SEA TURTLES DISEASE RISK ANALYSIS AND DISCOVERY OF THE FIRST AUSTRALIAN GREEN TURTLE (*Chelonia mydas*) PAPILLOMAVIRUS

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TO WOMEN IN SCIENCE

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The research presented and reported in this thesis was conducted with the approval of the James Cook University Research Ethics Committee and in accordance with the National Statement on Ethical Conduct in Human Research, 2007; Australian Code for the Care and Use of Animals for Scientific Purposes, 2007; and the Queensland Animal Care and Protection Act, 2001.

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STATEMENT OF THE CONTRIBUTION OF AUTHORS

This thesis was supervised by associate professor Ellen Ariel, Dr. Jennifer Elliman and associate professor Leigh Owens. As such they had significant input on the design, execution and analysis of this project as well as reviewing the individual chapters and resulted manuscripts. Dr. Graham Burgess was the expert consultant for chapter four, five and six of the thesis. He had provided valuable advice for the experiments enrichment and analysis along with reviewing the resulted chapters and manuscripts. Katrina Christy, Lauren Burchell and Kelly Johns provided editorial assistance on some chapters. Karina Jones provided the extracted DNA and the samples for the molecular survey in chapter six of the thesis. Alicia Maclaine assisted with some PCRs in chapter four and Wytamma Wirth with some PCRs in chapter six. The samples and data collection was done with the help of the JCU Turtle Health Reseach Team, Girringun and Gudjuda Aboriginal Rangers, Queensland Parks and Wildlife Service, Ecobarge, and the Turtle Hospital at Reef HQ Aquarium. Necropsies and histopathologies were done with the help of Dr. Jennifer Scott and Yissu Martinez at the veterinary pathology department. Outside the university, the necropsies were done in Queensland Parks and Wildlife Service facilities. BIV control DNA was kindly provided by Professor R. Whittington and A. Tweedie, Sydney University.

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ABSTRACT

Sea turtle populations are in decline, which has been attributed to threats such as predation, bycatch, unsustainable harvesting, habitat degradation, and pollution. The impact of these threats has resulted in the listing of six out of seven sea turtles in the IUCN red list of endangered animals. Infectious diseases are listed among the top five reasons for global species extinctions, but these have not been thoroughly evaluated in sea turtles. Suggested sea turtle conservation plans, based on risk and threat matrices, reported diseases of infectious aetiology with the least priority. Pollution and pathogens have been surveyed in less than half of regional management units (RMUs) and as such these threats have been identified as areas of data deficiency, warranting future investigations.

In this thesis, I have addressed the issue of increasing incidence of disease in sea turtles by carrying out a disease risk analysis (DRA) based on the published literature and with the contributions of a group of experts representing more than 20 regions around the globe. In **Chapter three** of the thesis a comprehensive list of health hazards is provided for all seven species of sea turtles. The risk these hazards possibly pose to the health of sea turtles were assessed and "*One Health*" aspects of interacting with sea turtles were also investigated. The general result of the DRA was the distinct lack of knowledge regarding a link between the presence of pathogens (either bacteria, fungi, parasites or viruses) and diseases of sea turtles. Among all pathogens, viruses were studied the least, although the debilitating disease of sea turtles, fibropapillomatosis (FP) is suggested to have a viral aetiology: *Chelonid alphaherpesvirus 5* (ChHV5).

The results of the DRA inspired efforts to improve methods for viral discovery in sea turtles. **Chapter four** of this thesis is focused on methods establishment. Three main virological methods: cell culture, molecular methods and histopathologial analysis, were used for this project. While the first two were developed and validated as part of this thesis, pathological analyses were done by a qualified veterinary pathologist. The aim was to increase the chance of finding new viruses using a combination of culture and non-culture methods. Sea turtle primary cell lines were established from different stages of green turtle embryos to provide a broad range of host tissues for viral isolation and propagation. PCR assays were designed to detect four DNA viruses previously described in chelonians: herpes, papilloma, adeno and iridoviruses. The methods developed were instrumental in identifying a papillomavirus associated with FP tumours in green turtles and led to the discovery of the first Australian *Chelonia mydas* papillomavirus (CmPV). First, cytopathic effects were observed in primary

cell lines inoculated with homogenates from eight FP tumours and subsequently PCR assays of the affected cultures and the original tissues confirmed presence of CmPV.

The Australian CmPV isolates were partially characterised and were examined further in **Chapter five**. Primer walking and Sanger sequencing revealed some features of the Australian CmPV isolates comparable to green turtle and loggerhead (*Caretta caretta*) turtle papillomaviruses from Florida, CmPV-1 and CcPV-1 respectively. The E1 gene partial sequencing and the L1 gene full sequencing suggested that Australian CmPV isolates are 100% similar to CmPV-1 and are whithin the same species as CcPV-1.

Chapter six is focused on investigating the relationship between CmPV and FP, as the eight CmPV-positive samples were found only in FP tumours of green turtles but not in normal skin, blood and cloacal samples of the same animals. To investigate the possibility of concurrent infection with ChHV5 and CmPV in sea turtles with FP, the samples were screened for both viruses. DNA from 131 tumour tissues and 36 normal skin samples from 89 green turtles afflicted with FP tumours and also 47 normal skin samples from asymptomatic green turtles were extracted and used for the survey. These samples were collected from different regions of Queensland, Australia. Out of 89 FP green turtle samples, 77.52% tested positive for ChHV5, 51.68% for CmPV, 46.06% for both viruses and samples from 15.73% turtles tested negative for both. From 36 normal tissues tested for presence of ChHV5 and CmPV, 50% samples reacted in PCR for ChHV5, 27.77% for CmPV, 8.33% for both viruses and 30.55% samples did not react for either of these viruses.

Six samples were collected from loggerhead turtles with FP and assessed for the presence of CcPV, CmPV and ChHV5. The samples were positive for CcPV and ChHV5 and negative for CmPV. The green turtle samples that were positive for CmPV, were negative for CcPV. Papillomaviruses of loggerhead and green turtles appear to be species specific, however further analyses on these samples and possibly more loggerhead samples are required to make sound conclusions about these findings.

The high level of co-infection of ChHV5 and CmPV in both tumour tissues and normal skins of sea turtles reported here, challenges the general opinion in the scientific literature for the past 30 years about the role of ChHV5 in FP.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

3D	Three Dimensional
ATCC	American Type Culture Collection
BF-2	Bluegill Fry fish cell line
BIV	Bohle Iridovirus
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CCL	Curved Carapace Length
CcPV	*Caretta caretta* papillomavirus
CFPHV	Chelonid fibropapilloma-associated herpesvirus
ChHV1	chelonid herpesvirus 1
ChHV5	*Chelonid alphaherpesvirus 5*
ChHV6	chelonid herpesvirus 6
CITES	Convention on International Trade in Endangered Species
CMEH	*Chelonia mydas* embryo head
CMEI	*Chelonia mydas* embryo internal organs
CMEM	*Chelonia mydas* embryo muscles
CMEW	*Chelonia mydas* embryo whole body
CmPV	*Chelonia mydas* papillomavirus
CPE	Cytopathic Effects
DBD	DNA Binding Domain
DCS	Decompression Sickness
DEHP	Department of Environment and Heritage Protection
DMSO	Dimethyl sulfoxide
Dpol	DNA polymerase
DRA	Disease Risk Analysis
DTS	Debilitated Turtle Syndrome
ELISA	Enzyme-linked immunosorbent assay
EPC	Epithelioma *Papulosum Cyprini* cell line
FBS	Fetal bovine serum
FHM	Fathead minow fish cell line
FP	Fibropapillomatosis
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBR	Great Barrier Reef
GBRMPA	Great Barrier Reef Marine Park Authority
GPD	Gray Patch Disease
HPV	Human papillomavirus
HSV	Herpes simplex virus
ICTV	International Committee on Taxonomy of Viruses
IUCN	The International Union for Conservation of Nature

