UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

HEALTH ASSESSMENT IN MADAGASCAR: INVASIVE ASIAN TOAD (*Duttaphrynus melanostictus*) AS A VECTOR FOR EMERGING PATHOGENS

INÊS ALVITO FÉLIX

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Ao meu Pai e à minha Mãe.

“Oh yes, the past can hurt.
But, the way I see it, you can either run from it… Or learn from it”

Rafiki, The Lion King

ACKNOWLEDGEMENTS

A quem me acompanhou nas aulas, nas festas e nas gazetas. Aos que passaram pelo mesmo e ao mesmo tempo. Obrigado Carolina por me manteres acordada às 8H da manhã com perguntas pré-exame e por nos intervalos da seriedade, em tom de musical, me amparares com gargalhadas e bons conselhos. Obrigado Rafa por me mostrares que é possível alguém surpreender todos os dias e por fazeres de mim uma melhor pessoa. Obrigado Xavi por seres direto, companheiro e conselheiro nas horas vagas. Obrigado Inês por sofreres sempre apenas a uma cadeira de intervalo e por todas as horas ao telefone que me fizeram sorrir e respirar de alívio. Obrigada Jodi, pela paixão que aplicas em tudo o que fazes e por salientares sempre os pontos fortes. Obrigado Vistas por seres um cartoon geek como eu, e por partilhares toda a animação que tens em ti.

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RESUMO

AVALIAÇÃO DA SAÚDE EM MADAGÁSCAR: SAPO ASIÁTICO INVASIVO (*Duttaphrynus melanostictus*) COMO VETOR DE AGENTES PATOGÉNICOS EMERGENTES.

O sapo asiático *Duttaphrynus melanostictus* foi recentemente introduzido em Madagáscar, apresentando uma rápida expansão. Para além da elevada taxa de fertilidade e toxinas, este sapo é potencialmente um reservatório de diferentes agentes patogénicos, podendo ameaçar a biodiversidade de Madagáscar. Este estudo tem como objetivo a avaliação dos agentes presentes, e se este sapo pode representar uma ameaça real para o ecossistema.

Sapos asiáticos (n=110) foram capturados vivos, perto do porto de Toamasina (Madagáscar), eutanaziados, e os seus fígados e pele foram colhidos e preservados em etanol a 98%. A captura decorreu na estação seca (Julho 2014) e chuvosa (Março 2015)

Os sapos foram testados para a presença de quitrídio (*Batrachochytrium dendrobatidis*), *Ranavirus*, clamídia, *Herpesvirus* e *Salmonella*. Os primeiros dois são agentes emergentes: o primeiro é responsável pela quitridiomicose e o segundo é um Iridovirus que causa a ranavirose. São consideradas a duas principais doenças que contribuem para o declínio e extinção de anfíbios.

Foram detetadas 10 amostras positivas para *Ranavirus* na estação seca, e nenhuma na chuvosa. Para Chlamydiaceae 9 amostras testaram positivas na estação seca e duas na chuvosa. Nenhuma amostra foi positiva para a quitridio ou *Herpesvirus*. O PCR para *Salmonella* detetou uma amostra positiva na estação seca.

Palavras-chave: Madagáscar; *Duttaphrynus melanostictus*; Quitridiomicose; *Ranavirus*; Chlamydiaceae; *Salmonella.*

ABSTRACT

HEALTH ASSESSMENT IN MADAGASCAR: INVASIVE ASIAN TOAD (*Duttaphrynus melanostictus*) AS A VECTOR FOR EMERGING PATHOGENS

Recently, the Asian common toad *Duttaphrynus melanostictus* arrived in Madagascar, presenting a high expansion rate. Besides the high fertility rate and toxins, it's likely that these toads may be infected with different pathogenic agents, posing a threat to the Malagasy species.

This study meets the need to identify which infectious agents are present, and if these toads are a real threat to the ecosystem.

Asian toads (n=110) were caught, close to the Toamasina port (Madagascar), euthanized, and the liver and skin were collected and preserved in 98% ethanol. The sample collection took place during the dry season (July 2014) and the rainy season (March 2015).

The toads were tested for the presence of chytrid fungus (*Batrachochytrium dendrobatidis*), *Ranavirus*, chlamydia, *Herpesvirus* and *Salmonella*. The first two are emerging infectious pathogens: chytrid leads to chytridiomycosis and the second is an Iridovirus responsible for ranaviral disease. Both are considered the two major diseases leading to amphibian declines and extinctions.

The *Ranavirus* screening detected 10 positive samples in the dry season, and none in the rainy season. 9 positive samples to Chlamydiaceae in the dry season and 2 in the rainy season. No sample was positive to chytrid or *Herpesvirus*. *Salmonella* PCR detected only one positive in the dry season.

Key words: Madagascar; *Duttaphrynus melanostictus*; Chytridiomycosis; *Ranavirus*; Chlamydiaceae; *Salmonella*.

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ABREVIATIONS AND SYMBOLS

% - Percent

≈ - Approximately

µL - Microliter

µm – Micrometer

AD – Anno domini (after Crist)

ARWH - Australian Registry of Wildlife Health

ATV - *Ambystoma tigrinum virus*

Bd – Batrachochytrium dendrobatidis

BIV - *Bohle iridovirus*

bp – Base pairs

Ca^{2+} - Calcium (ion)

CI - Confidence interval

CIISA – Centre for Interdisciplinary Research in Animal Health

CNS - Central nervous system diseases

Cph. - Chlamydophila

DC - direct contact

DNA – Deoxyribonucleic acid

dsDNA - double strain DNA

e.g – Example

EB - Elementary body

ECV - *European catfish virus*

EHNV - *Epizootic hematopoietic necrosis virus*

EtBr - ethidium bromide

FMV - Faculty of Veterinary Medicine

FV3 - *Frog virus 3*

FV4 - frog virus 4

g – Grams

GLXA - Glycolipid complex

HSP - Heat shock protein

HSV – *Herpes simplex*

ICTV - International Committee on Taxonomy of Virus

IFN γ - Interferon gama

IL – Interleukin

IUCN - International Union for Conservation of Nature

K^- – Potassium (ion)

Kbp – Kilo base pairs

kDa – Kilodaltons

LPS – Lipopolysaccharide

LPS - Lipopolysaccharide complex

Mb – Million base pairs

MCP - Major capsid protein

MHC I - Major histocompatibility complex I

mm – Millimeters

MOMP - Major Outer Membrane Protein

Na^+ – Sodium (ion)

NTS - nontyphoidal *Salmonella*

ºC – Degree Celsius

ORFs - open reading frames

PCR - polymerase chain reaction

PKR - protein kinase R

qPCR - quantitative PCR

RaHV-1 - ranid herpesvirus-1

RaHV-2 - ranid herpesvirus-2

RB - Reticulate bodies

RNA – Ribonucleic acid

rRNA – Ribossomal RNA

SCRV - *Santee-Cooper ranavirus*

T= - Triangulation Number

ULisboa - University of Lisbon

TRAINING PERIOD ACTIVITIES

This dissertation, carried out under the Master in Veterinary Medicine, was conducted in the Virology and Molecular Biology Laboratory of the Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine (FMV) of the University of Lisbon (ULisboa), under the guidance of Professor Ana Isabel Simões Pereira Duarte, and of the biologist Gonçalo Miranda Rosa. The accomplished training period lasted seven months, between October 2014 and May 2015 (approximately 1000 hours), during which I performed a molecular screening of the skin and liver from Asian toads samples from Madagascar.

LABORATORIAL WORK

During my training period I was introduced to different molecular diagnostic techniques, mainly to detect viral agents. I was able to analyse different kinds of biological samples, since I worked with animal groups from amphibians to large felines, tortoises, and small mammals.

CIISA/FMV, ULisboa is equipped with several laboratory instruments, used by researchers with scientific projects, masters or PhD degrees, and to answer the diagnostic requests from the teaching hospital of FMV or other clinics.

To develop my project I used three PCR (polymerase chain reaction) assays, quantitative PCR (qPCR), conventional PCR and nested PCR. The qPCR is used to detect and quantify the pathogen target sequence present in the sample. The conventional PCR, uses one set of primers, which amplify a specific target sequence. The amplicon is visualized in an agarose gel, after electrophoresis. The nested PCR is a modification of the conventional PCR technique, intended to reduce non-specific binding. It uses two sets of primers, in two successive runs of amplification. The results are visualized after electrophoresis, at the reaction end-point, similarly to the conventional PCR.

I also participated in two other projects, the first one referring to the study of immune mediators in sea turtles, *Chelonia mydas* and *Caretta caretta*, in which I amplified and cloned a fragment of the 18s RNA gene for both species.

The second study was conducted in biologic samples of lions and leopards of a game reserve in Niassa, Mozambique (provided by my college Miguel Lajas). I extracted DNA/RNA from collected faeces; performed the detection of viral nucleic acid, including canine distemper virus (CDV), feline coronavirus (FeCoV), and feline parvovirus (FPV). I also extracted DNA from blood samples, and analysed them for *Mycoplasma haemofelis.* Moreover, I participated in the serological detection of feline immunodeficiency virus (FIV), by western blotting and ELISA and amplification of FIV genomic regions, by conventional PCR.

1 THE STUDY

Human mediated introduction of alien species in naive habitats (accidently or deliberated) have been documented since the 1500's. This research topic has been seen as a serious conservation issue in many of those cases, where ultimately lead to the decline of autochthonous species and may even extinguished them. Animals can enter boats or trains, ending up in a different place, with or without favourable conditions for them to live and reproduce. Certain alien species, if the conditions are propitious, can become invasive and a plague, using up the resources, culminating in habitat loss and the decline of the more fragile species.

Madagascar is known for its communities of rare plant and animal species, and its rich ecosystem. The alien species in Madagascar expanded their range rapidly having negative and positive effects for the autochthonous biodiversity.

In recent times, the Asian common toad *Duttaphrynus melanostictus* has arrived in Madagascar, and quickly began its expansion. Afar from the high fertility rate and toxins, it's likely that these toads are carrying various pathogens and parasites potentially able to threaten the naive Malagasy species.

This study meets the need to understand which pathogens are present, and how prevalent and widespread they are, thus making it possible to evaluate the degree of potential impact of these toads to the ecosystem as vectors of new agents. 110 toads were caught alive on site, close to the Toamasina port, euthanized, and the liver and skin were collected and preserved in 98% ethanol. The collection took place in two distinct periods, during the dry season (July 2014), and the rainy season (March 2015), in order to detect any seasonality in the pathogens prevalence.

This study is just a small part of a larger plan, because when it comes to invasive species, to achieve eradication, is really important to have all the information. Currently, a team of experts on amphibians and invasive species is working on this goal, divided in 5 working groups:

i. Survey Education and Prevention group is delimitating the toad's expansion area, and informing the human population about the threats this species poses to the ecosystem, and to the human life, as its toxins are dangerous to both animals and humans;
ii. The Feasibility study group is trying to comprehend if the eradication is possible, and how to achieve it;
iii. The Chytrid and Samples Analysis group is looking for clues that indicate how this toad was introduced in the area, collecting samples and screening them to know which infectious diseases are present. This dissertation is integrated in this group action;
iv. Fundraising;
v. Communication group, to perform the interface between all the other groups and the Malagasy authorities.

1.1 OBJECTIVES

An invasive species can be a serious problem, and that's why this thesis is important, even if it's just a small part of the bigger plan. Actually, this study results can be decisive in the choice of strategy to achieve the eradication.

To see if eradication is an emergency, it's essential to know if these toads are a living threat to the health status of the native amphibian species.

This thesis has the objective of i) knowing if the Madagascar invasive toads are infected with amphibian chytrid fungus (*Batrachochytrium dendrobatidis*), *Ranavirus*, *Chlamydia*, *Herpesvirus* and *Salmonella*; ii) to estimate the infectious prevalence in this population; and iii) investigate the impacts of the presence of *Duttaphrynus melanostictus* in the Malagasy biodiversity.

2 STATE OF THE ART

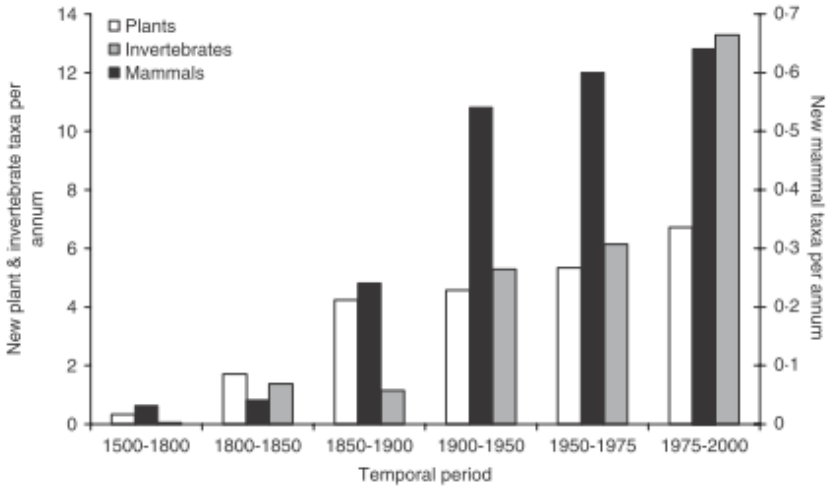
2.1 INVASIVE SPECIES

Alien species are organisms introduced in a new area, intentionally or not, by humankind (Hulme, 2009). Their invasiveness is characterized by the adaptive response to the new environments, regardless of the founder effect phenomenon (Phillips & Shine, 2005).

Several studies have shown that new species are introduced accordingly to the human trade and transports (e.g. Meyerson & Mooney, 2007; Perrings et al., 2005). Hereupon, taking a look into human history, there are three main eras when transportation peaked: the first one coincide with the rediscovery of the world by the Europeans (1500 AD), during this time new plants and animals with human interest were found, and imported to Europe; at the same time Europeans implanted the ones they already used in the new lands. The second era refers to the Industrial Revolution when the trades increased across all continents, even humans started to migrate more, taking with them new species. The importation growth stroke again, and now we are in the so called globalization era, that started in the 70's, where everything is at our reach, and the distance between continents was shortened due to the new means of transportation: ships, planes and improved railways and roads (Hulme, 2009).

But not all introductions of alien fauna and flora were/are intentional; some organisms can be

Figure 1 - Annual rates of increase in the establishment of alien species in Europe since 1500 AD (Hulme, 2009).



introduced in novel environments accidentally. Stowaways can be found in the transportation containers from boats, planes or trains. Aquatic species can enter the ballast water and arrive in a new location. Additionally to the relocation, it is necessary for an alien species to have easy access to different sites to establish new colonies, making roads and railways a convenient way to travel (e.g.: spatial patterns in the invasions of plants in China, insects in Europe and cane toads, *Bufo marinus*, in Australia are correlated to road density) (Hulme, 2009).

When talking about an invasive species, it is important to know that it's not the whole species that is invasive, but the specific population colonizing the new site, and that in its origin location the same species may be endangered. There isn't one accepted definition to the term "invasive species", some researchers highlight the negative impacts of the aliens (like threats to native biodiversity), others believe that invasive species are foreign, reproducing constantly and can extent to a vast area rapidly. However there are those who support that a species can have an invasive attitude but still be native, invasion is characterized by a specie that can overcome the others when natural obstacles to its proliferation are surpassed (Kull et al., 2014).

Summing up, an invasive species can be foreign or not, and can have invasive behaviour or not. The human exploitation of the environment unbalances the ecosystems, sometimes invasive behaviour is seasonal or transitory, for instance, *Lantana* invaded several islands having an initial outburst that subsided overtime (Kull et al., 2014).

Alien species can have positive and negative impacts in the ecosystem, they are often related to economic and ecological (predation, poisoning, competition, environmental damage and new diseases) losses but they can also bring benefits (Kull et al., 2014).

