Totivirus* | fungi | JN997474.2 |
| 321 | *Xingshan nematode virus 6* | XNV 6 | Unclassified | arthropod | KX882996.1 |
| 322 | *Yongshan Totivirus* | YoTV | Unclassified | arthropod | MN176215.1 |
| 323 | *Yuanmou Totivirus* | YuTV | Unclassified | arthropod | MN176214.1 |

***Appendix 5.2***

**List of Models generated by ProtTest3 and processed by BEAST2 for amino acids (Page 1)**
Yellow colour indicates for model that used for amino acid in BEAST2

| Model | deltaAIC | AIC | AICw | -lnL |
|---|---|---|---|---|
| VT+I+G+F | 0.00 | 568135.73 | 1.00 | 283533.87 |
| VT+G+F | 105.55 | 568241.28 | 0.00 | 283587.64 |
| Blosum62+G+F | 1243.01 | 569378.75 | 0.00 | 284156.37 |
| Blosum62+I+G+F | 1281.71 | 569417.44 | 0.00 | 284174.72 |
| VT+I+G | 1319.95 | 569455.68 | 0.00 | 284212.84 |
| VT+G | 1445.20 | 569580.94 | 0.00 | 284276.47 |
| Blosum62+I+G | 2479.41 | 570615.14 | 0.00 | 284792.57 |
| Blosum62+G | 2607.91 | 570743.64 | 0.00 | 284857.82 |
| VT+I+F | 10697.65 | 578833.38 | 0.00 | 288883.69 |
| VT+F | 10889.26 | 579024.99 | 0.00 | 288980.50 |
| Blosum62+I+F | 11687.91 | 579823.64 | 0.00 | 289378.82 |
| Blosum62+F | 11856.99 | 579992.73 | 0.00 | 289464.36 |
| VT+I | 12089.39 | 580225.12 | 0.00 | 289598.56 |
| VT | 12293.35 | 580429.08 | 0.00 | 289701.54 |
| HIVb+I+G+F | 12734.33 | 580870.06 | 0.00 | 289901.03 |
| HIVb+G+F | 12806.70 | 580942.44 | 0.00 | 289938.22 |
| Blosum62+I | 12914.83 | 581050.56 | 0.00 | 290011.28 |
| Blosum62 | 13085.20 | 581220.93 | 0.00 | 290097.46 |
| HIVb+I+G | 15830.05 | 583965.78 | 0.00 | 291467.89 |
| HIVb+G | 15920.68 | 584056.42 | 0.00 | 291514.21 |
| FLU+I+G | 16546.95 | 584682.68 | 0.00 | 291826.34 |
| FLU+G | 16650.76 | 584786.50 | 0.00 | 291879.25 |
| HIVw+I+G+F | 23489.01 | 591624.74 | 0.00 | 295278.37 |
| HIVw+G+F | 23593.66 | 591729.39 | 0.00 | 295331.70 |
| FLU+I+F | 26169.36 | 594305.09 | 0.00 | 296619.55 |
| FLU+F | 26391.56 | 594527.29 | 0.00 | 296731.64 |
| HIVb+I+F | 28062.65 | 596198.39 | 0.00 | 297566.19 |
| HIVb+F | 28310.95 | 596446.69 | 0.00 | 297691.34 |
| HIVb+I | 30757.50 | 598893.23 | 0.00 | 298932.62 |
| HIVb | 31034.18 | 599169.91 | 0.00 | 299071.95 |
| FLU+I | 31256.19 | 599391.92 | 0.00 | 299181.96 |
| FLU | 31554.63 | 599690.36 | 0.00 | 299332.18 |
| HIVw+I+G | 38406.61 | 606542.34 | 0.00 | 302756.17 |
| HIVw+G | 38542.57 | 606678.31 | 0.00 | 302825.15 |
| HIVw+I+F | 38698.65 | 606834.38 | 0.00 | 302884.19 |
| HIVw+F | 38956.03 | 607091.76 | 0.00 | 303013.88 |
| HIVw+I | 53979.54 | 622115.28 | 0.00 | 310543.64 |
| HIVw | 54350.54 | 622486.28 | 0.00 | 310730.14 |
| LG+I+G+F | 0.00 | 569722.74 | 1.00 | 284327.37 |
| LG+G+F | 82.06 | 569804.80 | 0.00 | 284369.40 |
| LG+I+G | 1371.42 | 571094.16 | 0.00 | 285032.08 |
| LG+G | 1455.64 | 571178.38 | 0.00 | 285075.19 |
| JTT+I+G+F | 2255.99 | 571978.73 | 0.00 | 285455.37 |
| JTT+G+F | 2346.52 | 572069.26 | 0.00 | 285501.63 |
| JTT+I+G | 3396.11 | 573118.86 | 0.00 | 286044.43 |
| JTT+G | 3477.31 | 573200.05 | 0.00 | 286086.03 |
| MtREV+I+G+F | 8892.49 | 578615.23 | 0.00 | 288773.62 |
| MtREV+G+F | 8992.50 | 578715.25 | 0.00 | 288824.62 |
| LG+I+F | 13671.95 | 583394.69 | 0.00 | 291164.35 |
| LG+F | 13877.09 | 583599.83 | 0.00 | 291267.92 |
| LG+I | 15042.15 | 584764.90 | 0.00 | 291868.45 |
| JTT+I+F | 15046.27 | 584769.01 | 0.00 | 291851.51 |