JCU	James Cook University
LCR	Long Control Region
LETD	Lung Eye Trachea Disease
LETV	Lung Eye Trachea Virus
LGRV	loggerhead genital-respiratory herpesvirus
LOCV	loggerhead orocutaneous herpesvirus
MCP	major capsid protein
MPP	membrane associated phosphoprotein
MTSG	Marine Turtle Specialist Group
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing platform
NLS	Nuclear Localization Signal
NTC	No template control
OIE	World Organisation for Animal Health
ORF	Open Reading Frames
qPCR	Quantitative Polymerase Chain Reaction
QPWS	Queensland Parks and Wildlife Service
PERT	Polymerase Enhanced Reverse Transcriptase
PV	Papillomavirus
PPE	Personal protective equipment
RMUs	Regional management unites
SSC	Species Survival Commission
STTV1	Sea Turtle Tornovirus 1
$TCID_{50}$	Median Tissue Culture Infectious Dose
TED	turtle excluding device
TEM	Transmission Electron Microscopy
TH-1	Terrapene Heart cell line

1. INTRODUCTION

Sea turtles are ancient inhabitants of the oceans and due to their longevity and high site fidelity, they are considered sentinels of habitat health (Aguirre and Lutz, 2004). Some feed on sea grass and potentially have a role in nutrient distribution (Kuiper-Linley *et al.,* 2007) while others contribute to the food chain by either feeding on invertebrates and other small prey or becoming food for larger predators (Wilson *et al.,* 2010). In addition to their environmental contributions, sea turtles have cultural significance that varies between societies and across geographical boundaries (Campbell, 2003). People interact with sea turtles for economic, recreational, scientific and traditional reasons, but currently, the common goal of most communities is to conserve sea turtles for future generations (Campbell, 2003).

The world population of sea turtles has declined over the past 100 years due to direct and indirect human interventions (Troëng and Rankin, 2005). The International Union for Conservation of Nature (IUCN) has listed six of the seven sea turtle species on the IUCN Red List of Threatened Species while the seventh species, the flatback turtle (*Natator depressus*), is reported as "*Data Deficient*" (IUCN, 2015).

Throughout their lives, turtles face both natural and man-made menaces. Terrestrial animals prey on the eggs and hatchlings on the beach and marine predators remain a threat to turtles during their aquatic existence. Anthropogenic effects are also life threatening and by-catch by trawlers before the implementation of turtle excluding devices (TEDs) was estimated to account for a mortality rate of 39% in 5000-6000 turtles caught in nets in the late 1980s in northern Australia (Department of the Environment 2008). Although these are examples of direct impacts, there are many more indirect effects from human activities including coastal development, light disturbance and pollution (DEWHA. 2008). Because of habitat degradation and associated stress, the incidence of disease is also increasing

in sea turtles (Milton and Lutz, 2002; Jones 2004) and therefore, this thesis attempts to clarify the knowledge and gaps in the field of sea turtle health.

Although there is a global awareness about wildlife health and conservation, health is not easily defined for wildlife and conservation is a controversial topic (Campbell, 2003).

According to Stephen (2014) describing **health** as "*the absence of disease*" is behind the modern concepts and general understanding. Wildlife health, like human health, is not a biologic state but rather a dynamic concept based on physical health and welfare of the animal (Stephen, 2014).

Roger and Walker (2017) explained that **disease** is not only biological dysfunction, as dysfunction is not a 0 or 100 concept but often differs by degree. The interpretation is based on the case and it is not correct to draw a line between healthy and sick individuals (Roger and Walker, 2017).

For health monitoring and assessment in the wild, the current strategy is surveillance, which encompass on-going monitoring of the species and recording diseases in animal populations with a view to disease prevention and management (Ryser-Degiorgis, 2013). Such strategies are also adapted for sea turtles; however, it is difficult to find and capture a diseased sea turtle in the wild (opportunistic sampling) and the disease diagnosis, frequencies, pathogenesis and clinical relevance are often not identified (Flint *et al.,* 2010a).

Assessment of impacts that disease might have on sea turtle populations is not considered systematically. In 2011, more than 30 institutes worldwide contributed to the latest edition of "*Global Conservation Priority for Marine Turtles*", including the IUCN/SSC Marine Turtle Specialist Group (Wallace *et al.,* 2011). This survey indicated that diseases were studied in less than half of the participating institutions. As a result, the threats of diseases have been reported as "*data deficient*" and a proposed knowledge gap for future assessments (Wallace *et al.,* 2014).

In 2017, Manire *et al.,* reviewed the current husbandry, health and rehabilitation information available, from a clinical view-point (Manire *et al.,* 2017). Such reviews are extremely valuable for treating individual turtles and clinicians can help give indications of common problems and emerging diseases. A management perspective for wild populations will have to include systematic monitoring and a broader approach in the dynamic context of an ecosystem to achieve long-term sustainability of populations (Fryxell and Sinclair, 2014). Monitoring and conservation interventions may require animal handling and translocation, which is accompanied by potential disease risk to the turtle population and/or humans. To document and manage these risks appropriately and effectively it is necessary to implement suitable investigation tools (Hartley and Sainsbury, 2017).

1.1. Disease risk analysis

Disease risk analysis (DRA) tools are designed to provide objective, repeatable and documented assessment of the risks for a particular case that is under investigation. A disease focused risk analysis was developed in 2014 by the joint contribution of IUCN and World Organisation for Animal Health (OIE) (Jacob-Hoff *et al.,* 2014) and has been used by multiple wildlife health investigators and conservationists (Jacob-Hoff *et al.,* 2017; Hartley and Sainsbury, 2017). It is stated in "*The Manual of Procedure for Wildlife Disease Risk Analysis*" that "*disease risk analysis is a structured, evidence-based process that can help in decision making in the face of uncertainty and determine the potential impact of infectious and non-infectious diseases on ecosystems, wildlife, domestic animals and people*".

A DRA can be adapted to analyse disease risk for the global population (Pacioni *et al.,* 2015) or disease risk for a specific scenario like translocating a population (ie clutch of egg) from region A to region B (Jacob-Hoff *et al.,* 2014). To the best of our knowledge, DRAs have not previously been published for sea turtles, although disease is reported to result in population decline (Milton and Lutz, 2002; Jones, 2004) and translocation is happening for conservation purposes. The first experimental chapter of this thesis is dedicated to a comprehensive disease risk analysis in collaboration with experts from around the globe with the aim of collating and analysing the current knowledge on sea turtle diseases and ranking the priorities for research and conservation.