Now taking an amphibian point of view, there are two well-known invasive toads, the American bullfrog (*Lithobates catesbeianus*) and the Cane toad (*Rhinella marina*).

Lithobates catesbeianus is a North-American non-poisonous species that was introduced in Europe, and is now stablished in France, Italy, Germany, Greece, Belgium and The Netherlands. The exerted threat to native amphibian fauna is through competition, predation and disease, since these bullfrogs are known reservoirs to many infectious pathogens, in particular, *Ranavirus* and amphibian chytrid fungus. These amphibians may also pose a threat to humans has they are known carriers of *Salmonella*, *Campylobacter, Escherichia coli*, among others (Martel et al., 2013).

The cane toad, *Rhinella marina* (formerly known as *Bufo marinus*), is a South American toad, and became a tremendously effective invader throughout the Caribbean and Pacific seas. However, is the Australian invasion the most known one (Phillips & Shine, 2005). Besides their extremely successful invasion and prolificacy, since introduction, these toads have killed many predators due to the poison they produce. They also competed with burrow nesting birds, disrupted the nutrient pools and the parasitic dynamics (Jolly et al., 2015). Over and more, the cane toad acts as a reservoir to infectious agents.

Rhinella marina wasn't an accidental introduction; due to its voracious predation on insects humans brought this species to Australia in attempts of fighting the pest beetles damaging the sugar canes in 1935 (Department of the Environment, 2010). These are large and toxic anurans, with high proliferative rates, that already colonized more than one million square kilometres of Australia, and invaded more than 20 countries (Phillips & Shine, 2005).

The impact of these anurans in Australia on predators is beyond death by poisoning, toxic cane toads induced morphological changes in the native species. Phillips & Shine (2004), proved

that Australian snakes became bigger with smaller heads, because feeding on large toads, with higher quantity of toxin, culminated in predator death, and a snake with a smaller head relative to its body mass couldn't ingest larger toads.

Islands' ecosystems are more susceptible to be invaded and threatened since they have higher importation rates (Hulme, 2009). Madagascar, as an island, is no exception, hosting 50-60 introduced animals and approximately 1,200 vascular plants species. 8.9% of these alien plants are considered invasive. However, not all foreign species are labelled as invasive, some contribute positively for the country's economy and culture (rice, vanilla, cloves, eucalyptus and zebu) (Kull et al., 2014).

Madagascar is a recognized ecological niche associated with high biodiversity, being one of the richest countries in the world with a wide number of amphibian species. In 2005 the Global Amphibian Assessment ranked Madagascar's as the 12th country with highest amphibian species diversity, and when analysing endemic data this island ranks 4th. Currently about 300 amphibian species are documented (AmphibiaWeb.org, 2015), with probably over 200 more waiting to be described (Perl et al., 2014); 100% of the native species and 88% of the genera only exists in Madagascar, and the International Union for Conservation of Nature (IUCN) listed 9 species as "Critically Endangered", and 21 as "Endangered".

Adding up to the fact that habitat loss has come to about 90% from the original vegetation (McConnell & Kull, 2014), and some Malagasy amphibian species have high international consumption and pet trade demands, its urgent to prevent loss of biodiversity, and avoid the entrance of alien amphibian species that can carry infectious agents (Kull et al., 2014).

Malagasy government made notable efforts with the implementation of national plans to early detection of chytridiomycosis (Weldon et al., 2013). However a new type of threat arrived to Madagascar in 2011, the introduction of the Asian toad *Duttaphrynus melanostictus*. This toad is a distant relative to the cane toad, with similar characteristics (e.g.: fertility rate and adaptability). This comes as a problematic situation, if the Asian toad turns out to invade Madagascar like the cane toad did already in Australia (Crottini et al., 2014).

2.2 *DUTTAPHRYNUS MELANOSTICTUS*

Duttaphrynus melanostictus[1] (Schneider 1799) is commonly known by the names Asian spined toad, Asian common toad or just Asian toad. Its IUCN status is “Least Concern”.

These toads are considered to have medium to large size; their snout-vent length can range between 57 and 150 mm, being the females usually larger than the males. The peak of sexual activity is right before the beginning of the rainy season. Per clutch, the females can lay up to 40,000 eggs and oviposition can occur up to two times in a year.

Breeding occurs in slow flowing rivers or stagnant water sources and even in cement cisterns, or brackish water up to 1% salinity. Hatching happens from 24 to 48 hours; larvae stage ranges between 34 to 90 days, and the metamorphosis can endure for 25-30 days. Young *D. melanostictus* can achieve sexual maturity at 23g of body weight, and their lifespan is 4-10 years in the wild.

These toads can be found and breed in urban and agricultural regions, riverbanks and upper beaches. The adults are terrestrial, and during the day they usually stay under ground cover.

Figure 2 – *D. melanostictus* by B. L. Richardson

Kingdom: Animalia
Phylum: Chordata
Class: Amphibia
Order: Anura
Family: Bufonidae
Genus: Duttaphrynus
Species: *Duttaphrynus melanostictus*

These toads have an opportunistic diet, ingesting invertebrates, mostly arthropods (ants and termites), but they can also eat scorpions and centipedes. Tadpoles diets resumes to phytoplankton.

Asian toads are found in subtropical and tropical habitats, in forest margins, riparian-areas, agrarian and urban areas.

This species is widely distributed in all Asia, being native to Pakistan, India through Indonesia. *D. melanostictus* are not a direct threat to human safety, but it produces toxins with lethal, hypotensive, hypertensive, neurotoxic, cardiotoxic, haemolytic and sleep inducing factors. In Timor-Leste at least one child has died and many other become sick after eating toads (Trainor, 2009).

They’re already considered an invasive species in some areas. It was first detected in Bali in 1958, and most recently in East-Timor where is responsible for ecological problems similar to the ones reported for the cane toad in Australia (Trainor, 2009).

[1]*Duttaphrynus melanostictus* first designation *Bufo melanostictus* (Schneider 1799), in 2006 a reevaluation in taxonomy changed this species genus (Frost et al. 2006). Synonyms: *Ansonia kamblei*, *Bufo tienhoensis*.

Currently *D. melanostictus* is also settled in Madagascar, in the Toamasina province (sea port city) and may have already been present for some years and potentially introduced prior to 2010. However, only in March 2014 its identification was released to the scientific community. Moore et al. (2015) showed the quick widespread that this species already has in Toamasina.

Like enunciated above, invasive species can be a serious problem when it comes to carrying infectious agents, and *Duttaphrynus melanostictus* is not an exception. In order to better evaluate the possible impacts that this species can have in the Malagasy naïve amphibian populations, it is essential to study some of the amphibian diseases with greater impact.

2.3 RANAVIRUS

2.3.1 Taxonomy

The family Iridoviridae includes the *Iridovirus* and *Chloriridovirus* genera which infect invertebrate hosts, and the *Megalocytivirus*, *Lymphocystivirus*, and *Ranavirus* genera that infect cold-blooded vertebrates (Reviewed by Gray & Chinchar, 2015).

The Iridoviridae family has 26 core genes in common, including viral structural proteins as well as proteins involved in the regulation of gene expression, virus replication, and virulence (Reviewed by Gray & Chinchar, 2015). These family genomes differ, in size, from 140 to 303 kbp. But because genomes are terminally redundant, range only from 105 to 212 kbp (Jancovich et al., 2012).

The viruses belonging to the *Ranavirus* genus have a wide range of hosts, as they can infect fish, amphibians and reptiles. It is believed that the first hosts were fish and then the virus made an evolutionary jump to amphibians and reptiles (Reviewed by Gray & Chinchar, 2015). *Frog virus 3* (FV3), *Ambystoma tigrinum virus* (ATV), *Bohle iridovirus* (BIV), *Epizootic hematopoietic necrosis virus* (EHNV), *European catfish virus* (ECV); and *Santee-Cooper ranavirus* (SCRV) are the six species recognized by the International Committee on Taxonomy of Virus (ICTV) within the *Ranavirus* genus (Jancovich et al., 2012). However there are more genetically distant ranaviruses not yet recognized as species.

2.3.2 Molecular Structure

Ranaviruses are large viruses, with or without envelope. The T=147 capsid is icosahedral, with 120-300 nm in diameter, and encloses a linear double strain DNA (dsDNA) genome (Figure 3) (Gregory et al., 2011). Most of the capsid is composed of a major capsid protein (MCP).

The species genome vary in size depending on the specific virus, ranging between 105 and 140 kbp, including 92 to 211 open reading frames (ORFs) (Table 1).

One of the first *Ranavirus* to be isolated was FV3, consequentially, becoming the type species of the genus. It has a circularly permutated and terminally redundant genome, which is a

characteristic of all viral genomes within the Iridoviridae family (Reviewed by Gray & Chinchar, 2015).

Ranaviruses encode 92 to 139 putative gene products; most of them recognized within the family, showing their importance in viral biogenesis. Besides the 26 core genes present in all the members of Iridoviridae, this genus has another 72 in common to all its species (Reviewed by Gray & Chinchar, 2015). Nevertheless the genomic diversity of the many Ranaviruses species is vast enough to allow differences in the pathogenicity and viral host range:

Ranaviruses genomes have repeated and variable regions which almost certainly regulate gene expression or ease the recombination process.

Also, it is known that viruses closely related to FV3 have different virulence, which can be explain by intragenic differences and variation within repeated sequences.

Figure 3 – Molecular structure of FV3 (source: *http://www.expasy.ch/viralzone).*

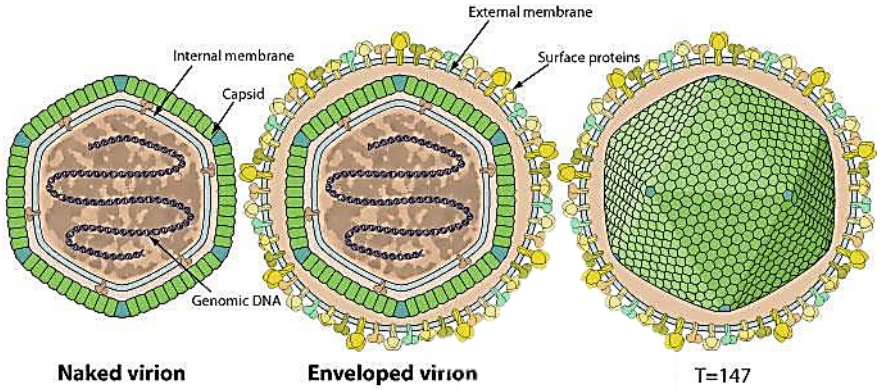


Table 1 – *Ranavirus* genomics (adapted from Gray & Chinchar, 2015)

Genus	Species	Size (bp)	Nº. ORFs
Ranavirus	FV3	105,903	97
	ATV	106,332	92
	EHNV	127,011	100

Frog virus 3 (FV3), *Ambystoma tigrinum virus* (ATV), *Epizootic hematopoietic necrosis virus* (EHNV).

2.3.3 Pathogenesis

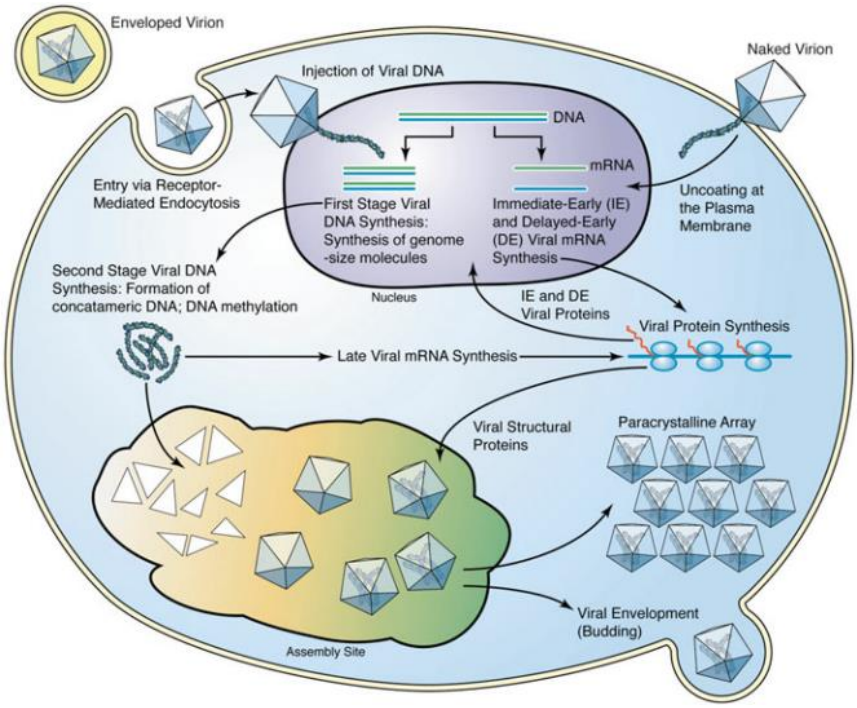
To understand the pathogenesis of this genus, it is important to have an insight of the cellular events that take place during the infection (Figure 4). The entry of a *Ranavirus* in a cell invariably leads to cell death, since both infectious and non-infectious virions have a shut-off mechanism, which inhibits the host DNA, RNA and protein synthesis. Apoptosis can occur consequently to the inhibition mechanism, or to the activation of protein kinase R (PKR) (Reviewed by Gray & Chinchar, 2015). Either way, it is the caspase activation that triggers this phenomenon, without it there's no apoptosis. Thus, if a caspase inhibitor is used, the process is blocked.

The viral replication, transcription and translation are not affected by the shut-off mechanism, producing high concentration of infectious virions within the first 24 hours.

Ranaviral disease in immunocompetent adult frogs is usually limited to the kidneys. Individuals are generally capable of clearing the virus in few weeks, which explains the lower mortality rates in this life stage. On the other hand, in tadpoles[2] and immunodeficient frogs the ranovirosis becomes systemic and reaches the liver, the gastrointestinal tract and the skin (Reviewed by Gray & Chinchar, 2015).

Macrophages are susceptible to FV3, as their capacity to process and present viral antigen grows affected with the presence of the virus within the cell. Over and more, these cells are an important source of persistently infected cells in the infected animal.

Figure 4 – *Ranavirus* replication process. (Gray & Chinchar, 2015)



"*Virions enter cells by one of two possible routes and initial events in virus replication (early viral transcription and the synthesis of unit-length genomes) take place within the nucleus. Viral genomes are subsequently transported into the cytoplasm where they are methylated and serve as templates for concatemer formation. Viral assembly sites contain viral DNA and a number of virus-encoded proteins and serve as the loci of virion formation. Newly synthesized virions are found free within the cytoplasm or within paracrystalline arrays, and, a minority, at least in vitro, bud from the plasma membrane and in the process acquire an envelope."* (Quoting - Gray & Chinchar, 2015)

[2] Tadpoles don't express the Major histocompatibility complex I (MHC I).

2.3.4 Clinical signs

Ranaviral disease affects mostly young amphibians and larvae; this group of animals usually has high mortality rates, making the outbreaks responsible for population decline. Though adults are able to fight the virus, more and more hosts are found, and with a high prevalence rate there is higher risk of a possible die-off. The die-off is characterized by mass and sudden mortality, with some scattered deaths due to secondary bacterial and fungal infection (Reviewed by Gray & Chinchar, 2015).

Table 2 enumerates some of the clinical signs exhibit by the hosts. Apart from these general findings, in adult frogs, two specific syndromes were described. The first one is characterized by systemic haemorrhages, and the other one by extensive cutaneous ulcerations. Both of them can coexist within the same individual.

Table 2 – Clinical signs of ranaviral infection (adapted from Gray & Chinchar, 2015).