## List of Models generated by ProtTest 2 and processed by BEAST2 for amino acids (Page 2)

| Model | deltaAIC | AIC | AICw | -lnL |
|---|---|---|---|---|
| JTT+F | 15264.30 | 584987.04 | 0.00 | 291961.52 |
| LG | 15269.66 | 584992.41 | 0.00 | 291983.20 |
| JTT+I | 16225.54 | 585948.28 | 0.00 | 292460.14 |
| JTT | 16445.13 | 586167.88 | 0.00 | 292570.94 |
| DCMut+I+F | 17767.88 | 587490.62 | 0.00 | 293212.31 |
| DCMut+F | 18018.93 | 587741.68 | 0.00 | 293338.84 |
| DCMut+I | 22445.88 | 592168.62 | 0.00 | 295570.31 |
| DCMut | 22754.96 | 592477.70 | 0.00 | 295725.85 |
| MtREV+I+F | 24892.80 | 594615.54 | 0.00 | 296774.77 |
| MtREV+F | 25129.18 | 594851.93 | 0.00 | 296893.96 |
| MtREV+I+G | 33218.76 | 602941.50 | 0.00 | 300955.75 |
| MtREV+G | 33280.26 | 603003.01 | 0.00 | 300987.50 |
| MtMam+G | 46261.61 | 615984.35 | 0.00 | 307478.18 |
| MtREV+I | 49916.38 | 619639.12 | 0.00 | 309305.56 |
| MtREV | 50126.51 | 619849.25 | 0.00 | 309411.62 |
| MtMam+I | 68447.13 | 638169.87 | 0.00 | 318570.94 |
| WAG+I+G+F | 0.00 | 569127.29 | 1.00 | 284029.64 |
| WAG+G+F | 72.52 | 569199.81 | 0.00 | 284066.90 |
| RtREV+I+G+F | 974.24 | 570101.53 | 0.00 | 284516.77 |
| RtREV+G+F | 1039.73 | 570167.01 | 0.00 | 284550.51 |
| WAG+I+G | 1457.31 | 570584.60 | 0.00 | 284777.30 |
| WAG+G | 1558.65 | 570685.93 | 0.00 | 284828.97 |
| CpREV+I+G+F | 2689.75 | 571817.04 | 0.00 | 285374.52 |
| CpREV+G+F | 2780.86 | 571908.15 | 0.00 | 285421.07 |
| CpREV+I+G | 5193.10 | 574320.39 | 0.00 | 286645.19 |
| Dayhoff+I+G+F | 5248.01 | 574375.30 | 0.00 | 286653.65 |
| CpREV+G | 5286.25 | 574413.54 | 0.00 | 286692.77 |
| Dayhoff+G+F | 5363.28 | 574490.57 | 0.00 | 286712.29 |
| RtREV+I+G | 6507.28 | 575634.56 | 0.00 | 287302.28 |
| RtREV+G | 6565.44 | 575692.73 | 0.00 | 287332.37 |
| Dayhoff+I+G | 9952.80 | 579080.09 | 0.00 | 289025.04 |
| Dayhoff+G | 10077.98 | 579205.27 | 0.00 | 289088.63 |
| WAG+I+F | 11471.84 | 580599.12 | 0.00 | 289766.56 |
| WAG+F | 11683.06 | 580810.34 | 0.00 | 289873.17 |
| WAG+I | 12878.71 | 582005.99 | 0.00 | 290489.00 |
| WAG | 13109.76 | 582237.05 | 0.00 | 290605.52 |
| RtREV+I+F | 14170.56 | 583297.84 | 0.00 | 291115.92 |
| RtREV+F | 14360.05 | 583487.34 | 0.00 | 291211.67 |
| CpREV+I+F | 15055.86 | 584183.15 | 0.00 | 291558.58 |
| CpREV+F | 15287.58 | 584414.87 | 0.00 | 291675.43 |
| MtArt+I+G+F | 17307.45 | 586434.74 | 0.00 | 292683.37 |
| CpREV+I | 17339.68 | 586466.96 | 0.00 | 292719.48 |
| MtArt+G+F | 17386.78 | 586514.07 | 0.00 | 292724.03 |
| CpREV | 17600.99 | 586728.28 | 0.00 | 292851.14 |
| Dayhoff+I+F | 18378.33 | 587505.62 | 0.00 | 293219.81 |
| Dayhoff+F | 18673.29 | 587800.57 | 0.00 | 293368.29 |
| RtREV+I | 19405.66 | 588532.95 | 0.00 | 293752.47 |
| RtREV | 19605.97 | 588733.25 | 0.00 | 293853.63 |
| Dayhoff+I | 23102.67 | 592229.95 | 0.00 | 295600.98 |
| Dayhoff | 23414.09 | 592541.38 | 0.00 | 295757.69 |
| MtArt+I+F | 37958.48 | 607085.76 | 0.00 | 303009.88 |
| MtArt+F | 38172.47 | 607299.76 | 0.00 | 303117.88 |
| MtArt+I+G | 45044.92 | 614172.21 | 0.00 | 306571.10 |
| MtArt+G | 45088.15 | 614215.44 | 0.00 | 306593.72 |
| MtArt+I | 66578.85 | 635706.14 | 0.00 | 317339.07 |
| MtArt | 66761.86 | 635889.14 | 0.00 | 317431.57 |