Although one of the most debilitating diseases of sea turtles, fibropapillomatosis (FP), is believed to have a viral cause (Jones *et al.,* 2016), the slow progress in the field of sea turtle virology is apparent by comparing comprehensive reviews published 9 years apart by Manire *et al.* and Alfaro *et al.* (Manire *et al.,* 2017; Afaro *et al.,* 2008). Only five families of viruses have so far been described in sea turtles and the link to pathogenesis and disease manifestation for these viruses is not fully understood (Mashkour *et al.,* 2018, Marschang, 2011). One reason behind the knowledge gap in sea turtle virology is the lack of suitable methods for viral diagnosis in these animals. Hence, the second experimental chapter focuses on virological method establishment with the aim of studying the presence and impacts of viruses in green turtles from the Great Barrier Reef.

1.2. Method establishment to study sea turtle viruses

In general, reptilian virology is in its nascent stages with a growing interest in discovery of novel viruses (Rivera *et al.,* 2009; Lang *et al.,* 2011; O'Dea *et al.,* 2016; Mashkour *et al.,* 2018), pathogenesis analysis (Work *et al.,* 2004; Kang *et al.,* 2008; Maclaine *et al.,* 2018) and establishing diagnostic tools (Hyndman and Shilton 2016). However, limited funding impedes research and development of specific methods

for reptilian virology, although it is important to test reptiles (especially pets) for infectious diseases (Rivas *et al.,* 2014) and to increase the awareness about health and hygiene of reptilian pet collections (Carmel and Johnson, 2014). The current methods for studying reptilian and sea turtle viruses are adapted from the procedures developed for human and mammalian species, but in most cases specific adjustment and modification are required simply because of the natural differences between ectothermic and endothermic animals. Due to their economic importance in fisheries and aquaculture, diagnosis of viral diseases in fish has a growing commercial focus, which has benefitted the study of viruses in other ectothermic hosts (Chinchar, 2011).

The early stages of reptilian virology were based on pathology and cell culture. However, since the early 2000s, advances in molecular techniques have played a crucial role in virology (Cann, 2001).

1.3. Histopathology

Outbreaks of gray patch disease (GPD), lung eye trachea disease (LETD) and mainly FP were the starting point for histopathology of infectious diseases in sea turtles. Histopathological findings of FP were firstly compared with herpesviruses in human and other animals as this was the first report of herpes-viral infection in sea turtles (Herbst *et al.,* 1999b). In green turtle FP, the hyperplasia of epidermis and proliferation in mesenchyme were similar to a neoplastic disease in horses caused by bovine papillomavirus types 1 or 2. Also, the proliferation of the dermis was similar to cattle and deer FP (Jacobson *et al.,* 1989). In these comparisons due to differences in the infected hosts minor variations were observed (Herbst *et al.,* 1999b).

The pathology of herpesvirus and papillomavirus viral infections in sea turtles have been described but tornovirus, retrovirus and betanodavirus sporadic reports are yet to be attributed to any disease state in marine turtles (Casey *et al.,* 1997; Herbst *et al.,* 1999b; Manire *et al.,* 2008; Ng *et al.,* 2009; Stacy *et al.,* 2008; Fichi *et al.,* 2016).

1.4. Cell culture

Mammalian cell lines are incubated at 37°C, which is a prohibitive temperature for cultivating many reptilian viruses. Cell lines originating from poikilothermic vertebrates such as fish, are more likely to support replication of reptilian viruses *in vitro* (Lu *et al.,* 2000, Leland and Ginocchio 2007) and have been used in attempts to propagate viruses isolated from sea turtles (Coberley *et al., 2002*). TH-1; ATCC No. CCL 50 is the only commercially available cell line from a turtle and was established from a

box turtle, *Terrapene carolina* (Clark *et al.,* 1967). This adherent cell line has been used to propagate different reptilian viruses such as adenovirus from corn snake (*Pantherophis guttatus*) (Juhasz *et al.,* 1993) and lung eye trachea virus (LETV) from green turtle (Curry *et al.,* 2000). A limited number of primary cultures originating from either terrestrial or marine turtles have been established (Koment *et al.,* 1982; Moore *et al.,* 1997; Lu *et al.,* 1999). LETV was the first virus to be isolated from sea turtles and the disease aetiology has been studied in great detail (Henle-Koch postulate has been fulfilled) (Coberley *et al.,* 2001). Despite several attempts the FP-associated herpesvirus (*Chelonid alphaherpesvirus 5* (ChHV5)) was not isolated until recently when Work and his colleagues successfully replicated ChHV5 in three-dimentional cell culture (Work *et al.,* 2017). These primary cultures are not available for commercial use and international transport of material originating from IUCN listed animals requires comprehensive permits (Mashkour *et al.,* 2018).

1.5. Molecular techniques

Conventional molecular methods were used for taxonomic identification of ChHV5 and ChHV6 (Herbst *et al.,* 1999a; Jacobson *et al.,* 1986; Lackovich *et al.,* 1999; Quackenbush *et al.,* 1998). The polymerase chain reaction (PCR) primers were designed based on the predicted homology of the turtle herpesvirus DNA polymerase gene and using the information published about mammalian herpes-viral DNA polymerase. PCR products were amplified from lesion or tumour samples and were subjected to sequencing and cloning in vectors (Queckenbuch *et al.,* 1998). This strategy did not work for papillomavirus; the degenerate PCR primers that successfully detected mammalian papillomaviruses were not suitable to diagnose reptilian papillomavirus in 1999 and 2000 (Brown *et al.,* 1999; Lu *et al.,* 2000).

Molecular techniques such as conventional reverse transcriptase were conducted in 1997 and led to discovery of retrovirus in green sea turtles (Casey *et al.,* 1997), although the sequence references are not available for further investigations. The first incidences of infection with loggerhead genital-respiratory herpesvirus (LGRV), loggerhead orocutaneous herpesvirus (LOCV) (Stacy *et al.,* 2008), *C. mydas* papillomavirus 1 (CmPV-1) and *C. caretta* papillomavirus 1 (CcPV-1) (Herbst *et al.,* 2009) were confirmed by PCR after histopathological investigation. Viral metagenomics approaches were used in discovery of sea turtle tornovirus 1 (STTV1). The virus was completely sequenced (Ng *et al.,* 2009). But similar to retrovirus the study was discontinued and there is not enough evidences to associate the virus to any diseases or to consider it commensal.

Molecular analyses have been used to survey the distribution of Chelonid fibropapilloma-associated herpesvirus (CFPHV) (unified as ChHV5) in sick and healthy marine turtles worldwide. In 2014, Alfaro-Núñez and his colleagues used singleplex PCR primers designed to target highly conserved regions for three different genes to screen sea turtles for the presence of ChHV5. They reported that the virus was present in all species of marine turtles if they were afflicted with FP and 15% of healthy turtles were also positive for ChHV5. They concluded that the disease may be a result of co-evolution with sea turtles and latent infection in turtles (Alfaro-Núñez *et al.,* 2014).