Class	Lesion
Amphibian larvae	Loss of buoyancy; erratic swimming; anorexia; swelling (oedema) of the body, head, legs, and internal soft tissues; external haemorrhages (especially around the vent, periocular, gular region, legs); occasional internal haemorrhages (especially pronephros, liver, spleen).
Anuran adults	Lethargy; anorexia; loss of buoyancy and erratic swimming (aquatic species); swelling (oedema) of the legs, feet, body, and internal soft tissues; skin ulcers; dermal, oral and internal haemorrhages (ecchymotic, petechial); friable (necrotic) organs.
Caudate adults	Lethargy; anorexia; loss of buoyancy and erratic swimming (aquatic species); haemorrhages (especially on tail and plantar surfaces of feet); swelling (oedema); skin ulcers internal haemorrhages (ecchymotic, petechial); friable (necrotic) organs; necrosis of extremities (Chinese Giant Salamanders).
It can also be found rough discoloured areas, spleen and hepatomegaly and intestinal haemorrhage (in mortality events.) The necrosis is associated with apoptosis or virus replication, and according to the extension of this phenomenon, it can be general or discrete pale foci scattered throughout an organ	

Some animals, known as subclinical individuals, are infected with *Ranavirus* without any of the clinical signs mentioned above. Subclinical infections result from the early stage of infection or persistent infected animal, which already had signs of disease (Reviewed by Gray & Chinchar, 2015)

2.3.5 Transmission and Persistence in the Environment

This genus can be transmitted by two general paths: the indirect and the direct transmission. The indirect transmission is characterized by exposure to virions in contaminated waters, or fomites. Infection occurs across the epithelium cells of the skin, gills and intestine. However, contamination through skin is mostly restricted to larvae, since adult amphibian are able to produce antimicrobial peptides which inactivate numerous pathogens, including FV3 and ATV. After exposure, the infection can be noticed in 3 hours (Reviewed by Gray & Chinchar, 2015). Direct transmission includes direct contact, mostly during breeding, skin to skin contact (an adult can contaminate larvae in only one second of contact), and ingestion of infected individuals, e.g.: cannibalism or predation (Reviewed by Gray & Chinchar, 2015).

Ingestion is the most effective way of contamination, and the mortality can be twice as fast as in indirect routes. The several paths of transmission result in exposure to different amounts of virus; larger doses are associated to higher and faster mortality, which can explain the effectiveness of infection by ingestion.

Ranaviruses center their persistence in their capacity to endure in the environment or in subclinical hosts (reservoirs).

The virions resist well in adverse conditions, including freezing and drying circumstances. However they are quickly destroyed in water by native microbes and zooplankton. For instance, the persistence of ranaviruses in the dry soil is 30 to 48 days (with loss of 90% of its infectivity - T-90 value), and the T-90 value in unmanipulated pond water is only one day (Reviewed by Gray & Chinchar, 2015).

2.4 AMPHIBIAN CHYTRID FUNGUS

2.4.1 Taxonomy

Chytridiomycosis is a fungal disease caused by the zoosporic fungus *Batrachochytrium dendrobatidis* (*Bd*). This is one of the two fungi species belonging the Chytridiomycota Phylum that infects vertebrates, and the only one that thrives in keratinized amphibian cells (Fisher et al., 2009).

Chytrids are ubiquitous in aquatic environments, and can even persist in damp soil. Hyphae are not present, and they exploit cellulose, chitin and keratin (Reviewed by Lawrence, 2008). During the life cycle, chytrids undergo a motile stadium, in which the zoospore has a flagellum, enabling the movement in water. They can parasite algae, plants, other fungi, invertebrates and vertebrates, and sometimes they are free-living or commensal fungi (Fisher et al., 2009).

Since *Bd* is one of the two species of Chytridiomycota infecting vertebrates, it's safe to say that this agent has unique structural and genomic characteristics, and for that it was placed in a new genus, *Batrachochytrium* (Rooij et al., 2015).

Kingdom: Fungi
Phylum: Chytridiomycota
Class: Chytridiomycetes
Order: Rhizophydiales
Family: incertae sedis
Genus: Batrachochytrium
Specie*: Batrachochytrium dendrobatidis*

2.4.2 Molecular structure

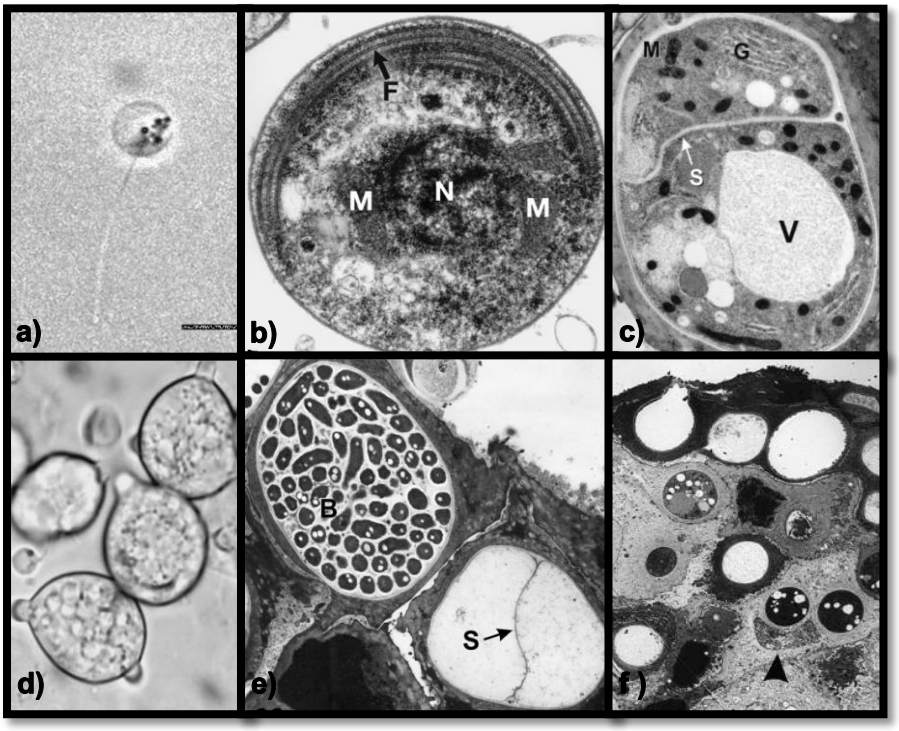
Morehouse et al. (2003), conducted the first molecular epidemiological study of *Bd*, where multilocus sequence typing was used to survey 35 strains from different geographical locations. *Bd* is a diploid organism, since the study has shown 2 alleles per locus, and it has low genetic variation, only 5 variable nucleotide positions were identified within 10 loci (5918 bp). These findings point to a clonal reproduction. When analysing different genes, it was possible to assume that these fungi are highly heterozygous, suggesting that the actual *Bd* resulted from the crossing between two different parental strains.
This genotyping study also projected 14 to 20 chromosomes in *Bd*'s genome, sized between 0.7 to 6.0 million base pairs (Mb), ranging the full genome from 35 to 40 Mb. The lack of genomic diversity was also confirmed by sequencing a >11kb segment of mitochondrial genome without finding any variations (Fisher et al., 2009).

2.4.2.1 Morphology (Berger et al., 2005)

During the mobile life stadium the *Bd* organism is called zoospore; it has a flagellum that offers aquatic mobility but does not have a cellular wall, (Figure 5a). The zoospore shape can go from globular to amoeboid (just after the discharge of the zoosporangium), and its diameter ranges from 3 to 5µm, with a total length of 19 – 20 µm.
The *Bd* zoospore has some characteristics in common with other members of its order, such as the disassociation between the nucleus and kinetosome, the endoplastic reticulum surrounds the ribosomes, which are aggregated. Despite these similarities there are unique features, like the presence of lipid droplets linked with the ribosomatical agglomeration, which make *Bd* distance itself from Rhizophydiales.
The zoospore enters the epidermis and encysts in a skin cell, the flagellum is reabsorbed and a cell wall is formed, yielding the zoosporangium, (Figure 5b). Some rhizoids can develop, being more frequent in culture than in live amphibian skin.
During zoosporangium maturation, multiple mitotic divisions happen to form new zoospores, and one or more discharge papillae develop, which are blocked by a plug, (Figure 5d). Some sporangia are divided by a septum, (Figure 5c) - colonial growth – but usually there's no division – monocentric growth. When the zoospores are entirely developed, already with

flagellums, the plug dissolves and the zoospores are discharged in the environment, leaving the zoosporangium empty, (Figure 5e).

Figure 5 – Six stages of *Bd* development – Cell contents (Berger et al., 2005)



a) Zoospore, note the presence of a flagellum.
b) Presence of a cell wall - (F) reabsorbed flagellum, (N) nucleus, (M) mitochondria
c) Recent colonial zoosporangium, a septum (S) yields 2 compartments. (V-vacuole; G-Golgi complex; M- mitochondria)
d) Discharge papillae in zoosporangia.
e) Empty sporangia. Bacterial (B) invasion through an old discharge tube.
f) Multiple layers of dark infected keratinized cells. Some nuclei of infected cells are degenerate and chromatolytic (arrowhead).

2.4.3 Pathogenesis

Bd is a skin fungus that thrives in the keratinized skin cells of amphibian epidermis, with a highly pathogenic impact, causing a global population decline, being also suspected of some extinctions. This fungus is known for its fast spread, worldwide distribution[3], and high mortality rate. Despite chytridiomycosis impact in the amphibian population, its pathogenesis is not yet clear, since several body systems enter in failure earlier to death (Voyles et al., 2009).

The amphibian skin is more than a physical barrier from the surrounding environment, it's responsible for respiratory gas trades, and the transport of electrolytes and water. This permeability requires the maintenance of the osmotic balance[4], essentially with the help of sodium channels and energy consuming Na^+/ K^- pumps (Voyles et al., 2011).

[3] *Bd* was isolated in every continent except Antarctica.
[4] An Interior (skin) hyperosmotic compared to an exterior (environment) hyposmotic.

Bd has two life stages, one mobile and exterior – flagellated zoospore – and the other inside the amphibian skin cells – zoosporangium. The zoospore reaches the epidermis when the host is in direct contact with infected water or skin to skin with an infected amphibian (OIE, 2012). The life cycle is completed in approximately 5 days, if temperature and nutrients conditions are optimum (Johnson & Speare, 2003).

The fungal entry in the epidermal cell is not clear; one theory suggests that the zoospore encysts on a cell and only then introduces the *Bd* nuclear material, using a germ tube (reviewed by Voyles et al., 2011).

The two hypotheses for the cause of death from *Bd* are: damage to skin function disrupting osmotic balance, or the release of proteolytic enzymes or other compounds absorbed by the amphibians. The first one is the most accepted by the scientific community (OIE, 2012).

Voyles et al. (2009), demonstrated the first hypothesis right, comparing diseased individuals (of *Litoria caerulea*) epidermal electrolyte transport, blood and urine biochemical parameters, and cardiac electrical activity with uninfected ones. The conclusion was that the "*pathophysiology of chytridiomycosis appears to be disruption to the osmoregulatory functioning of the skin* – by sodium channels disturbances – *and consequent osmotic imbalance that leads to cardiac standstill*".

Nevertheless, Voyles et al. (2009) couldn't clarify the biomechicals of the sodium channel problem, and a toxin could be the stressor. In culture, *Bd* secretes extracellular proteases, which degrade casein and gelatin, furthermore molecular studies indicated the expression of serin protease and fungalysin metallopeptidase, responsible for enzymatic penetration of host cells (Voyles et al., 2011).

The chytridiomycosis fungus optimal growth temperature rounds 17-23ºC, at 30ºC the cultures die, and at lower temperatures, 7-10ºC, zoospores take longer to encyst and mature (Voyles et al., 2011). Thus the higher rate of morbidity and mortality in tropical areas are seen during the winter, or in higher altitudes, when the temperature is lower/moderate (OIE, 2012).

Adult amphibian are the preferred targets, however *Bd* can also infect tadpoles, but only within the mouthparts, since everywhere else keratin is absent (Lawrence, 2008).

2.4.4 Clinical signs

Amphibians with chytridiomycosis have multiple skin lesions, existing a positive correlation between the quantity of zoosporangia and the magnitude of the clinical signs, and eventual death (Voyles et al., 2011). Chytridiomycosis is hard to characterize since the infection is restricted to the superficial skin layers, with a minor immune response, and no regular pathological findings while examining the internal organs (Voyles et al., 2009).

Table 3 enumerates some of the most important and prevalent clinical signs in chytridiomycosis.

Table 3 – Signs of *Batrachochytrium dendrobatidis* infection. (Voyles et al., 2011) and (Mader & Divers, 2014).

SIGNS OF *BATRACHOCHYTRIUM DENDROBATIDIS* INFECTION	
MACROSCOPIC	The skin lesions usually appear on the ventral body and feet and include: Epidermal hyperplasia; Hyperkeratosis; Cutaneous erythema; Irregular skin sloughing, Epidermis ulcer, Skin discoloration, Granular changes in skin texture.
BEHAVIOUR	Lethargy; Inappetence; Hind legs abducted; Loss of righting reflex.
MICROSCOPIC	In scattered cells in deeper epidermis layers: Cytoplasm degeneration and vacuolization.

2.4.5 Environment persistence and treatment

Bd doesn't need a host to complete its life cycle; it was successfully cultured in laboratory with absence of keratin, it also prevailed up to 3 months in moist sterile sand, and it could mature in dead algae, arthropod exoskeletons and on sterile feathers. *Bd* was found thriving in geese toe scales, being the zoospores able to survive a desiccation period of 30 minutes, this proves that these fungi can persist as saprobes (Garmyn et al., 2012).

It was reported that *B. dendrobatidis* can be carried by terrestrial reptiles, waterfowl and nematodes, but it was not proven the transmission or the growth of the pathogen in these reservoirs (McMahon et al., 2013).

A study by McMahon et al. (2013), showed that crayfish may be a host for *Bd* during the low prevalence season in amphibian in some areas of North America.

Asymptomatic amphibians are frequent since *Bd* was proved to be well adapted to its hosts, and there are some species that can co-exist with the fungus without clinical signs of disease (Berger et al., 2005).

Aside the bird reservoirs, because *Bd* can't resist desiccation, and the crayfish, the blame of global dispersion falls mostly on imported amphibians for human profit (food, scientific research, pets), becoming the quarantine and the disinfection strategies (Appendix 1) the most important course of action to control *Bd* prevelance (Johnson & Speare, 2003).

Bd is susceptible to some antifungal agents and high temperatures (>30ºC). The use of voriconazole is acknowledged as safe and effective, spraying the diseased animals once a day for a week. The heat baths worked in two amphibian species, and this technique should be optimized for the different hosts (OIE, 2012). More treatments are presented in Table 4.

Infected amphibians may also profit from fluid and electrolytic therapy, since chytridiomycosis affects the skin osmoregulation system. Ringer's solution for amphibians can be added to the environment or in the drinking water, if the host is light to moderately infected. However, animals seriously diseased should be treated via intracoelomic administration. Furthermore, antibiotic may also be an asset in a successful treatment, covering the secondary infections (Mader & Divers, 2014).

Table 4 – Different substances used in the *Bd* treatment (Mader & Divers, 2014).

***Bd* TREATMENTS**	
Antifungals in the Azole Family	Inhabitation of ergosterole (component of fungus cell membranes)
Itraconazole	
Miconazole	Safe and effectiveness in tadpoles wasn't studied
Fluconazole	Reduces prevalence but doesn't eliminate the agent
Voriconazole	
Malachite green and Formalin	
The two compounds are known as teratogenic/carcinogenic substances	
Benzalkonium chloride	
Fungistatic quaternary ammonium disinfectant	
Chloramphenicol	
Antibiotic	
Terbinafine	
Antifungal	
Elevated environmental temperature	
Constant rather than intermittent temperature elevations	
There's still no standard protocol to eliminate *Bd*, thus after the chosen treatment it's essential to test the animals using qPCR.	