1.6. Viruses of green turtles

Of the seven species of sea turtles, green turtles have received the most attention in terms of viral infections. Different herpesvirus associated diseases such as GPD, LETD and FP were first described in green turtles (Lackovich *et al.,* 1999; Herbst *et al.,* 1999b; Aguirre and Lutz 2004; Santos *et al.,* 2015). Retroviruses were isolated from a group of FP-afflicted Hawaiian green turtles (Casey *et al.,* 1997). One of the papillomaviruses was found in a green turtle and was therefore named *C. mydas* papillomavirus 1 or CmPV1 (Manire *et al.,* 2008, Herbst *et al.,* 2009). The only case of tornovirus infection was discovered in a Florida green turtle (Ng *et al.,* 2009). Betanodavirus is the only sea turtle virus that was found in captured loggerheads, but this study examined only one green turtle (Fichi *et al.,* 2016).

As methodologies develop and scientists continue their quest for viral discovery, many more viral families are bound to be revealed in sea turtles and adding to our knowledge of sea turtle health and disease. To contribute towards the knowledge of health and diseases of sea turtles, especially viral infectious diseases several aims were set for this project.

1.7. The aims of the project

The aims of each experimental chapter were explained in the introduction and are listed here:

1. To do a structured sea turtle Disease Risk Analysis (DRA) in collaboration with the experts in the field (Chapter three)

2. To develop and assess virological methods to study the presence and impacts of viruses in green turtles from the northern Great Barrier Reef (Chapter four)

3. To characterise the Australian *Chelonia mydas* papillomavirus isolates (Chapter five)

4. To determine the level of co-infection of ChHV5 and CmPV in fibropapillomas and normal skin biopsies of green turtles from the northern Great Barrier Reef (Chapter six)

2. LITERATURE REVIEW

The Great Barrier Reef (GBR), the world's largest coral reef, stretches 2,300 km along the Queensland coast and is listed under Natural World Heritage for its outstanding universal value (GBRMPA, 2018). It has long been known to and used by the Aboriginal Australian and Torres Strait Islander peoples (GBRMPA, 2018). Sea turtles are of great value for indigenous cultures as well as sustainable ecotourism (Tisdell & Wilson, 2002). The Great Barrier Reef is home to six of the seven species of sea turtles: Green, Loggerhead, Hawksbill (*Eretmochelys imbricata*), Flatback (*Natator depressus*), Olive ridley (*Lepidochelys olivacea*), and Leatherback turtle (*Dermochelys coriacea*) (GBRMPA 2018). Unfortunately, the population of sea turtles has declined due to direct and indirect interventions (Troëng & Rankin, 2005). The International Union for Conservation of Nature (IUCN) has listed five of these sea turtles on the IUCN Red List of Threatened Species while the flatback turtle (*Natator depressus*), is reported as "*Data Deficient*" (IUCN, 2015). A green turtle (*Chelonia mydas*) is shown in figure 2-1.

Figure 2-1 A juvenile green turtle (Chelonia mydas), courtesy: Triggerfish images

Sea turtles are generally hard to survey due to their complex life history and the distances they migrate (Jensen, 2010). Turtles face natural predation throughout their lives from both terrestrial and marine predators (foxes, goannas, birds, sharks and fish). In addition, intentional hunting by humans as well as fisheries by-catch may cause a mortality rate of up to 39%. There are many more indirect effects influencing their survival including coastal development, light, pollution and boat strike (Department of the Environment, 2008).

Disease outbreaks also contribute to the morbidity and mortality of sea turtles. Turtles respond to stressful situations by a decrease in physiological and immunological function. Subsequently an increase in the incidence of different diseases such as bacterial, mycotic or viral infections is expected (Milton and Lutz, 2002; Jones, 2004).

2.1. Are infectious diseases a threat for endangered animals?

Infectious diseases, whether caused by bacteria, fungi, parasites or viruses, can be a critical threat to some species and they are listed among the top five reasons for global species extinction (Smith *et al.,* 2006; Wiethoelter *et al.,* 2015). Emerging infectious diseases are threats to both human and animal populations, although in most studies the knowledge about transmission pathways, infection impacts and dynamic interfaces between species is limited (Wiethoelter *et al.,* 2015). Emerging infectious diseases have also been confirmed to be increasing globally (Stevenson and Firestone, 2015; Wiethoelter *et al.,* 2015) and can be the sole or contributing threats for wildlife, although in many cases the disease aetiolgy is not well defined (Smith *et al.,* 2006).

The pillars of the current global strategy for limiting the impacts of infectious diseases in humans and animals are monitoring and reporting via veterinary and medical services (Deem *et al.,* 2001; Troëng and Rankin, 2005). Such surveillance is highly efficient and applicable for humans and livestock, where regular access and cooperation is assumed. Due to challenges of sampling in remote locations, these monitoring strategies are not feasible for wildlife (Daszak *et al.,* 2000).

It is difficult to track aetiopathogenesis of infectious disease for extinct species prior to 1800 due to the lack of baseline data on the role of infectious disease in extinctions prior to this date (Smith *et al.,* 2006). However, high rates of emerging infectious disease is a concern for current endangered species (Smith *et al.,* 2009; Smith *et al.,* 2006). To address this issue the disease drivers and contribution of other threats should be defined (Smith *et al.,* 2009), because stressors such as habitat loss, climate change and overexploitation have been shown to increase the negative impact of disease at population levels (Daszak *et al*., 2000; Smith *et al.,* 2009).

Generally two hypotheses describe the emergence of infectious diseases: 1) Novel pathogen hypothesis; and 2) Endemic pathogen hypothesis (Rachowicz *et al.,* 2005). The novel pathogen hypothesis considers the exposure of naïve individuals to infectious diseases, leading to observation of infection, while the endemic pathogen hypothesis emphasises pathogenicity increase or transmission possibilities when infectious agents are already present. In both scenarios, background knowledge about possible pathogens of vulnerable species is required (Rachowicz *et al.,* 2005). This poses a challenge for sea turtles as reptiles have rarely been the topic of conservation efforts or thorough health investigations due to relatively low commercial importance and problematic husbandry and sampling (Ariel, 2011).

2.2. Why study sea turtle viruses?

In this project, the focus is on viruses of sea turtles because of personal interests and also because a review of the literature shows that knowledge about viruses of sea turtles has improved little in the last 30 years:

Other research has examined viral infections in chelonians and found that reported chelonian viruses were from heterogeneous families and therefore classification was difficult (Jacobson *et al.,* 1982; Ahne, 1993). In 1993, Ahne categorised chelonian viruses into two groups: arthropod transmitted and chelonian originated viruses. In his classification, arthropod transmitted viruses found in chelonians consisted of toga-, flavi-, rhabdo- and bunyaviruses, and chelonian originated viruses included papova-, herpes-, irido- and paramyxoviruses. In the case of arboviruses, the interest in poikilothermic animals has historically been related to virus overwintering during the hibernation periods of poikilothermic vertebrates and its emergence the following spring. They concluded that turtles and other ectotherms were possible reservoirs for arboviruses. In the second category where viruses specifically targeted chelonians, the most important pathogens were herpesviruses. Herpesviruses have been reported to impact chelonia and have caused epizootic incidences, but in many cases the aetiological roles of herpesviruses were not clear (Ahne, 1993).