2.5 CHLAMYDIACEAE

2.5.1 Taxonomy

The species belonging to the Chlamydiales order are gram negative obligatory intracellular bacteria, infecting a wide range of vertebrate hosts, from herpetofauna[5] to humans (Everett et al., 1999). The families belonging to this order are Chlamydiaceae, Parachlamydiaceae, Simkaniaceae, and Waddliaceae. This order is characterized by its high genomic diversity, and most likely more families are to be included (Everett et al., 1999).

The first family to be identified was Chlamydiaceae in 1957, including nine species: *Chlamydia pneumoniae*, *Chlamydia pecorum*, *Chlamydia felis*, *Chlamydia caviae*, *Chlamydia abortus*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Chlamydia muridarum*, and *Chlamydia suis* (Mitchell et al., 2010). However, a new taxonomy was proposed, due to antigenic and genetic diversity, by Everett et al. (1999), and these species were divided in two genera *Chlamydia*, that includes the last 3 species mentioned above, and *Chlamydophila*.

In herpetofauna Chlamydia-like organisms may be the cause of some deadly diseases, with high mortality rates. In amphibians 6 species were identified: *Chlamydia psittaci*, *C. pneumoniae*, *C. abortus*, *C. suis* and species belonging to a new genus, *Amphibiichlamydia,*

[5] Amphibians and reptiles.

the *Candidatus Amphibiichlamydia salamandrae* and the *Candidatus Amphibiichlamydia ranarum.* This new genus most probably includes chlamydial species adapted to amphibians, which may represent new emerging diseases (Martel et al., 2012).

Molecular assessments showed that disparities within the Major Outer Membrane Protein (MOMP) gene allowed the distinction of *Cph. pneumoniae* in 4 genotypes (A to D). Genotype A is human specific, nonetheless it was found in Blue mountain tree frogs. Genotype B is restricted to horses. Genotype C is identified in Koalas, and was also found in Great barred frogs. Genotype D was recognized in two frog species.

Kingdom: Bacteria

Phylum: Chlamydiae

Order: Chlamydiales

Family: Chlamydiaceae

Genera: Chlamydophila and Chlamydia

2.5.2 Molecular Structure

The life cycle of Chlamydiaceae is composed of two cell types, the infectious “elementary bodies” (EB) and the multiplying “reticulate bodies” (RB). The first cell enters the eukaryotic cell, and differentiates to RB, which divide by binary fission and eventually become EB again; when it does EB leave the infected cell to find a new, uninfected one (Corsaro & Venditti, 2004). The cell invasion mechanism can vary according the strain of Chlamydiaceae in question. Nevertheless, there are 2 features that are constant: the invasion usually targets nonprofessional phagocytes, and after entrance the chlamydial cell acquires a vacuolar appearance, residing in the eukaryotic cell as an cytoplasmic inclusion (Moulder, 1991).

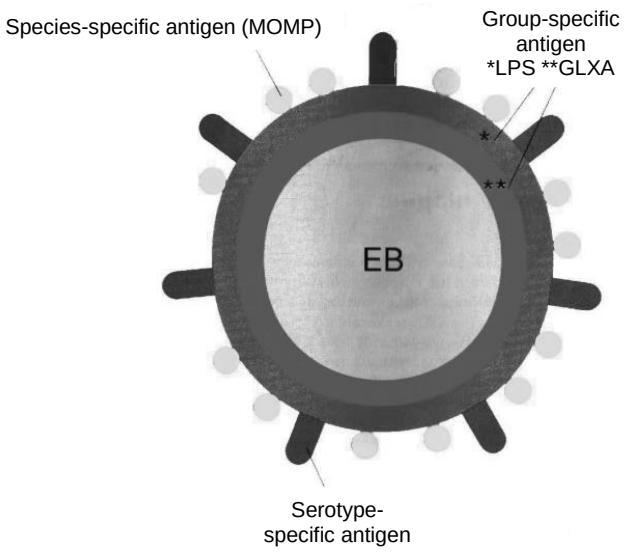
The entry ability is associated to the EB wall structure. EBs have 0.2-0.4µm in diameter and can’t replicate, being only infectious (Zdrodowska-Stefanow et al., 2003). EB wall is similar to gram-negative cells wall, but without peptidoglycan. To confer rigidity to the wall, chlamydia-like organisms have a disulphide bond system between the outer membrane proteins (Moulder, 1991).

Chlamydia trachomatis, is one of the most studied Chlamydiaceae, probably because of its impact in humans, since this species is responsible for one of the sexual transmitted diseases with higher pathogenic impact (Corsaro & Venditti, 2004). The EB has 4 antigen groups, one which is common to all Chlamydia species, the second is species-specific, the third is respective to *C. trachomatis* serotypes, and the last one refers to subspecies-specific antigens (Zdrodowska-Stefanow et al., 2003), Table 5 and Figure 6.

Table 5 - Antigenic Structure of *C.trachomatis* (Zdrodowska-Stefanow et al., 2003)

ANTIGENIC STRUCTURE OF *C.TRACHOMATIS*	
ANTIGEN GROUPS	CHARACTERISTICS
Group-specific	Thermostable polysaccharide complex – lipopolysaccharide (LPS) + glycolipid (GLXA))
Species-specific	Protein structure and is thermolabile MOMP – 38-42 kDa (60% all outer membrane proteins) Chlamydial heat shock proteins (HSP – 10 and 60 kDa)
Serotype-specific	Thermolabile polypeptides of 30 kDa
Subspecies-specific	Polypeptide structure Depending on the structure the serotype is categorized subspecies group B or C

Figure 6 - Antigenic structure of *C.trachomatis* (Zdrodowska-Stefanow et al., 2003)



2.5.3 Pathogenesis

Chlamydial diseases can be acute or chronicle. The most worrisome form is the chronic, responsible for the characteristic clinical symptoms of the disease.

An infection by Chlamydiaceae has an inflammatory process; the affected area shows a profuse infiltrate of neutrophils and lymphocytes. The infiltrate evolves and macrophages and infection plasma cells take on. At this stage, lymph nodes centre is populated with macrophages and B cells, and peripherally with T cells.

If this severe inflammatory reaction is maintained, the host cells can suffer necrosis, activating epithelial proliferation yielding the formation of scar tissue. For instance, this scarring process is responsible for the corneal impairment and probable blindness, or infertility secondary to *C. trachomatis* infection.

Hereupon, the maintenance of a chronic inflammatory phenomenon is essential to the pathogenesis of chlamydia-like organisms, since the scarring can take years of injury to develop (Stephens, 2003).

During chlamydia life cycle (Figure 7) the RB can stay arrested, without evolving to EB for years – Cryptic form (Corsaro & Venditti, 2004).

There are two hypothesis to explain chlamydial pathogenesis, the "immunological pathogenesis paradigm" and the "cellular pathogenesis paradigm" (Stephens, 2003).

The first one is based on the premise that a persistent or a reinfection triggers an increased inflammatory response, culminating in tissue injury and scarring – chronical pathological host response. There are two features to this hypothesis, a delayed-type hypersensitivity or an autoimmune response through molecular mimicry. In both features, the trigger for the exacerbated inflammatory response is an antigen, which already has been in contact with the host (Stephens, 2003).

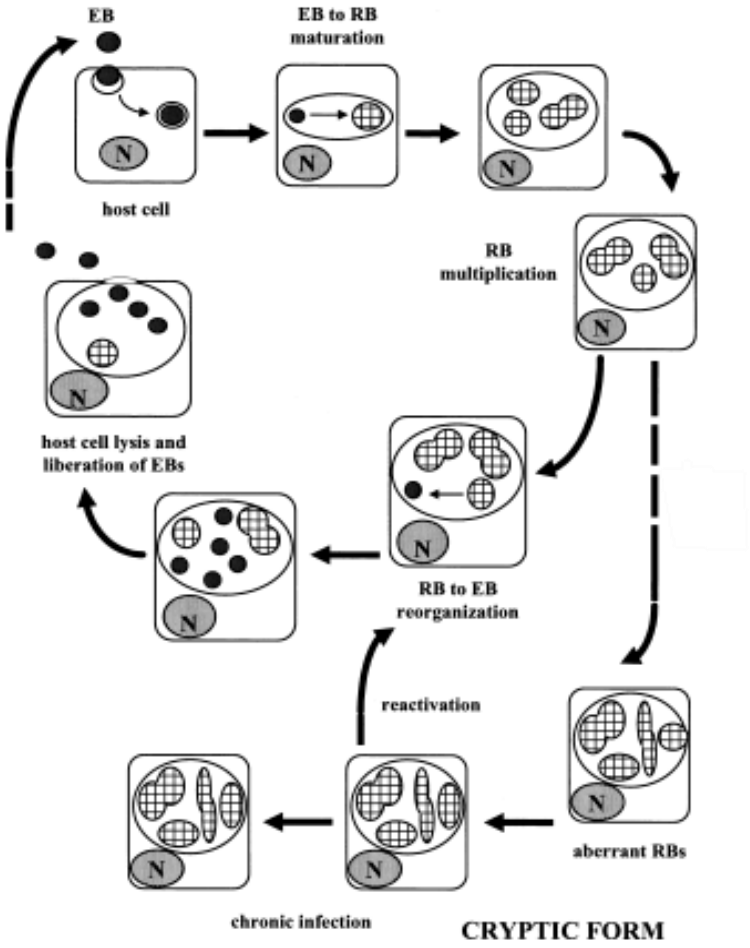
The delayed-type hypersensitivity reaction is sustained by the antigen, if the stimulus disappears the reaction ceases. Hosts with pronounced scar response had higher anti-chlamydial antibody totals and low cell-mediated proliferative responses, clarifying that the Th1-type responses are beneficial to terming the disease, but Th2-type response culminates in scarring (Stephens, 2003).

On the other hand, the autoimmune response doesn't need chlamydia to unfold, the antigen epitopes, identified by antibody or T cells during first contact, are responsible for this response. These antigen epitopes cross-react with certain host epitopes causing the chronic pathological host response. In humans HSP60 cross-reacted with chlamydial HSP60, however, the stimulation of autoreactive antibodies or T cells wasn't proved (Stephens, 2003).

The cellular pathogenesis paradigm is sustained by the secretion of large amount of proinflamatory chemokines after infection; the reinfection intense inflammatory response is caused by adding of the Chlamydia-specific immune cells to the initial proinflamatory chemokines (Stephens, 2003).

This kind of inflammatory response can cause tissue remodeling and scarring, since infected host cells produce IL-11, known for inducing tissue fibrosis. Over and more, during the inflammatory response, growth factors are produced, which lead to tissue remodeling and scarring (Stephens, 2003).

Figure 7 - Life cycle of chlamydia (Corsaro & Venditti, 2004)



The elementary body (EB) small extracellular infectious cell, attaches to and enters the eukaryotic cell.
Maturation, occurs in a few hours, to a vegetative form, the Reticulate Body (RB).
RB multiplies (binary fission) forming an intravacuolar microcolony – inclusion.
RBs reorganize into EBs, which leave the host cell, initiating a new infection.
The cycle completes in 36 - 96 hours.
The microcolony may enter in a cryptic form. This form maintain persistent infections.
Reactivation is possible.

2.5.4 Clinical signs

The members of the Chlamydiales order can affect different systems. For instance, respiratory infections can be caused by: *Chlamydophila pneumoniae* in a wide diversity of animal species, *Cph. psittaci* in birds and humans (zoonotic), in cats (bird and humans are also affected due to its genetic similarities to *Cph. psittaci)*, *C. muridarum* in mouses, *C. suis* in swine, *C. trachomatis* in new-born humans, *Cph. pecorum* in ruminants and humans (zoonotic), *Parachlamydia acanthamoebae* in *Acanthamoeba* spp. isolated from human nasal mucosa, and *Simkania negevensis* in humans. The symptoms range from nasal and ocular discharge to bronchitis and pneumonia, depending on the chlamydial specie or the host (Corsaro & Venditti, 2004).

Chlamydial-like organisms can also be the cause for ocular infections from conjunctivitis, keratoconjunctivitis to blindess; urogenital infections and abortion, cardiovascular diseases (arteriosclerosis) and central nervous system diseases (CNS) (Table 6).

In amphibians, chlamydial infection was documented in 1999 by Berger et al. (1999), in free-ranging giant barred frogs (*Mixophyes iteratus*) from Australia, which presented petechiation and sloughing of skin, abdominal swelling due to hydrocoelom, accumulation of excess fluid in lymphatic sacs, lethargy, and cutaneous depigmentation. Table 7 shows with detail the effects of *Cph. pneumoniae* infection in different amphibians.

The new species of genus *Amphibiichlamydia* were identified in some species of salamanders and in *Lithobates catesbeianus* (North American bullfrogs), although not successfully cultured. Despite the absence of clinical disease in the bullfrogs ('*Candidatus Amphibiichlamydia ranarum'*), *Candidatus Amphibiichlamydia salamandrae*'s hosts presented anorexia, lethargy, oedema, and marked abnormal gait (Martel et al., 2012).

Table 6 – Chlamydial infections (adapted from Corsaro & Venditti, 2004)

TAXON	HUMAN INFECTION	ANIMAL INFECTION	TRANSMISSION
C. trachomatis	Ocular, urogenital, joint respiratory (neonate)		Eyefly, direct contact (DC) sexually transmitted
C. muridarum A	Respiratory	Rodents: respiratory	Aerosol
C. suis		Swine: respiratory, ocular	Feco-oral
Cph. pneumoniae B		Marsupials: ocular, urogenital Horse: respiratory Herpetofauna: systemic	Aerosol, direct contact
Cph. pecorum	Respiratory?	Ruminants: respiratory, ocular, urogenital, joint, CNS Swine: respiratory, urogenital Marsupials: ocular	Aerosol, feco-oral
Cph. felis	Ocular, respiratory?	Cat: ocular, respiratory	Aerosol, DC
Cph. caviae C	Ocular?	Guinea pig: ocular	DC
Cph. abortus D	Respiratory, abortion	Ruminants and Swine: respiratory, urogenital	Aerosol, DC, feco-oral
Cph. psittaci E	Respiratory	Birds: systemic Mammals: mainly respiratory	Aerosol, DC

A – Model for urogenital infection;
B – Associated to chronicle diseases like atherosclerosis and Alzheimer's disease; Able to multiply within *Acanthamoeba;*
C – Model for ocular infection;
D – Important cause of zoonotic abortion;
E – Highly infectious.

Table 7 - *Chlamydophila pneumoniae* in amphibians (adapted from Corsaro & Venditti, 2004).

SPECIES	GEOGRAPHY	DISEASE
Blue mountains tree frog (*Litoria citropa)*	Australia	Chronic nephritis
Great barred frog (M*ixophyes iteratus)*	Australia (free-ranging)	Severe pneumonia, anaemia, pancytopenia
Cryptohylax greshoffi	Africa (imported)	Acute hepatitis, haemorrhagic pneumonia
African clawed frogs (*Xenopus tropicalis*)	West Africa (imported)	Lethargy, active hepatitis

2.5.5 Environment persistence and treatment

Presence of Chlamydia was reported in protists, arthropods and in various environmental habitats (reviewed by Corsaro & Venditti, 2004).

Acanthamoeba is an example of a free-living amoebae, which is a natural host for parachlamydia, probably being reservoir for other families in the Chlamydiales order (experimentally can host for *Cph. pneumoniae*). Moreover, the relationship between amoebas and chlamydia, when infecting vertebrates, sometimes appears symbiotic. Laboratory findings showed an amplified cytopathogenicity in acanthamoebae infected with parachlamydia reviewed by Corsaro & Venditti, 2004.

New species of chlamydia were found to infect arthropods, but it's unclear if this new species can infect vertebrates. Nonetheless, *Musca sorbens* is a known vector for *C. trachomatis*, as is *Musca domestica*. Experimentally, *M. domestica*, could infect guinea pigs with viable *Cph. caviae* (reviewed by Corsaro & Venditti, 2004).

In addition, hematophagous arthropods can also act as vectors. For instance, a mass mortality event in turkeys, by *C. psittaci*, began with a plague of black flies *Simulium* spp.. A laboratory study proved that *Ornithodoros coriaceus*, with chlamydia, can act as a vector up to 48 days after contacting in the agent (reviewed by Corsaro & Venditti, 2004).

To treat frogs with chlamydiosis, a broad-spectrum antibiotic has to be used, usually doxycycline or oxytetracycline (Mader & Divers, 2014).

2.6 HERPESVIRUS

2.6.1 Taxonomy

According to the ICTV the Herpesvirales order has 3 families: Alloherpesviridae, Herpesviridae, and Malacoherpesviridae. The Alloherpesviridae infects fishes and amphibians and is divided in 4 genus: *Batrachovirus*, *Cyprinivirus*, *Ictalurivirus* and *Salmonivirus*; the Malacoherpesviridae includes the *Aurinivirus* and *Ostreavirus* genus, and infects bivalves. The Herpesviridae family infects mammals, birds and reptiles, and is subdivided in 3 subfamilies - *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gamaherpesvirinae*. (Table 8 for more details on Herpesviridae).

The *Herpesvirus* family has a wide range of hosts, as it can infect mammals, birds, herpetofauna, insects and invertebrates. Additionally, some *Herpesvirus* can infect different species, and there are more than 200 recognized *Herpesvirus* species (Pellett & Roizman, 2013).

Two species of frog *Herpesvirus* are recognized, ranid herpesvirus-1 (RaHV-1), also known as Lucké tumour herpesvirus, and ranid herpesvirus-2 (RaHV-2) or frog virus 4 (FV4), being the first one assigned as type species by the ICTV. RaHV-1 was identified by Lucké in 1934, as intranuclear acidophilic inclusion bodies, alike the ones characteristic from herpes simplex infections, in renal adenocarcinoma cells from *Rana pipiens*. The virus was quickly associated to the origin of the adenocarcinoma (Densmore & Green, 2007).

Table 8 – Herpesvirales order, classification by ICTV.

HERPESVIRALES		
Family	**Subfamily**	**Genus**
Alloherpesviridae		*Batrachovirus* *Cyprinivirus* *Ictalurivirus* *Salmonivirus*
Malacoherpesviridae		*Aurinivirus* *Ostreavirus*
Herpesviridae	*Alphaherpesvirinae*	*Iltovirus* *Mardivius* *Scutavirus* *Simplexvirus* *Varicellovirus*
	Betaherpesvirinae	*Cytomegalovirus* *Muromegalovirus* *Proboscivirus* *Roseolovirus*
	Gamaherpesvirinae	*Lymphocryptovirus* *Macavirus* *Percavirus* *Rhadinovirus*

2.6.2 Molecular Structure

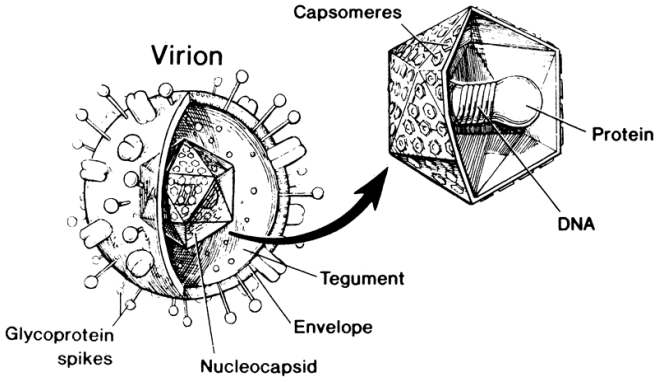
Herpesviruses (Figure 8) have a linear DNA double strand genome, with approximately 124-295 Kb, involved by a T=16 icosahedral capsid, ≈125 nm in diameter, composed by 161 capsomeres. The core and the capsid altogether are named “nucleocapsid” (Kaplan, 1973).

The capsid is composed by 4 main structural elements, the major capsid protein (MCP), the monomer and dimer proteins of the triplex and the small capsomere-interacting protein (Pellett & Roizman, 2013).

The tegument, a proteinaceous structure, is often surrounding the nucleocapsid, and can exist in different quantities depending on the virion. The proteins that comprise it adjust the cellular environment, even without transcription or translation, blocking the host protein synthesis, lowering cellular defences culminating in empowered viral expression (Pellett & Roizman, 2013).

The virion structure includes an envelope, consisting of a lipid structure, including viral glycoproteins and cellular proteins, that can vary in number and in function (Davison & Preston, 2008).

Figure 8 - Molecular structure of *Herpesvirus*
(Source: http://www.oculist.net/downaton502/prof/ebook/duanes).



Amphibian *Herpesvirus*, RaHV-1 and 2, have a large genome (Table 9), with families of genes, and genes captured from cells or other viruses. These frog *Herpesviruses*' genome is methylated due to an encoded DNA cytosine-5-methyltransferase. The two RaHV share 40 genes (Davison & Preston, 2008).

The *Alloherpesviridae* members have low rate of conserved genes, meaning that they are not as closely related as the *Herpesvirus* infecting higher vertebrates. These genes encode proteins responsible for *Herpesvirus* replication (capsid morphogenesis, nucleotide metabolism, DNA replication and DNA packing) (Davison & Preston, 2008).

RaHV-1 and 2 homologous genes have the same organization, apart from a group of genes with different genomic positioning and orientation (Davison & Preston, 2008).

Table 9 – Characteristics of amphibian *Herpesvirus* genomes (adapted from Davison & Preston, 2008)

	GENOME SIZE (BP)	GENES	GENES FAMILIES
RaHV-1	220 859 [636]	132	15
RaHV-2	231 801 [912]	147	15

2.6.3 Pathogenesis

The herpesviral infections are mostly benign and asymptomatic in immunocompetent animals. However, in immunocompromised, young, old or when species barrier is crossed, these viruses can have a high mortality rate (Pellett & Roizman, 2013).

The replication cycle has not been studied in all *Herpesvirus*, therefore the cycle of the type species *Herpes simplex* (HSV) virus is the model for *Herpesvirus* infections (Davison & Preston, 2008). Despite some lack of information regarding the replication cycle, there are known standards to this order: all viruses have a high number of replication enzymes; Replication, transcription and nucleocapsid assembly occurs in the cell nuclei, and the tegument is acquired in the cytoplasm; lytic replication culminates in cell death; all herpesviruses have a latency mechanism; the infection is characterized by the presence of intranuclear acidophilic inclusion bodies (Pellett & Roizman, 2013).

The replication cycle can be compartmentalized in three stages, the initiation of infection, the lytic replication and the latency (Pellett & Roizman, 2013). To initiate the infection the virus envelope has to merge with the cell membrane, enabling the nucleocapsid-tegument complex entrance in the cell. The viral DNA enters the nuclei through a channel formed by one capsomeric structure, and as soon as the linear double strain DNA exits the capsid it circularizes into the host nuclei (Davison & Preston, 2008).

The viral genome is transcribed into mRNA by the RNA polymerase II of the cell. First the immediate early genes (independent of protein synthesis) are transcribed and regulate the expression of early and late genes. The early genes encode structural proteins and DNA replication proteins.

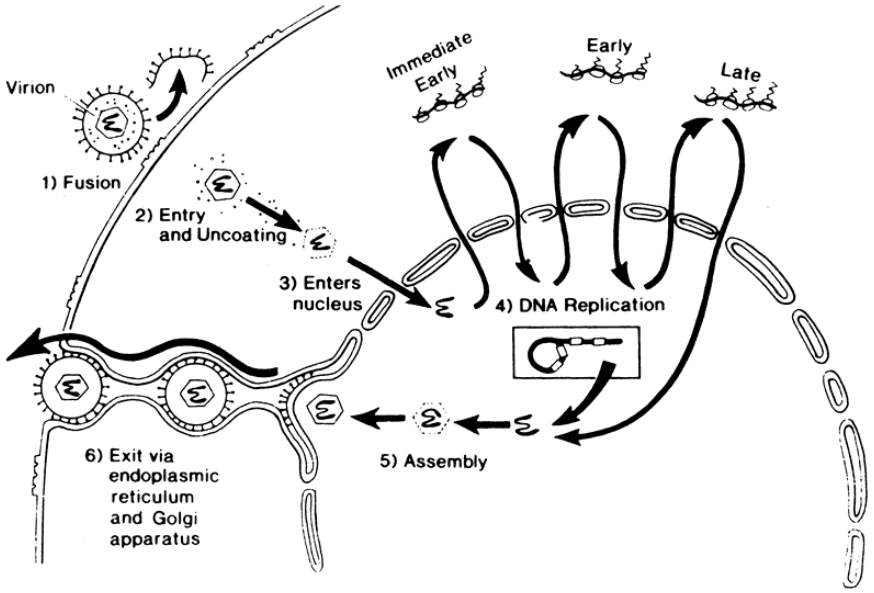
During the lytic replication, the structural and genomic compounds of the virion are mass-produced. The nucleocapsid assembles in the nuclei, and some proteins attach to it forming an initial tegument. The nucleocapsid goes to the cytoplasm by budding through the nuclear membrane, obtaining a transitory envelope. In the cytoplasm more proteins attach to the tegument, and the nucleocapsid-tegument complex acquire the envelope, by budding into a Golgi compartment (Davison & Preston, 2008).

As for the amphibian *Herpesvirus*, RaHV-1 occurs naturally in *Rana pipiens* (Davison & Preston, 2008). However, researchers haven't managed to reproduce the infection in vitro through tumour or urine samples, making it difficult to study the replication cycle. During one of these attempts RaHV-2 was isolated from urine. This virus has a cytopathic effect, but no tumour effect, in embryo cells and in *Rana pipiens* adult renal cells at 25ºC after 10 to 21 days from exposure (Davison & Preston, 2008).

RaHV-1 is temperature dependent, which means it has a seasonal action. At 11.5ºC or less intranuclear inclusions are found in the tumour cells, however, at higher temperatures the cells

are clear. The mechanism for this phenomenon is not yet described, but studies showed that the hosts' immune system is not responsible for it (Davison & Preston, 2008).

Figure 9- Herpes simplex replication cycle (source: http://www.oculist.net/downaton502/prof/ebook/duanes)



2.6.4 Clinical signs

Herpesviruses have a wide range of hosts, and within every host the affected system may change, as does the clinical signs. Taking *Herpes simplex* species for example, HSV-1 and HSV-2 infect humans in all ages and the clinical signs may vary form epithelial lesions to CNS disease (Davison & Preston, 2008).

In childhood the HSV-1 infections are usually silent, however disease may occur, normally characterized for gingivostomatitis (gums, tongue, mouth, lip, facial area and pharynx infection). In adults, on the other hand, the infection targets the upper respiratory tract (pharyngitis or/and a mononucleosis-like syndrome). After latency, the reactivated HSV-1 cause mucosal ulceration or lip vesicles (in mucocutaneous junctions) that endure for 4 days to a week. Other skin lesions can occur, but HSV-1 can also be responsible for ocular infections (stromal keratitis, chorioretinitis) (Davison & Preston, 2008).

HSV-2 infects the genitalia, and in women the vesicles in the labia and the vagina can evolve to ulcers, over and more, the infection can develop systemic symptoms like fever, headache, photophobia, malaise, and generalized myalgia. In males the infection isn't so severe, the vesicles can appear in the shaft of the penis, the prepuce and the glans penis, possible accompanied by urethritis and dysuria (Davison & Preston, 2008).

Hereupon, the same species of virus can infect a wide age range and can induce diverse lesions; taking into account the amount of species in this order, the enumeration of all clinical signs would become too extensive.

RaHV-1 is a tumour producing type of virus, the affected *Rana pipiens* have renal adenocarcinoma and ascites. The ascites fluid, if inoculated in embryos, infects the cells and causes tumours. The cytopathic effect of RaHV-2 infection includes cells rounding, vacuolization, enlargement of nuclei and syncytia[6] (Davison & Preston, 2008).

2.6.5 Environment persistence

Although the distinct adaptation to its host enunciated above, it is the herpesviruses latency mechanism the mainly cause for the permanence of the virus in the populations.

After epithelial cells of skin are infected, the virus can infect sensory or autonomic nerve endings, enabling the invasion of ganglia (trigeminal in HSV-1 and sacral in HSV-2) – retrograde transport. Here the viral DNA is maintained as an episome and it's not transcript. When reactivation occurs there is reverse axonal transport of virus to near the entry site – anterograde transport. The reactivation can be unleashed by fever, axonal injury, sunlight exposure, stress, hormonal irregularities (Davison & Preston, 2008).

Latency, adaptability to the host, wide dispersion of host species, and efficient transmission, are essential to the virus permanence in populations. Furthermore, a review by Kramer et al., (2006), enunciates that *Herpesvirus* (HSV-1 and 2) can persist on dry inanimate surfaces for 4.5 hours to 8 weeks, and that low humidity and temperature rates are consistent with longer persistence.

2.7 *SALMONELLA*

2.7.1 Taxonomy

Salmonella are Gram-negative, flagellated, facultative anaerobic bacilli, belonging to the family Enterobacteriacea. This species has three major antigens: H or flagellar antigen; O somatic lipopolysaccharide (LPS) antigen; and Vi antigen (present in only a few serovars – e.g.: *S. thyphi*) (Giannella, 1996).

The genus *Salmonella* has only two species (Table 10), *S. enterica* and *S. bongori*, however more than 2,600 serovars are included in this genus. These serovars are characterized by the expression of O antigens and H antigens (Cooke et al., 2007). In 2005, *Salmonella enterica* was approved as the type species of the genus *Salmonella*. In addition, *Salmonella bongori* was recognized in the genus (Agbaje et al., 2011).

Roughly, *Salmonella* serovars are distributed in two groups, the ones mainly infecting humans, causing enteric fever (*S.* Typhi and *S.* Paratyphi A, B and C), and the rest is known by "nontyphoidal *Salmonella*" (NTS), usually triggering a milder gastroenteritis, frequently self-limiting (Cooke et al., 2007).

[6] Multinucleated cytoplasm mass, without cell compartmentalization.

Salmonella serovars have a widespread host range, infecting warm and cold-blooded animals (Agbaje et al., 2011). Amphibians are known carriers of these bacteria, being first isolated in 15 of 27 cane toads in 1958 (reviewed by Chambers & Hulse, 2006). The most two common serovares infecting amphibians are *Salmonella enterica* Typhimurium and Enteritidis (Chambers & Hulse, 2006).

Table 10 – *Salmonella* nomenclature (adapted from Agbaje et al., 2011)

Genus	Species	Subspecies	Serovar (example)
Salmonella	*enterica*	I – *enterica*	Choleraesuis, Enteritidis, Paratyphi, Thyphi, Thyphimurium
		II - *salamae*	
		III_a - *arizonae*	
		III_b - *diarizonae*	
		IV - *houtenae*	
		VI - *indica*	
Salmonella	*bongori*	V	

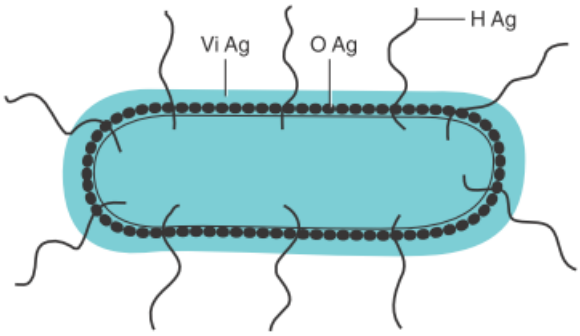
2.7.2 Molecular structure

Salmonellae are generally motile with peritrichous flagella, aerogenic, non-lactose fermenting, oxidase-negative, urease-negative, citrate-utilizing, acetylmethylcarbinol-negative and potassium cyanide-negative (reviewed Agbaje et al., 2011).