In addition to Ahne's classification, other sporadic investigations reported viruses such as: Poxvirus attribution in an amelanotic Californian desert tortoise (*Xerobates agassizi*); pox-like viral infection in a captive Hermann's tortoise (*Testudo hermanni*); papilloma-like infection in Bolivian side-neck turtles (Jacobson *et al.,* 1982); papilloma-like particles in a Russian tortoise (*Agrionemys horsfieldii*) (Drury *et al.,* 1998); and evidence of retrovirus in a green sea turtle (Casey *et al.,* 1997).

Since 2000, adenovirus infections have been documented in turtles (Wilkinson *et al.,* 2004; Farkas and Gál, 2009; Rivera *et al.,* 2009). The majority of adenoviruses described in chelonians have clustered together and are hypothesised to represent a new genus: proposed "*Testadenovirus*" (Doszpoly *et al.,* 2013). Molecular based virological studies also revealed two Papillomaviruses in *C. mydas* and *C. caretta*. These two viruses were shown to be distinct from other papillomaviruses and they were also different from one another (Manire *et al.,* 2008). Paramixoviruses of the genus *ferlavirus* were characterised in a range of reptilian species including from a Hermann's tortoise and a leopard tortoise with history of pneumonia (Marschang *et al.,* 2009; Papp *et al.,* 2010). A single report of reovirus infection in a spur-thighed tortoise and several incidences of picornaviruses in different European tortoises have also been reported (Marschang, 2011).

At present, the most important infectious diseases caused by viruses that are known to afflict turtles are suggested to be caused by herpesviruses and ranaviruses. Herpesviruses are responsible for direct losses in turtles and tortoises, although the transmissions are under investigation (Ariel, 2011; Marschang, 2011). Iridoviruses of the genus *Ranavirus* have also caused mortality and morbidity in tortoises (Ariel, 2011). Although the short- and long-term effects on chelonian populations are unknown, according to the loss of adult females and the fact that tortoises reproduction is slow, viral infection can pose a possible risk to populations over time (Farnsworth and Seigel, 2013). The growing interest in discovery of novel viruses in reptiles (Rivera *et al.,* 2009; Lang *et al.,* 2011; O'Dea *et al.,* 2016; Zhang *et al.,* 2018; McKenzie *et al*., 2019) is a driver to study more reptilian hosts, including turtles, and to establish effective diagnostic tools (Hyndman and Shilton, 2016).

2.3. The current information about viruses of sea turtles

Techniques used in the Identification of viruses in sea turtles and the regions where they were isolated vary. Table 2-1 lists five families of sea turtle viruses, as discussed above, techniques used to identify them and the geographic region where the turtle was examined. As can be seen from the table, there is no single identification technique used in all viral identification, although PCR is the most common and most common regions are around the United States of America. There is still uncertainty in proposing these viruses as the main reason leading to the diseases. In some cases only one or two turtles were investigated.

Table 2-1 Viruses of sea turtles reported in the literature

Virus Family	Nomenclature	Identification Techniques						Reported regions and Species	Key References
		C	T	P	M				
					PCR	Sequencing	Protein Analysis		
Herpesviridae	Chelonid fibropapilloma-associated herpesvirus (CFPHV) (*Chelonid alphaherpesvirus 5*)	✓ ?	✓	✓	✓	✓		Reported worldwide in tropical waters and captivity; all seven species of sea turtles	(Smith and Coates, 1938; Harshbarger, 1991; Limpus *et al.,* 1993; Barragan and Sarti, 1994; Aguirre *et al.,* 1999; D'Amato and Moraes-Neto, 2000; Huerta *et al.,* 2002; Jones *et al.,* 2016)
	Gray-patch disease (GPD) (Chelonid herpesvirus 1)		✓					Cm	(Haines and Kleese, 1977; Mahy, 2009).
	lung, eye, trachea disease (LETD) (Chelonid herpesvirus 6)	✓	✓	✓	✓	✓			(Coberley *et al.,* 2002; Klein and Jacobson, 2001; Ritchie, 2006)
	loggerhead genital-respiratory herpesvirus (LGVR)				✓	✓		Florida Cc	(Stacy *et al.,* 2008)
	loggerhead orocutaneous herpesvirus (LOCV)				✓	✓			
Papillomaviridae	*Chelonia mydas* papillomavirus 1 (CmPV-1)			✓	✓	✓		East Central Coast of Florida Cm	(Herbst *et al.,* 2009; Manire *et al.,* 2008)
	Caretta caretta Papillomavirus 1 (CcPV-1)			✓	✓	✓		North East Florida Cc	
	?		✓		✓				(Lu *et al.,* 2000)
Not assigned to a family	sea turtle tornovirus 1 (STTV1)				✓	✓		Lake Worth Lagoon, FL Cm	(Ng *et al.,* 2009)
Retroviridae			✓		✓		✓	Hawaiian islands Cm	(Casey *et al.,* 1997)
Nodaviridae	Betanodavirus	✓			✓			Cc	(Fichi *et al.,* 2016)

*C: Cell culture; T: TEM; P: Pathology; M: Molecular; PCR: Polymerase chain reaction; Cm: *Chelonia mydas*; Cc: *Caretta caretta*

2.3.1. Herpesviruses

Herpesviruses are large DNA viruses (100-200 nm in diameter) that infect a broad range of species and are well-known to cause latent infections in survivors from the first infection (Hernandez-Divers, 2006). Immunocompromised hosts are reported to experience severe infections and secondary bacterial or viral infections are common (Ritchie, 2006). Herpesviruses can cause different disease presentations in chelonians and are frequently observed in animals facing stressful situations and with lower immune responses (Kirchgessner and Mitchell, 2009). Despite various studies, the aetiology of herpesvirus associated diseases in sea turtles are not yet well understood (Chaves *et al.,* 2013; Coberley *et al.,* 2002).

The assessment based on partial DNA polymerase gene sequences puts all chelonian herpesviruses in a monophyletic clade within alphaherpesviruses. Five genera has been described in this subfamily and the genus *Scutavirus* is the only one described that includes a chelonian virus: *Chelonid alphaherpesvirus 5* (ChHV5) which is the type species of the genus (ICTV, 2015). The phylogenetic analysis so far has suggested the coevolution of chelonian hosts and herpesvirus lineages (McGeoch and Gatherer, 2005).

2.3.1.1. Chelonid herpesvirus 1; Gray-patch disease associated virus (GPD)

Gray patch disease manifests as grayish lesions on the head, neck and flippers of green sea turtles in captivity. GPD is a disease of reared green turtles aged between 6-8 weeks and 1 year (Haines, 1978). Stressed animals are prone to clinical manifestations. Overcrowded hatchling environment and elevated temperature could lead to clinical signs in captivity (Haines and Kleese, 1977).

Inoculation of naïve sea turtles with bacteria-free GPD patches led to clinical signs in 100% of the inoculated turtles (Haines, 1978). Electron microscopy showed 160-180 nm virus particles with distinct nucleocapsids and envelopes. Virus isolation in cell culture was unsuccessful. GPD-associated virus was named chelonid herpesvirus 1 (ChHV1) with unknown genus (Mahy, 2009).