As mentioned above, these bacteria have three main antigens, H, O and Vi (Figure 10). The flagellar antigen, H, it's a heat labile protein extremely immunogenic, inducing a strong humoral response. Somatic antigen, O, integrates the cellular outer membrane, containing long chain polysaccharides, and it's heat stable. Vi is also a superficial heat labile antigen, covering the O antigen; it prevent phagocytosis and the activation of the complement (Walker et al., 2014). The antigenic structure analysis (in particular O and H antigens), by serotyping, helps categorizing the serogroups. There are different kinds of O antigens (designed by Arabic numeration), and more than one type can be present in the same bacterial strain. In addition, the H antigen can be present during phase 1 and phase 2[7]. During phase 1 flagellar antigens exists only in some serotypes, determining its immunologic profile. Phase 2 H antigens are more common (Walker et al., 2014). The LPS present in the cellular wall can act as an endotoxin, and affects virulence has does antigen Vi (Giannella, 1996).

[7] Bacteria can alternate between two phenotypes, as a method for dealing with rapidly varying environments. It's a reversible switch with variation in the level of expression of some proteins (Woude & Bäumler, 2004).

Figure 10 – Structural antigens in *Salmonellae*. (Walker et al., 2014)



2.7.3 Pathogenesis

Salmonella's pathogenic strains are considered a life-threatening bacterium, being one of the first causes of food-borne bacterial illnesses in humans. Primarily these bacteria are spread by the host faeces, and can contaminate water and food (meat, eggs, and vegetables). Generally people infected with *Salmonella* suffer from self-limiting gastroenteritis, but sometimes the infection becomes invasive (usually in the elderly, youngsters and immunocompromised individuals) and antibiotic therapy is required (Mahmoud, 2012).

The symptoms can range from mild gastroenteritis to septicaemia. The fever usually drops within 48 to 72 hours, and the diarrhoea within 3 to 7 days, the most common complications are severe dehydration, shock, collapse, and/or septicaemia (Mahmoud, 2012).

Reptiles and amphibians act as reservoirs for this agent being asymptomatic, but usually the serotypes associated with these animals are infectious to humans, and *Salmonella* outbreaks can begin with direct or indirect contact with herpetofauna (Mermin et al., 2004).

Reptile associated *Salmonella* is frequently invasive disease, usually in infants, culminating in hospitalization. Fortunately, less than 1% of *Salmonella* infections are caused by contact with these animals (Mermin et al., 2004).

Mermin et al. (2004), surveyed *Salmonella* infections, in the USA between 1996-1997, concluding that contact with herpetofauna was related to infection (serogroups B, D and non B or D), with an estimationof 74,000 infections per year. Additionally, the U.S. Centers for Disease Control and Prevention (CDC) reported 241 cases of human salmonellosis caused by *Salmonella* Typhimurium in 42 states by contact with African dwarf frogs in 2010-2011 (Walker et al., 2014).

2.7.4 Clinical signs

Salmonella is a major source of foodborne infection all through the world. Nonetheless, it's a commensal organism in the gastrointestinal tract of humans and animals.

There are two common manifestations of disease, the gastroenteritis and typhoid fever.

Salmonellosis generally manifests 12 to 72 hours after infection and is characterized by acute abdominal pain, diarrhoea, nausea and vomit, fever, meningitis, osteomyelitis, peritonitis and pleurisy (Chambers & Hulse, 2006; WHO, 2013).

Disease can endure for 4 to 7 days, and most people recover without treatment; antibiotics may be used when the bacteria enter the bloodstream and in risk age groups (WHO, 2013).

2.7.5 Environment persistence

Salmonella transmission from amphibians to humans may vary, especially because these bacteria can survive well in the environment. *Salmonella* was isolated in 6 months reptile faeces, and in turtle's aquarium water after 6 weeks. Amphibian terrariums also contain water probably contaminated with *Salmonella* (Pfleger et al., 2003).

However, *Salmonella* is more easily found, when screening faecal samples, in arid to mesic reptile environments than in aquatic amphibian terrariums. Also, the risk of an amphibian to excrete the bacteria was approximately 5 times lower than reptile excretion (Pfleger et al., 2003).

Reptiles and amphibians are asymptomatic hosts and the morbidity rate in reptiles can go as high as 94% (Mermin et al., 2004) probably because they have *Salmonella* as a part of their normal flora (Pfleger et al., 2003). In amphibians the prevalence in asymptomatic animals is usually higher than 10%, and can reach the 60% (ARWH, 2012). Chambers & Hulse (2006), tested 92 amphibians and the prevalence was 39.1%, concluding that in salamanders a lower occurrence was present, since they lowered the rate.

Salmonella is found in the intestinal tract of clinical normal animals, is excreted in faeces intermittently, and the maintenance of the agent is due to coprophagy, ingestion of infected water and eating undercooked meat. The bacteria are generally transmitted to humans through consumption of contaminated food of animal origin, mainly meat, poultry, eggs and milk (Rabsch et al., 2013).

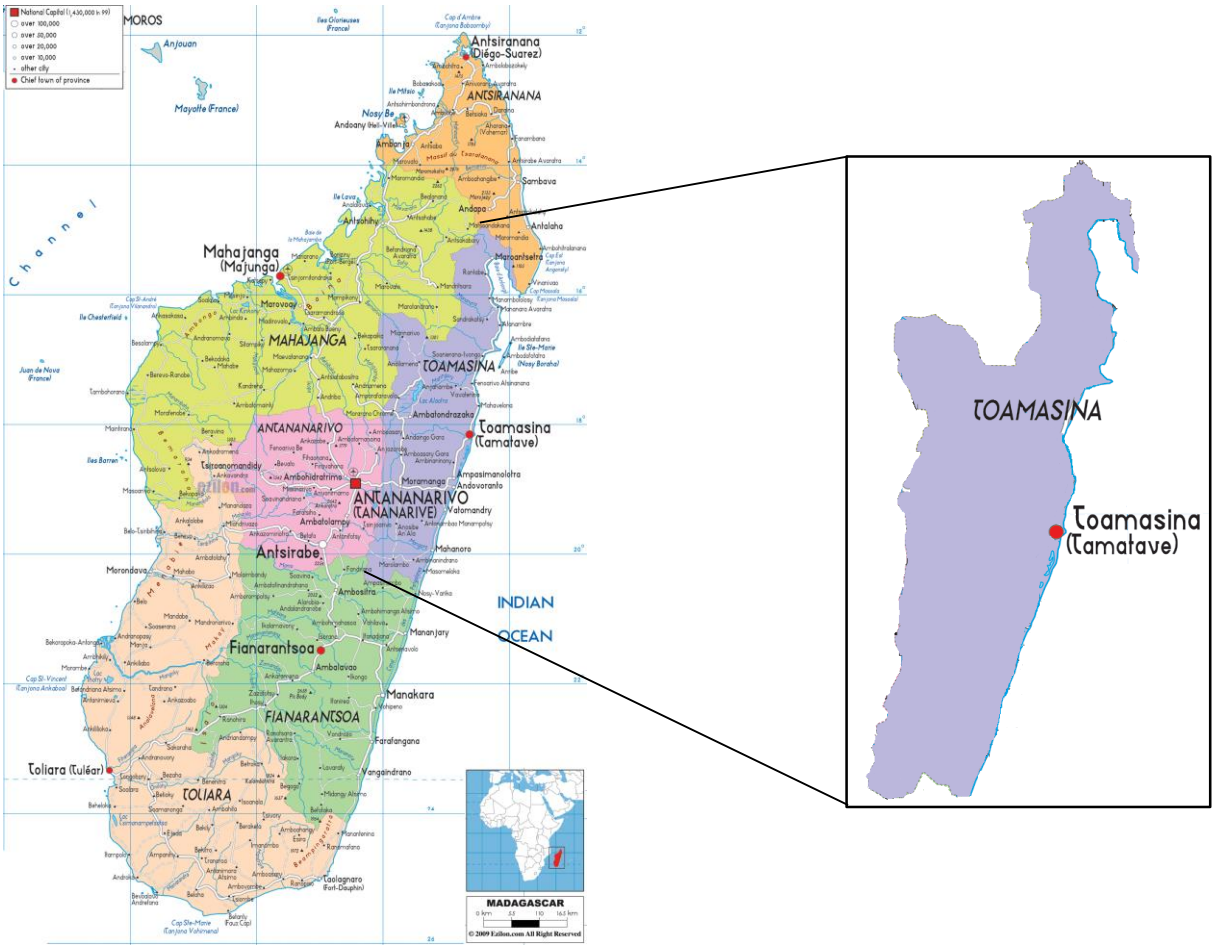
Salmonella serovars prevalence differ in geographic regions and in time, meaning that the same serovar can be predominant in a country and has low prevalence in another country. New *Salmonella* serovars are easily introduced in a different location by food and animals importation (Agbaje et al., 2011).

3 MATERIALS AND METHODS

3.1 POPULATION AND COLLECTION OF SAMPLES

The toads were caught in the region of Toamasina, Madagascar (Figure 11), in two time periods: 60 samples in the dry season of 2014, and 50 in the rainy season of 2015. In total 110 toads were caught and euthanized for sample collection of liver, skin and skin swabs, all preserved in ethanol 98% at room temperature.

Figure 11 - Madagascar's political map. See Toamasina region in purple. (Source: http://www.ezilon.com/maps/africa/madagascar-maps.html)



3.2 SAMPLE PROCESSING

Before DNA extraction, 1 cm^3 of each sample was hydrated in 4 successive baths, of fifteen minutes, at room temperature, with increasing water concentration, 15% per bath (Table 11).

Table 11 - Hydration protocol.

	[EtOH]%	[H_2O]%	
Room Temperature 15 minutes	75	25	375µl EtOH + 125µl H_2O
	50	50	250µl EtOH + 250µl H_2O
	25	75	125µl EtOH + 375µl H_2O
	-	100	500µl H_2O

3.3 NUCLEIC ACID EXTRACTION

The nucleic acid extraction from the hydrated samples, was performed with the commercial DNeasy® Blood & Tissue Kit (Qiagen, Germany), following the manufacturer protocol for total DNA purification. Briefly, 20-30 mg of each hydrated sample was placed in a 2 mL microtube, with 200µl of lysis buffer (ATL), and a stainless steel bead; the tissue was physically disrupted in the TissueLyzer (Qiagen), during 20 seconds at 15 hertz. To complete the cellular digestion, 20µl of proteinase K were added to the mixture and incubated at 56ºC during 3-12h hours, with occasional vortexing to disperse the sample, until complete digestion; 200µl of AL buffer and 200µl of EtOH 100% were added into the lysate, to achieve nucleic acids precipitation. The mixture was loaded onto the silica-based column, which provides selective retention of the dehydrated nucleic acid, after centrifugation, at 6000×g for 1 minute and the flow-through was discarded with the collection tube. After two washing steps with buffers with decreasing EtOH concentrations, the elution of the nucleic acids was performed with 200 µl Buffer AE directly onto the membrane, incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000×g. The DNA yield was quantified using Nanodrop 2000c (Thermo Scientific) and the samples were stored at -20ºC.

3.4 NUCLEIC ACID DETECTION THROUGH QUANTITATIVE PCR

Quantitative real-time PCR (qPCR) was used to detect viral, bacterial and fungi nucleic acids. This method is based on PCR techniques (Polymerase Chain Reaction), requiring a pair of specific primers, for amplification of a specific DNA sequence. The detection of the amplification and of the quantitation of the target sequence, in real time is enabled by the thermal cycler system that measures the fluorescence emission during the amplification of the target region.

Although there are several methods based on fluorescence emission, the use of TaqMan™ probes was the one chosen in this screening. The Taqman™ probe is a third primer, complementary to an inner region, within the amplicon, labelled with a fluorophore (reporter) in the 5’ end, and a quencher in the 3’ end. Due to the probe length, the quencher blocks the fluorescence emission by the reporter. The probe only binds to a specific DNA region, within the forward and reverse primers targeted region. Without its attachment the reporter and the quencher remain together, allowing the quencher to inhibit the reporter’s fluorescence. Once the binding occurs, the probe is hydrolyzed by the Taq polimerase’s exonuclease 5’-3’ activity, during the primer extension, releasing the reporter from the suppressor action of the quencher and inducing fluorescence emission, which is detected by the thermocycler system. This fluorescence is proportional to the quantity of target sequence amplified. If the target sequence isn’t present, the probe does not bind, therefore no fluorescence is detected, meaning that the sample is negative for whatever we are looking for (VanGuilder et al., 2008).

The commercial mix used in the qPCR screening was SensiFAST™ Probe Hi-ROX Kit, which includes the reaction buffer, the enzyme and the dNTPs needed to amplify the target sequence. The *Ranavirus*, Chlamydiaceae and Chytridiomycosis target DNA amplification reactions were performed in the 7300 real-time thermocycler – Applied Biosystems.

The RACE (Risk Assessment Chytridiomycosis to European amphibian biodiversity) protocol was followed to examine if the samples were infected with Chytridiomycosis, since it's the standard test for this disease. The primers and probe used are based in *Batrachochytrium dendrobatidis* strain UM142 clone L internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, see Table 12. In this protocol the commercial mix used is the Taqman® Gene expression MasterMix. However, in this study, only the rainy season samples were tested with this mix, the others were tested using the SensiFAST™ Probe Hi-ROX Kit.

The primers and probe used in *Ranavirus* qPCR were calculated with the Primer designing tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), targeting based on the conserved MCP gene from FV3 (Table 12)(Mao, Hedrick, & Chinchar, 1997).

The primer set and the probe used to target the 23S rRNA gene, specific for all known Chlamydiaceae, were reported by Everett et al. (1999) (Table 12).

All the probes were used at a final concentration of 0.25µM, and the primers at 0.9µM. The qPCR reaction volume oscillated between 20 and 25µL; the DNA concentration was 100ng/µL, for the *Ranavirus* and Chlamydiaceae screening, and 150ng/µL for Chytridiomycosis test. The amplification conditions were the same for the all the three screenings (Table 13).

The positive recombinant controls used for the *Ranavirus* and Chlamydiaceae screening were previously prepared for use in the Virology Laboratory of FMV/ULisboa. Chytridiomycosis positive control was provided by RACE.

The DNA extracted from the liver samples was used in the *Ranavirus* and Chlamydiaceae assays, and for the *Bd* screening the template was skin DNA.

Table 12 – Primers and probes used in qPCR, to amplify the agents under study.

Agent	Primers and probes sequences (5'→ 3')		Product length
Chlamydiaceae Everett et al., 1999	Forward Reverse Probe	GAAAAGAACCCTTGTTAAGGGAG CTTAACTCCCTGGCTCATCATG FAM-CAAAAGGCACGCCGTCAAC-TAMRA	99 bp
Chytridiomycosis	Forward (ITS-1) Reverse (5.8S) Probe	CCTTGATATAATACAGTGTGCCATATGTC AGCCAAGAGATCCGTTGTCAAA FAM-CGA GTC GAA CAA AAT-	145 bp
Ranavirus Mao et al., 1997	Forward Reverse Probe	AACCAGGCGTTGAGGATGTAA TTCGGGCAGCAGTTTTCG FAM-CCCGACCTGGGAACGCCGA-TAMRA	62 bp

Table 13 – qPCR amplification conditions for the three agents (*Ranavirus*, *Bd*, and Chlamydiaceae).

Denaturation	Annealing	Extension
95°C / 10 min	90°C / 15sec	60°C / 1min
	45 cycles	

3.5 CONVENTIONAL AND NESTED PCR ASSAYS

The *Salmonella* screening was performed with primers which targeted the OMPC gene, encoding for protein C, involved in the invasion of epithelial cells (Table 14, Freitas et al., 2010). The *Salmonella* DNA used for positive control was extracted from *Salmonella typhimurium* pure culture, with DNeasy® Blood & Tissue Kit (Qiagen, Germany).

The OMPC primers were used at a final concentration of 10 pmol, and 100 ng of template (liver DNA) was added to the reaction. The amplification cycle included 29 cycles, each one with a denaturation step at 95 °C for 2 min, annealing at 57 °C for 2.5 min, and an extension step at 72 °C for 2.5 min (Freitas et al., 2010).

Table 14 - Primers used in the *Salmonella*'s screening (conventional PCR).

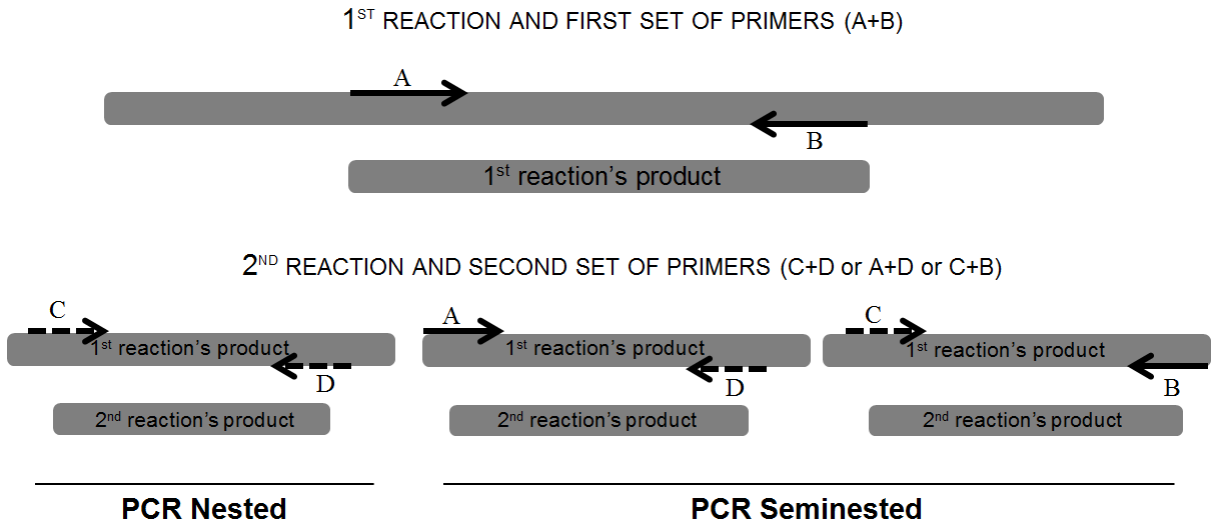
Agent	Primers sequences (5'→ 3')		Product length
Salmonella **de Freitas et al., 2010**	Forward OMPC Reverse OMPC	ATCGCTGACTTATGCAATCG CGGGTTGCGTTATAGGTCTG	204bp

The PCR nested technique was chosen to investigate the presence of *Herpesvirus* in the samples. It was necessary to use two assays, since the first one proved inconclusive.

This technique offers a lower probability of nonspecific amplification, due to the two sets or primers required. The first pair amplifies a longer target region, and the second pair has a target region within the first amplicon. In the seminested PCR, for the second round, one of the primers was reused, and the other primer was located within the first amplicon (Figure 12).

The first system was reported by (Murakami et al., 2001), and targeted the DNA polymerase gene of a tortoise H*erpesvirus*, by a semi-nested PCR.

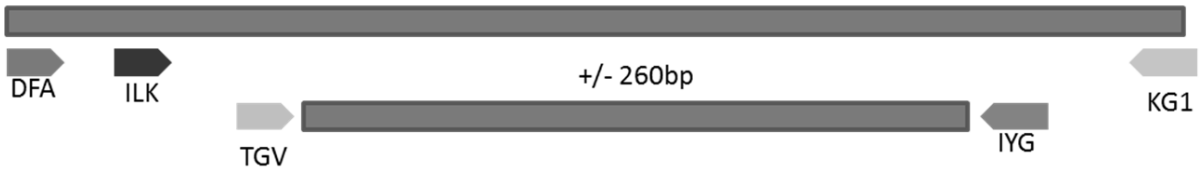
Figure 12 – Illustrative PCR nested and seminested procedure (Original).



All the primers were used at a final concentration of 0.2µM in a reaction volume of 25µL, in both reactions. The amplification cycle included an initial denaturation at 94º C for 3 min, followed by 35 cycles of denaturation at 94º C for 30 sec, annealing at 54º C for 1 min, and extension at 72º C for 1 min, followed by a final extension period at 72º C for 10 min, see Table 15.

The second procedure was a nested PCR based on VanDevanter et al. (1996) (Figure 13).

Figure 13 – Primers used in *Herpesvirus* nested PCR (VanDevanter et al., 1996)



Description: DFA, ILK and TGV are primers forward. KG1 and IYG are primers reverse.

This system uses degenerated primers (Appendix 2), designed based on a region containing three conserved coding motifs within an approximately 800bp region of the DNA polymerase gene, allowing the amplification of highly diverse *Herpesvirus*, being considered a panherpesvirus amplification approach, Table 15.

In the first round reaction all the primers were used at the final concentration of 0.4µM, whereas in the second round, primers were used at 0.08µM. The amplification reaction was performed in 25µL final volume, with 150ng of template (skin DNA) and 10 µL of 5 PRIME MasterMix (2.5x) The amplification cycle included 45 cycles for the first round PCR and 30 cycles for the second round PCR. Each cycle included a denaturation step at 94ºC for 30 seconds, annealing

at 46ºC for 1 minute and extension at 68ºC for 30 seconds, followed by an extension step 68ºC for 7 minutes.

Table 15 – Nested assays to the screening for *Herpesvirus*

HERPESVIRUS PCR Nested				
Procedure	**Reaction**		**Primers sequences (5'→ 3')**	**Product length**
Murakami, Matsuba, Une, Nomura, & Fujitani, 2001	1st	Forward	U-73 AGGCGGGAAAGGATTATGTC	535bp
		Reverse	L-588 AGTTTGATAGGGGATTTGAA	
	2nd	Forward	U-289 GATTTACTGGCGTGGCTATG	319bp
		Reverse	L-588	
VanDevanter et al., 1996	1st	Forward	DFA GAYTTYGCNAGYYTNTAYCC ILK TCCTGGACAAGCAGCARNYSGCNMTNAA	215-235bp
		Reverse	KG1 GTCTTGCTCACCAGNTCNACNCCYTT	
	2nd	Forward	TGV TGTAACTCGGTGTAYGGNTTYACNGGNGT	
		Reverse	IYG CACAGAGTCCGTRTCNCCRTADAT	

The amplified fragments, yielded by each conventional PCR, were analysed in an agarose gel electrophoresis that enables the separation of the amplified DNA according to its size.

A marker, with a predetermined set of products with known sizes, was run alongside with the PCR products, to ascertain by comparison, their approximated size.

A staining agent to allow the DNA visualization was added to the agarose. Gel Red is an intercalating fluorescent nucleic acid dye, designed to replace the carcinogenic ethidium bromide (EtBr). Its optical properties are identical to EtBr, when exposed to UV light it will fluoresce with an orange colour.

Each set of primers amplified a specific region of known size. Only the positive samples will have a band with the predicted size.

In this study, the PCR products were separated by electrophoresis in an agarose gel at 2% TAE, stained with 0,05µL/mL, 0,005% GelRed Nucleic Acid Gel Stain (Biotium®), during approximated 1 hour at 90-100V. The results were visualized with Image Master® VDS – Pharmacia Biotech at a 330nm wavelength, and the amplified DNA fragments size was compared with the bands from the 100bp marker, NZYDNA Ladder V from Nzytech. All the results were photo documented.

3.6 STATISTICAL ANALYSES

Fisher's exact test was used to assess the association between the positivity to the three agents (*Ranavirus*, chytrid fungus and *Salmonella*) and the gender, age and season. The test was evaluated with a statistical significance of 5% (α=0.05), being stablished a statistical association between variables and the positivity to the three agents when $p<0.05$.

These analysis were performed in the statistical program SPSS® 20 (Statistical Package for the Social Sciences, IBM Corporation, Chicago, EUA).

Sterne Exact Confidence Level was used to calculate the prevalence of the positive samples to *Ranavirus*, Chlamydiaceae and *Salmonella,* with a confidence interval (CI) of 95% and a test sensitivity and specificity of 1. Using http://epitools.ausvet.com.au to do the calculations.

4 RESULTS

The dry season samples were the first to be collected and processed, therefore, the code assigned to the liver samples was L01 to L60, and to the skin samples S01 to S60. For the rainy season samples, the identification ranged from L61 - L110 for the liver samples and S61-S110 for the skin samples (Appendix 3).

The DNA quantification went between 13 and 2118 ng/µl, with a mean of 287.95 ng/µl.

In 110 toads 38 were males (34.5%), 43 were females (39.1%) and 29 undetermined (26.4%), with the following distribution in the dry season, 27 males (45%), 22 females (36.7%) and 11 indeterminate (18.3%); and 11 males (22%), 21 females (42%) and 18 undetermined (36%) in the rainy season sampling.

For the life stage, 88 were adults (80%) and 21 were sub-adults, and one sample lacked information; in the dry season, 42 adults (70%) and 18 sub-adults (30%). In the rainy season 46 adults (92%), 3 sub-adults (6%) and 1 undetermined (2%), Table 16.

Table 16 – Samples specifics.

		DRY SEASON	RAINY SEASON	BOTH SEASONS
GENDER	Male	27	11	38
	Female	22	21	43
	Undetermined	11	18	29
LIFE STAGE	Adult	42	46	88
	Sub-Adult	18	3	21
	Undetermined	-	1	1

The *Ranavirus* screening detected 10 positive samples in the dry season, and none in the rainy season. The Chlamydiaceae diagnosis revealed 9 positive samples in the dry season and 2 in the rainy season. No sample was positive to chytrid fungus or *Herpesvirus*. Salmonellosis PCR detected one positive sample in the dry season test (Appendix 4), (Table 17 and Table 18).

Table 17 – Positive samples in the screening.

SEASON	*RANAVIRUS*	CHLAMYDIACEAE	*SALMONELLA*
DRY	L05 L08 L13 L17 L21 L27 L38 L42 L45 L54	L06 L21 L32 L33 L35 L36 L39 L56 L60	L03
RAINY		L29 L32	
TOTAL	10	11 (9 dry+2 rainy)	1

Table 18 – Agents prevalence percentages.

SEASON	*RANAVIRUS*	CI%	CHLAMYDIACEAE	CI%	*SALMONELLA*	CI%
DRY	16.7%	[8.9 – 28.2]	15%	[7.8 – 26.5]	1.7%	[0.1 – 8.9]
RAINY	-		4%	[0.7 – 13.7]		
BOTH	9.1%	[4.9 – 16.2]	10%	[5.3 – 17.2]	0.9%	[0 – 4.9]

From the 10 toads positive to *Ranavirus*, 5 (50%) were male, 4 (40%) were female and 1 (10%) unknown gender. Within the same animals, 6 (60%) were adults and 4 were subadults.

The Chlamydiaceae screening showed 11 positives, of which 7 (≈64%) were males, 3 (≈27%) were females and 1 (≈9%) unknown gender. 8 (≈73%) were adults and the other 3 (≈27%) were sub-adults.

An adult female was positive to *Salmonella*.

No statistical association was found between the gender or age and the positivity to the three agents (p value>0.05). However, a statistical association between *Ranavirus* positivity and the collection during the dry season (p=0.002), Table 19.

Table 19 – Association between gender/age and positivity to the agents.

FISHER'S TEST α=0,05			
	Ranavirus	**Chlamydiaceae**	***Salmonella***
Gender	p=0.728	p=0.177	p=1
Age	p=0.098	p=0.440	p=1
Season	p=0.002	p=0.064	p=1

5 DISCUSSION

Ranavirosis and chytridiomycosis are considered the two major diseases contributing to amphibian decline and extinction; the other causes are anthropogenic, including habitat destruction or alteration, introduction of alien species, pet trade and consumption (Stuart et al., 2004).

The 110 samples of *D.melanostictus* sampled in this study were negative to *Bd*.

Bd prevalence is low in Madagascar, mostly because studies previous to 2014 didn't identify positive samples, despite the global distribution of this agent. Madagascar was considered a chytrid fungus-free country until recently, regardless the finding of *Bd* in pet trade of Malagasy frogs to the USA in 2012, which only alerted for the possible presence of this agent in Madagascar, since the origin of infection wasn't proved (Bletz et al., 2015).

In 2014, a study was published presenting data obtained during the years of 2005 to 2014 (Bletz et al., 2015), tracking *Bd* in different Malagasy locations. The first evidence of the presence of *Bd* in Madagascar was detected in frogs in 2010.

Currently there is no information regarding the susceptibility of Malagasy frogs to chytridiomycosis, and no positive animal showed signs of clinical chytridiomycosis (Bletz et al., 2015). Due to the devastating effects of *Bd* in amphibian populations, serious control measures are triggered for the prevention of infection, attempting to avoid the possible extinction or decline of several Malagasy species, as already happened in Australia (Daszak et al., 1999; Lawrence, 2008 and Berger et al., 1998).

Prior to 2010 no records of *Bd* were found, and the most likely reasons include the introduction of invasive species or from infected water/soil. In 2003 foreign crayfish was accidentally introduced in Madagascar, as was *D.melanostictus* recently (Bletz et al., 2015). These animals are considered a serious threat to the amphibian Malagasy biodiversity, since they can be carriers of *Bd*.

The absence of positive samples in this survey was not surprising since Kolby et al., (2015) swabbed 508 Malagasy amphibians, including *D.melanostictus*, and found no positives.

Similarly to *Bd*, ranaviruses are emerging pathogens, with a worldwide distribution mostly due to human transportation, pet trades and consumption, and their host range continues expanding (reviewed by Gray & Chinchar, 2015).

Kolby et al. (2015), demonstrated that ranaviral infection was present in Madagascar in 2014, with a prevalence of infection of 6.4% (5/97) IC of 2.6% to 14.4%.

Ranaviruses epidemics occur mostly during mid-to-late summer (in temperate habitats) in late stage tadpoles and young adults, with high (close to 90%) mortality rates, and even higher morbidity (Gray & Chinchar, 2015). According to this information an association between ranaviral infection and age was expected. However, in this survey, no significant statistic association was identified, most probably because within de 110 samples, only 21 were subadults versus 88 adults.

When analysing the two seasons a statistical association (p=0.002) was detected relating the dry season and ranaviral infection, since no animal was infected during the rainy season.

The 10 toads positive to *Ranavirus* were sampled during the dry season from 8 to 10 of July of 2014. The mean temperature registered in Toamasina – Madagascar during these days was 24ºC (accuweather.com), which is considered an optimal temperature to ranaviral replication (replication rates in culture cells increased to optimum when exposed to temperatures between 24 to 28ºC, Gray & Chinchar, 2015). In this sense, the detection of *Ranavirus* was, theoretically, propitious in this period, which probably explains the higher prevalence comparing to Kolby et al. (2015).