2.3.1.2. Chelonid alphaherpesvirus 5; Fibropapilloma associated herpesvirus[1]

Fibropapillomatosis (FP) has been documented worldwide (Lackovich *et al.,* 1999; Quackenbush *et al.,* 1998) and in all seven species of sea turtles (Jones *et al.,* 2016). Juvenile turtles are more prone to showing the signs and sex has not been shown to be a contributing factor in disease prevalence (Work *et al.,* 2004). FP is a neoplastic disease, internal and external tumours develop in turtles and are categorised as forms of papillomas, fibropapillomas and fibromas (Figure 2-2) (Kang *et al.,* 2008). These benign tumours can affect the general health of the turtle and are not considered just cosmetic problems. In severe cases, vision and locomotion may be interfered with or normal organ functioning if visceral tumours develop (Lu *et al.,* 2000; Ng *et al.,* 2009). In addition, affected turtles are expected to have an increased vulnerability to secondary or opportunistic pathogens (Curry *et al.,* 2000). Work *et al*. (2004) on the other hand, considers the disease to adversely affect the turtle if there is a pre-existing condition of anaemia, immunosuppressive diseases and bacterial infection. In either case, whether FP is a trigger of immunosuppression or if it is primarily manifested in turtles with pre-existing conditions of immunosuppression, turtles affected by FP are often found with other ailments.

Figure 2-2 A green turtle with external tumours being examined and sampled by Narges Mashkour (Courtesy of Karina Jones)

[1] Other nomenclature: Green turtle fibropapillomatosis-associated herpesvirus (GTHV) or Fibropapilloma-associated turtle herpesvirus (FPTHV) or *Chelonia mydas* herpesvirus or *Caretta caretta* herpesvirus

Research to date has suggested a herpesvirus as the aetiological agent for FP (Mansell *et al.,* 1989; Lackovich *et al.,* 1999; Kang *et al.,* 2008), other contributing factors are not known. The impact of climate, pollution and seasonality are still unclear (de Deus Santos *et al.,* 2015). The virus has been un-cultureable (Ackermann *et al.,* 2012; L. Herbst *et al.,* 1995) until recently when it was propagated *in vivo,* in three-dimensional skin grafts (Work *et al.,* 2017).

2.3.1.3. Chelonid herpesvirus 6; Lung-eye-trachea associated herpesvirus (LETV)

Lung-eye-trachea disease is characterised by caseous debris over the respiratory tract and exudate covering the eyes. These signs develop over two to three weeks. Harsh respiratory sounds and buoyancy problems are also reported in infected turtles. The disease is generally diagnosed in turtles more than one year old (Jacobson *et al*., 1986; Coberely *et al.,* 2001; Marschang and Diver, 2014).

In 1986, the virus was isolated from an infected green turtle (Jacobson *et al*., 1986) and then propagated in terrapene heart cells (TH-1; American Type Culture Collection CCL 50). LETV is the only sea turtle herpesvirus to be isolated in cell culture (Coberely *et al.,* 2001)

Transmission electron microscopy has revealed the presence of herpesvirus virions in lesions (Rebell *et al*., 1975). The disease was first described in captivity, but in 2001 Klein and Jacobson described the disease in free ranging turtles (Coberley *et al.,* 2002; Klein and Jacobson, 2001). The disease mortality rate is higher than GPD. LETV is shown to be stable and infectious after exposure to salt water for a long time (Curry *et al.,* 2000).

2.3.1.4. Loggerhead genital-respiratory herpesvirus (LGVR) and loggerhead orocutaneous herpesvirus (LOCV)

Two other herpesvirus related diseases were found in six moribund Atlantic loggerhead turtles (Stacy *et al.,* 2008). The turtles were found stranded in different regions of Florida, USA, during 2001 and 2006 and after unsuccessful rescue the carcasses were investigated to determine the cause of stranding. The common clinical signs were oral, respiratory, genital, and cutaneous lesions. The lesions of six necropsied turtles were investigated by histopathology and molecular techniques (Stacy *et al.,* 2008).

Two herpesviruses were found, one was associated with lesions in genital organs and respiratory tracts (loggerhead genital-respiratory herpesvirus (LGVR)) and the other caused ulcers in oral cavity

and skin (loggerhead orocutaneous herpesvirus (LOCV)). These viruses are genetically similar, but LGVR contains a conserved region that is considered significant enough by Stacy *et al.* (2008) to report them as two different alphaherpesviruses.

2.3.2. Papillomaviruses

Papillomaviruses are small (45-50 nm) DNA viruses with (7–8 kb) circular genomes. These non-enveloped, icosahedral viruses form highly diverse groups. Papillomaviruses are generally host-specific pathogens, many species causing benign muco-cutaneous and cutaneous epithelial proliferation, and skin tumours (papillomas) Herbst *et al.,* 2009). Transmission is horizontal and viruses are persistent in the environment. Papillomaviruses are difficult to isolate in cell culture and identifications are generally based on molecular methods directly from lesions (Ritchie, 2006).

2.3.2.1. Chelonia mydas PV (CmPV-1) and Caretta caretta PV (CcPV-1)

Papillomaviruses have been described in two sea turtles: green (*C. mydas*) and loggerhead (*C. caretta*) turtles. Both cases were found stranded in the Florida region, USA. The green turtle was afflicted with FP and underwent surgery to remove the tumours. Both cases showed cutaneous white lesions several weeks after admission to the Sea Turtle Rehabilitation Hospital, Sarasota, Florida. Lesion biopsies were taken for histopathology, TEM and molecular investigations. The lesions of both turtles healed while still in care and those overlying hard surfaces left indented scars (Manire *et al.,* 2008).

Histopathology revealed areas of hyperplasia and cell degeneration in the epidermis. Nuclear changes and eosinophilic inclusion bodies were also noted in biopsied lesions. Virions (50-55 nm) were observed by electron microscopy. These findings were consistent with molecular analysis and proved the existence of papillomaviruses in skin lesions. Papillomaviruses were detected in samples using polymerase chain reaction (PCR) and further analyses of papillomavirus E1 gene through sequencing and phylogeny studies proposed two distinct viruses (*C. mydas* PV (CmPV-1) and *C. caretta* PV (CcPV-1)) in the family *Papillomaviridae* (Manire *et al.,* 2008).

In their following study, Herbst *et al.,* provided supplementary information about CmPV-1 and CcPV-1 and elucidated the genomics characterisation. These two sea turtle PVs have the smallest genome among PVs. CmPV-1 and CcPV-1 were included with the non-mammalian amniote papillomaviruses. They are closely related to avian PVs but form different clades (Herbst *et al.,* 2009).

1.3.2.2. Un-specified papillomavirus report

Prevalence of papillomaviruses in sea turtles was examined in cell cultures established from an FP afflicted turtle. In that study, the researchers induced tumour-like aggregates in healthy cells derived from the turtle using cell-free media from tumourous-like cells. Papova-like particles were detected in the tumourous cells and suggesting a possible correlation between papova-viruses and disease (Lu *et al.,* 2000). The term papova is no longer used in taxonomy, the family is divided into *papillomaviridae* and *polyomaviridae* (Van Regenmortel *et al.,* 2000).

2.3.3. Retroviruses

Retroviruses are enveloped viruses, 95-110 nm in diameter and consist of single stranded RNA molecules. The effect of infection on the hosts varies from zero clinical signs to benign neoplasia and acute neurologic diseases. The horizontal transmission occurs with direct contact between the animals, aerosol and excrement. Overcrowding and stress appears to enhance infection susceptibility (Hernandez-Divers, 2006).

The interest in surveying retroviruses in FP afflicted turtles arose from their tumour-inducing nature. The presence of reverse transcriptase is an indicator for retrovirus infection. To study reverse transcription, samples were purified via sucrose gradient, and examined with polymerase enhanced reverse transcriptase (PERT). PERT analysis confirmed positive reverse transcriptase activity in all samples. Conventional reverse transcriptase assay showed quantitative results with expected high activity in tumourous tissue samples. Protein analyses and electron microscopy supported the evidence for the presence of retroviruses in green turtles. This survey indicated that green turtles may be a host for retroviruses. Reverse transcriptase activity was higher in FP turtles but the information was insufficient to confirm the correlation between FP disease and retrovirus infection (Casey *et al.,* 1997).

2.3.4. Tornovirus

Tornoviruses are small single stranded DNA viruses with partial capacity to code proteins. These viruses encounter rapid evolutionary changes and may reproduce new single stranded DNA viruses as possible eukaryotic pathogenic viruses (Rosario *et al.,* 2012). The tornoviruses found in sea turtles were first reported as a subfamily of viruses in the family *anelloviridae*, which was highly diverged

from there rest of viruses in this family (Ng *et al.,* 2009). The virus is now reported as an unclassified single stranded DNA virus (GenBank: EU867816.1).

Metagenomic analyses have been used to study single stranded viruses (Rosario *et al.,* 2012). Virion purification and shotgun sequencing was used by Ng *et al.,* and led to the discovery of sea turtle tornovirus 1 (STTV1). Direct samples were collected from 27 FP-afflicted green turtles and 35 healthy turtles. Two turtles were found positive for STTV1 and viral particles were detected in blood and major organs. Sea turtle tornovirus 1 is not associated with FP disease, but this virus may act as an opportunistic, secondary pathogen (Ng *et al.,* 2009).

2.3.5. Betanodavirus

Betanodaviruses are non-enveloped, single stranded RNA viruses causing nervous system disease in marine and freshwater fish and growing numbers of hosts are showing susceptibility to this virus. A worldwide distribution has been reported for the virus. The virus was detected by electron microscopy and molecular methods as well as immunological assays; the virus has been cultured in several cell lines (Shetty *et al.,* 2012).

In 2016, betanodavirus was found in four loggerhead turtles stranded along the coast of Tuscany in the Mediterranean Sea. The findings suggested sea turtles as possible carriers for fish pathogens, although there is no evident pathogenic effect on examined sea turtles (Fichi *et al.,* 2016).

2.4. Methods for surveying viruses in sea turtles

Gradual improvements from the discovery of tissue culture and viral proliferation *in vitro* to state-of-the-art molecular methods have shaped virology and facilitated viral discoveries. Conventional methods and modern biological techniques play complementary roles to isolate, identify, and characterise viruses (Mokili *et al.,* 2012).

In this section, the virological methods and their application in sea turtle virology is reviewed.

2.4.1. Transmission electron microscopy (TEM)

Transmission electron microscopy is used to visualise objects in the realms of micro (1 micron/1µm) to nano (1 nanometer/nm) using an intensive beam of high energy electrons. In 1938, Ruska *et al.,* visualised tobacco mosaic virus with EM. Since then electron microscopy has led to new viral discoveries and confirmations. Early viral classifications were highly dependent on morphological evidence shown by EM (Goldsmith and Miller, 2009).

Even today, viruses are demonstrated with EM figures in taxonomy reports. Table 2-2 compares different virological methods with TEM in terms of viral detection feasibility. Some techniques require probes or reagents to study a virus, and when investigating a disease caused by unknown viruses it is difficult to select the appropriate reagents. TEM is used to provide a general overview of viruses present in the infected tissue but is not used to define the viruses beyond the family level (Goldsmith and Miller, 2009), because viruses may share the same morphology even if they are from completely different genera.

*Table 2-2 Comparison between TEM and other virological methods**

	Cell Culture	Serology	Pathology	Molecular Techniques
Advantages of TEM in comparison with other virological methods	1. To do TEM there is no need for live viruses but cell culture propagates live cells 2. TEM can detect viruses in samples preserved in unknown solutions after long time	1. TEM can detect viruses in samples preserved in unknown solutions after long time 2. TEM does not require specific reagents/probes 3. TEM helps in bservation of whatever might be present in the sample	TEM can detect viruses in samples preserved in unknown solutions after long time	1. TEM can detect viruses in samples preserved in unknown solutions after long time 2. TEM does not require specific reagents/probes 3. TEM helps in bservation of whatever might be present in the sample
Disadvantages of TEM in comparison with other virological methods	1. Cell culture is cheaper than TEM 2. Cell culture requires less system maintenance and is not sensitive	1. Serology is cheaper than TEM 2. Serology requires less system maintenance and is not sensitive	1. Patholgy is cheaper than TEM 2. Pathology requires less system maintenance and is not sensitive	1. TEM is not assisting in defining the virus beyond the family level 2. Molecular techniques are cheaper than TEM

		3. Serology requires less training to avoid image artifacts		
Contributions of TEM to other methods	TEM is used to visualise cultured viruses		TEM can assist in the confirmation processes after pathological analysis	TEM can assist in the quality control processes after using molecular techniques

*Adapted from "*Modern Uses of Electron Microscopy for Detection of Viruses*" by Goldsmith *et al.,* 2009, Clinical Microbiology Reviews, 22, 552-563; and "*Applications of transmission electron microscopy to virus detection and identification*" by Vale *et al.,* 2010, Microscopy: Science, Technology, Applications and Education, 128-136.

2.4.1.1. TEM and sea turtle virology

Chelonian virology, like other fields of virology, benefits from TEM. The first virus of sea turtles visualised by electron microscopy was associated with gray patch disease. Samples were collected from scraping lesions and viral particles were observed by TEM (Rebell *et al*., 1975).

FP causes lesions with intracytoplasmic vacuoles. In 1989, Jacobson *et al.,* observed electron-dense particles (155 to 190 nm) in the vacuoles within epidermis (Jacobson *et al.,* 1989). Previous surveys to visualise FP-associated virus by light microscope and HE staining revealed intracellular inclusions. These findings were consistent with TEM pictures of GPD-associated herpesviruses. Jacobson *et al.,* reviewed previous electron microscopies on reptilian herpesviruses to confirm the connection between herpesviruses and the intracytoplasmic particles they found (Jacobson *et al.,* 1989; Rebell *et al*., 1975).

Electron microscopy requires specific skills in sample preparation and micrograph analysis. In the first analysis of FP, Jacobson *et al.,* evaluated the graphs in comparison with normal skin electron microscopy (visualised by Matoltsy and Huszar in 1972). Even the normal epidermis contains various granules merging in cytoplasmic zone and releasing into the extracellular margins. These granules are mucous vacuoles that form the stratum corneum and are not related to viral infections. These granules appear to have electron dense centres. In some cases the observed particles were herpesviruses of 155-190 nm in diameter (Jacobson *et al.,* 1989). Normally TEM is coupled with molecular techniques for further investigations of the suspected virus.