Despite the recognized presence of ranaviruses in Malagasy amphibians, no epidemic was registered in this country. The absence of reported die-offs from *Ranavirus* infections in Madagascar can have two explanations: the first one is associated with the vast Malagasy forest area, meaning that an unnoticed ranaviral epidemics could have happen in isolated locations; the second reason is based on the idea that the ranaviruses found in Madagascar are endemic, and evolved with the Malagasy amphibian biodiversity, causing relatively minimal virulence to native wildlife (Kolby et al., 2015), enhancing the importance of surveying invasive species, potentially harboring different strains of *Ranavirus*, more virulent to the Malagasy species. In this survey, we detected the presence of *Ranavirus* in *D. melanostictus* specimens, however it was not possible to assess if *D. melanostictus* were infected already in Madagascar, or if they carried the agent from their place of origin, spreading a different strain of *Ranavirus* into Malagasy amphibians.

Although chytridiomycosis and ranavirosis in amphibians have been identified in several studies, due to their corrosive impact in amphibian populations, there is still some unexplained and enigmatic deaths.

Recently, two new members of the family Chlamydiaceae were associated to amphibian disease (Martel et al., 2012 and Martel, Adriaensen, Sharifian-Fard, et al., 2012). The primer set and probe used in this survey target the 23S rRNA gene (Everett et al., 1999), allowing the identification of all families in the order Chlamydiales, including the Chlamydiaceae. This wide-range of detection was chosen in an attempt of identify all chlamydia-like bacteria, since the information regarding specific amphibian chlamydia is recent, and it's still under study. Although the two new identified species, mentioned above belong to the Chlamydiaceae family, there is no information regarding other families within the order that may infect amphibians.

Similar to *Ranavirus*, bacterial infection with Chlamydiaceae recorded higher prevalence during the dry season sampling, however, no statistically association was made. Chlamydia-like bacteria's seasonality patterns have been shown in humans and amoebas (Schroeder et al., 2001; Pizzetti et al., 2015), however the pattern wasn't attributed to the bacteria *per se*, but

to the hosts behaviors. For instance, in humans a higher sexual activity was recorded during autumn and summer, increasing the rates of Chlamydia infections (Schroeder et al., 2001).

Myers et al. (2009), proposed that "*C. pneumoniae* was originally an animal pathogen that crossed the species barrier to humans through ongoing reductive evolutionary processes and has adapted to the point where human isolates of *C. pneumoniae* no longer require an animal reservoir for transmission". Mitchell et al. (2010) data suggested two lineages of *C. pneumoniae*, both of them with amphibian isolates as the origin. Hereupon, and knowing that several species of Chlamydiaceae infect amphibians, it is important to be aware of possible zoonotic implications.

Salmonella are other source of zoonotic infections, and as mentioned above, amphibian are a well-known reservoir to these bacteria, as they shed it intermittently for long periods of time, contaminating the environment (Srikantiah et al., 2004).

In amphibians the prevalence in asymptomatic animals is usually higher than 10%, and can reach the 60% (ARWH, 2012), however, in this survey, the prevalence was below 1% in the Asian toad.

A low number of positives was not expected, since *Salmonella* is considered a commensal gastrointestinal organism in amphibians, and the prevalence rates are usually higher (Chambers & Hulse, 2006). The conventional PCR assay was conducted thoroughly, respecting the protocol described in Freitas et al. (2010), and using a positive control.

The preferable samples to detect *Salmonella* are cloacal swabs, faeces, or gastrointestinal samples (tissue or content), since it's location it's mainly intestinal, but heart and liver are also used (Freitas et al., 2010). In this survey the liver samples were preserved in alcohol. Møretrø et al. (2009) proved that the best control method against *Salmonella* cells, cells attached to surfaces or embedded in biofilms were disinfectants containing 70-80% of ethanol.

Thus, the absence of a higher prevalence may be associated to the preservation method, and the only positive sample, L03, could have a higher bacterial concentration, resisting the alcoholic preservation.

Herpesviruses cause a wide range of diseases, with a wider range of hosts. The best characterized effect of these viruses on amphibians is Lucké's renal adenocarcinoma in Leopard Frogs (*Rana pipiens*) caused by RaHV-1. RaHV-2, isolated from urine of RaHV-1 infected frogs, still has no clinical significance. Other than these two amphibian herpesviruses, only reports of herpesvirus-like particles by electron microscopy, without virus isolation or molecular characterization (Mader & Divers, 2014).

In this survey, no positive sample to *Herpesvirus* was detected, but without a positive control, it is not possible to claim, that none of the 110 *D. melanostictus* was infected with *Herpesvirus*. Two functional systems were used, the first one targeted the DNA polymerase gene of a tortoise *Herpesvirus*, and no positive samples were detected. The second assay is considered

a panherpesvirus amplification approach, but similarly to the previous assay, no samples tested positive in this system.

As RaHV-1 and 2 only were reported in *Rana pipiens*, and other amphibian reports related to *Herpesvirus* haven't confirmed the presence of these viruses in other amphibian species, no solid conclusion is possible.

6 FINAL CONSIDERATIONS

Amphibians are the most threatened group of animals on the planet. Approximately one in four species are considered endangered being the primary threat to these animals mankind, by destruction of habitats, over-harvesting (pet and food trades), pollution and climate changes, and of course the net of infectious diseases and invasive species which depend mostly on efficient transportation.

Madagascar still has an astonishing amphibian diversity and endemism, and the preservation of this ecological niche is of main concern. Yet, human devastation is still the most direct danger to the Malagasy amphibians. Therefore it is essential to identify and isolate possible threats as rapidly and effectively as possible.

Duttaphrynus melanostictus is the most recent invasive amphibian specie identified in Madagascar. As this toad has a high fertility rate, and a generically diet it can prevail in Malagasy soil, possibly endangering the native species, competing for food, and territory. Further, this species produce a venom that may lead to predators' death. Therefore, *Duttaphrynus melanostictus* is not only an amphibian threat, but is also a threat to its predators, including humans due to feeding habits (in this area of the globe, natives eat amphibians).

Moreover, this alien toad may carry several pathogens that can unleash epizootic or zoonotic outbreaks, stressing the need of surveying pathogens that most likely can trigger an outbreak. This assay tried to meet this need.

In this screening chytridiomycosis was found negative. However it is essential to keep surveying for this agent, since it has one of the highest impacts in amphibian declines and extinctions world-wide, with mass mortalities and high infection rates.

Other of these world-wide amphibian decline and extinction diseases is ranavirosis. And although no ranavirosis die-off was reported, the *Ranavirus* positivity of this invasive toad, which can probably act as reservoir to these viruses, is a serious threat to amphibian and reptile health.

The ranaviral infection prevalence is worrisome not only because it threatens the amphibian diversity in Madagascar, but also because *Ranavirus* can also infect reptiles, in particularly chelonians, which are also important to Malagasy biodiversity.

This Asian toad is also infected with Chlamydia. Besides its pathological effects in amphibians, this agent has epizootic and zoonotic potential. Therefore a strict control, with rapid identifications of possible species jumps is important.

Salmonellosis is one of the most important food-borne bacteria, and amphibians are known reservoirs to this pathogen, causing disease in humans. The sample preservation, in this assay, wasn't compatible with the detection of these bacteria. To fully assess *Salmonella* distribution in *D. melanostictus* a new study, aiming the detection of *Salmonella* in more appropriate samples would be of interest. Due to the invasive behaviour of *D .melanostictus,* as its population thrives in Madagascar, more will be the opportunities of human contact.

Although no positive sample was detected for *Herpesvirus*, and because this pathogen as a great impact in chelonians, it is also significant to explore the possible infection of the Asian toad. Madagascar shelters *Astrochelys yniphora*; which is considered to be the world's rarest tortoise and one of the most endangered reptiles, among other species.

This survey only addressed the pathogens detection, but for future studies, it would be essential to genotype the viruses and bacteria infecting the invasive *D. melanostictus*, providing a better assessment of the real threat placed by this toad and answering the questions: "Does *D. melanostictus* carries zoonotic agents"; "Are the pathogens that this toad carries virulent enough to trigger an amphibian outbreak?" and "Was the Asian toad infected in Madagascar, or was it already infected?".

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8 APPENDIX

8.1 APPENDIX 1 – Disinfection strategies suitable for killing *Bd* (reviwed by OIE, 2012)

Application	*Disinfectant*	*Concentration*	*TIme*
Disinfecting surgical equipment and other instruments (e.g. scales, callipers)	Benzalkonium chloride	2 mg ml^{-1}	1 minute
	Ethanol	70%	1 minute
Disinfecting collection equipment and containers	Sodium hypochlorite	1%	1 minute
	Path X or Quaternary ammonium compound 128	1 in 500 dilution	0.5 minutes
	Trigene	1 in 5000 dilution	1 minute
	F10	1 in 5000 dilution	1 minute
	Virkon	2 mg ml^{-1}	1 minute
	Potassium permanganate	1%	10 minutes
	Complete drying		>3 hours
	Heat	60°C	30 minutes
	Heat	37°C	8 hours
Disinfecting footwear	Sodium hypochlorite	1%	1 minute
	Path X or Quaternary ammonium compound 128	1 in 500 dilution	0.5 minutes
	Trigene	1 in 5000 dilution	1 minute
	F10	1 in 5000 dilution	1 minute
	Complete drying		>3 hours
Disinfecting cloth	Hot wash	60°C or greater	30 minutes

8.2 **APPENDIX 2** - IUPAC system for degenerated nucleotide nomenclature (source: http://bitesizebio.com/18992/a-primer-for-designing-degenerate-primers).

Single letter code	Nucleotide/s	Explanation
R	A or G	pu**R**ine
Y	C or T	p**Y**rimidine
M	A or C	a**M**ino
K	G or T	**K**eto
S	C or G	**S**trong interaction
W	A or T	**W**eak interaction
H	A or C or T	not G, **H** follows G in alphabet
B	C or G or T	not A, **B** follows A in alphabet
V	A or C or G	not T/U, **V** follows U in alphabet
D	A or G our T	not C, **D** follows C in alphabet
N	A or C or G or T	a**N**y

8.3 **APPENDIX 3** – Samples quantification and features.

Liver sample	[DNA] ng/µl	Age	Sex	Skin sample	[DNA] ng/µl
L01	92,1	Adult	Male	S01	44
L02	168,9	Adult		S02	41,6
L03	303	Adult	Female	S03	50,7
L04	291,9	Adult	Female	S04	53,2
L05	295,8	Sub-Adult		S05	85,8
L06	285,9	Adult	Female	S06	26,1
L07	126,8	Adult	Male	S07	158,5
L08	123	Adult	Male	S08	32,6
L09	307,1	Adult	Male	S09	35,6
L10	176,7	Adult	Female	S10	49
L11	258,1	Adult	Male	S11	39,2
L12	120	Sub-Adult		S12	45,5
L13	103,8	Adult	Female	S13	50,2
L14	173,2	Adult		S14	65,8
L15	127	Sub-Adult	Male	S15	51,6
L16	149,9	Adult	Female	S16	119,9
L17	12,6	Adult	Female	S17	140,3
L18	145,2	Sub-Adult		S18	76,4
L19	450,5	Adult	Female	S19	96,7
L20	86,1	Adult	Male	S20	104,9
L21	57,9	Sub-Adult	Male	S21	124,3
L22	86,4	Adult	Female	S22	119,5
L23	198,3	Adult	Female	S23	65,7
L24	135,4	Adult	Female	S24	70

Appendix 3 (continuation) – Samples quantification and features

Liver sample	[DNA] ng/µl	Age	Sex	Skin sample	[DNA] ng/µl
L25	148,2	Adult	Female	S25	82,4
L26	172,5	Adult	Female	S26	131,9
L27	139	Adult	Female	S27	119,3
L28	187,3	Adult	Female	S28	70,4
L29	124,2	Sub-Adult		S29	67,4
L30	225,1	Sub-Adult		S30	56,3
L31	243,7	Adult	Male	S31	33,2
L32	95,7	Adult	Male	S32	37
L33	172,9	Adult	Male	S33	51,8
L34	139,8	Sub-Adult	Male	S34	36,5
L35	168,4	Sub-Adult	Male	S35	69,3
L36	124,1	Sub-Adult	Male	S36	107,7
L37	150	Adult	Male	S37	121,6
L38	176,9	Adult	Male	S38	62,6
L39	102,1	Adult	Male	S39	42,7
L40	142,1	Adult	Male	S40	104,1
L41	44,3	Adult	Male	S41	61
L42	148,4	Sub-Adult	Male	S42	101,9
L43	63,3	Adult	Male	S43	72,3
L44	183,9	Sub-Adult		S44	118,6
L45	100,3	Sub-Adult	Male	S45	117,5
L46	70,2	Sub-Adult	Male	S46	69
L47	102	Adult	Male	S47	108,8
L48	151,6	Adult	Male	S48	120,7
L49	148,8	Adult	Female	S49	71,3
L50	102	Adult	Female	S50	107,2
L51	98,6	Sub-Adult		S51	85,7
L52	143,9	Sub-Adult		S52	89,2
L53	91,5	Sub-Adult	Male	S53	83,5
L54	141	Adult	Female	S54	136,3
L55	113,3	Adult	Female	S55	119,6
L56	154,4	Adult	Male	S56	147,9
L57	109	Sub-Adult		S57	108,4
L58	153	Adult	Female	S58	105,5
L59	113,4	Adult	Female	S59	121
L60	74,4	Adult	Female	S60	121,5
L61	215,4	Adult	Female	S61	89,5
L62	314,8	Adult		S62	103,3
L63	1129,9	Adult	Female	S63	125,6
L64	297,8	Adult	Female	S64	64,2
L65	340,7	Adult	Female	S65	106,7
L66	51,5	Adult	Male	S66	101,6
L67	546,3	Adult	Male	S67	94,6
L68	366,5	Adult	Female	S68	91,3

Appendix 3 (continuation) – Samples quantification and features

Liver sample	[DNA] ng/µl	Age	Sex	Skin sample	[DNA] ng/µl
L69	584,8	Adult	Male	S69	114,1
L70	626,2	Adult	Female	S70	94
L71	552	Adult		S71	149,6
L72	351,6	Adult	Female	S72	96,1
L73	149,8	Adult	Female	S73	90,6
L74	464,5	Adult	Male	S74	55,6
L75	669,9	Adult	Female	S75	83,6
L76	308,3	Adult	Male	S76	82,7
L77	534,8	Adult	Female	S77	129,4
L78	2118,4	Adult	Male	S78	68,5
L79	123,7	Adult	Male	S79	78,4
L80	248,5	Adult	Male	S80	196
L81	276,6	Adult		S81	11,9
L82	126,3	Adult		S82	107,1
L83	472,7	Adult	Female	S83	104,9
L84	349,9	Adult		S84	156,4
L85	182,2	Adult		S85	167,3
L86	541,8	Adult	Female	S86	189,7
L87	390,3	Adult	Male	S87	133,4
L88	309,8	Sub-Adult		S88	160,5
L89	435,3	Adult	Female	S89	228,7
L90	510,4	Adult		S90	110,3
L91	114,4	Adult	Male	S91	110,4
L92	188,3	Adult		S92	121,5
L93	359	Adult		S93	93,7
L94	609,2	Sub-Adult		S94	95,5
L95	492,4	Adult	Female	S95	110,2
L96	265,3	Adult	Female	S96	130,4
L97	251	Adult		S97	144,5
L98	524	Adult	Female	S98	160,1
L99	538,3	Sub-Adult		S99	208,5
L100	351,5	Adult	Male	S100	91,2
L101	301,6	Adult	Female	S101	150,6
L102	728,6	Adult	Female	S102	119,7
L103	602	Adult	Female	S103	138
L104	382,7	Adult		S104	159,1
L105	425,6	Adult	Female	S105	202,2
L106	66,1			S106	57
L107	418,4	Adult		S107	59
L108	738,1	Adult	Female	S108	92
L109	299,7	Adult		S109	173,6
L110	1336,3	Adult		S110	94

8.4 APPENDIX 4 – L03 electrophoresis results for *Salmonella*'s conventional PCR.