2.4.2. Pathology

Pathology is a key tool in the early recognition and control of infectious disease outbreaks. Studying the morphological changes occurring at different stages of infection can increase our understanding of the pathogenesis for that infection. Although morphologic alterations do not uncover all the reasons of how and why disease occurs, data generated by other virological methods can remain meaningless without pathology (Caswell and Callanan, 2014). The knowledge of veterinary pathology is limited in exotic animals such as reptiles. Although, research on reptilian pathology occurred in the late 19th century, the progress has been gradual and more work is required (Hoff, 2012).

2.4.2.1. Pathology and sea turtle virology

The principles of reptile necropsy are the same as for other species: a systematic anatomy method, attention to detail, analysing and sampling tissues with unusual appearance. The information acquired during necropsy sessions is followed up with histopathology of samples (Hanley and Hernandez-Divers, 2003). For sea turtles, histopathological analysis of infectious diseases increased greatly with the outbreaks of GPD, LETD and mainly FP (Rebell *et al*., 1975; Jacobson *et al*., 1986; Herbst *et al*., 1999b).

Previous incidences of infectious diseases are valuable references during carcass and sample investigation. Pathological findings of FP were firstly compared with herpesviruses in humans and other animals (due to lack of pathological references in sea turtles). Although minor differences in manifestation existed between species, degenerative changes and cleft formation in green turtles were similar to those described in erythema multiforme, which is caused by a herpesvirus infection in humans (Herbst *et al.,* 1999b). The epidermal hyperplasia and mesenchymal proliferation in green turtle FP was similar to Sarcoid, a neoplastic disease of horses which is caused by bovine papillomavirus types 1 or 2. The morphology of the dermal proliferation was also similar to cattle and deer FP. However, in all cases minor differences were observed, such as "*koilocytotic atypia*" in mammalian papillomavirus infections that is absent in FP (Jacobson *et al.,* 1989).

Herbst *et al.,* examined large numbers of spontaneous and experimentally induced green turtle FPs from Hawaii and Florida (n=247). The main reason for this study was to clarify the histological features of FP and its aetiological agent (Herbst *et al.,* 1999b). The first virological infections in a new species or region are normally described by histology. The uniformity of findings when compared to previous reports of the disease in other species or regions helps confirm the presence of a viral disease in new

cases. In 2012, FP was described for the first time in Principe Island, West Africa. The histological features were consistent with those observed elsewhere (Duarte *et al.,* 2012).

Sea turtle herpes and papilloma-viral infections have been documented with pathological features, but tornovirus, retrovirus and betanodavirus sporadic reports are not yet attributed to any viral diseases in sea turtles (Casey *et al.,* 1997; Herbst *et al.,* 1999a; Manire *et al.,* 2008; Ng *et al.,* 2009; Stacy *et al.,* 2008).

2.4.3. Cell based viral isolation

The history of growing viruses in cell culture dates back to 1913, when vaccinia virus was successfully propagated in rabbit cornea cells for 34 days and reported to remain active throughout this time (Steinhardt *et al.,* 1913). Since then different viruses have been grown in cells for the purpose of identification and live vaccine production. Cell culture refers to the *in vitro* proliferation and maintenance of eukaryotic cells, specifically of animal origin under sterile laboratory conditions and specific pH, temperature, and growth conditions (Helgason and Miller, 2005). Cell culture is a successful *in vitro* method which offers an alternative to *in vivo* laboratory experiments (Zurlo *et al.,* 1994).

Diagnostic virology has classically relied on cell culture because viruses can only replicate in live cells. Cultured cells can amplify the viruses and may exhibit cytopathic effects (CPE) in the form of cell death which can be detected microscopically (Leland and Ginocchio, 2007).

Animal cell culture like most biological experiments has a component of failure, success and development. Recently numerous innovations have occurred in culture formats and technologies:

2.4.3.1. Centrifugation-enhanced inoculation

This process enhances cell infection by low speed centrifugation i.e. two rounds per minute. Different viruses have been shown to propagate faster by means of spinoculation. However, the mechanism of accelerated infectivity in this inoculation system has been questioned. A group of scientists believe that centrifugation stresses the monolayer cells and increases cell susceptibility (Leland and Ginocchio, 2007). Others have focused on viral fusion enhancement or viral deposition on the surface of monolayers (O'Doherty *et al.,* 2000).

2.4.3.2. Pre-CPE assays or NON-CPE

Conventional cell culture assay relies on observing the CPE caused by viral infection. The CPE-independent technique involves detecting the infection before or in the absence of CPE. The infected monolayer is stained with immunofluorescent antibodies to detect specific antigens in the culture. Pre-CPE assays enable cell culture to specifically detect a virus of interest in a shorter time or a virus that does not cause CPE (Leland and Ginocchio, 2007).

2.4.3.3. Co-cultivated cell lines

Co-cultivation of established cell lines increases the host diversity for viral propagation. Multicellular cultures express certain cell-surface viral receptors and mimics the three-dimentional (3D) model of body tissues. The cells are normally genetically close enough to form a stable culture system (Straub *et al.,* 2007). Co-cultivation is even being commercialised. R-Mix is a hybrid cell line of A549 and mink lung (Mv1Lu) cells which is successfully propagating respiratory system viruses (Leland and Ginocchio, 2007).

2.4.3.4. Transgenic cell lines

Genetically modified cells embrace cellular pathways favoured for viral replication. The modification should be stable, specific and detectable. Transgenic cells have been successfully implemented in clinical virology (Leland and Ginocchio, 2007). Horvat *et al.,* generated transgenic mice cells expressing human measles viral receptors. The lung and kidney cells permissively supported viral growth and emphasised the benefits of using specific host cell-type in cell culture systems (Horvat *et al.,* 1996).

2.4.3.5. Cell culture and sea turtle virology

Cell lines established from lower vertebrates are more likely to support the cultivation of reptilian viruses. Furthermore, many of reptilian viruses grow at temperatures which are incompatible with the 37°C required for a mammalian cell culture (Coberley *et al.,* 2002).

Cell lines of chelonian origin are limited. Different laboratories have established primary cultures and published sporadic viral isolations in these cells. The only commercially available chelonian cell line is

TH-1 (TH-1; ATCC No. CCL 50) which was established from the heart tissue of a box turtle, *Terrapene carolina*. Clark and Karzon established this epithelial, adherent cell line in 1967 (Clark and Karzon, 1967). TH-1 has been used to propagate different reptilian viruses such as adenovirus from corn snake (*Pantherophis guttatus*) (Juhasz and Ahne, 1993). LETV was also propagated in this cell lines (Curry *et al.,* 2000) after being first isolated in primary sea turtle embryo fibroblastss (Jcobson *et al.,* 1986). The disease aetiology of LETV was studied in details because of the ability of TH-1 to support the growth of the virus (Coberely *et al.,* 2001).

Several scientists have trialed culture innovations to isolate viruses of sea turtles especially ChHV5 (Curry *et al.,* 2000; Jacobson *et al.,* 1986). Coberley *et al.,* established several cell lineages from FP-tumours of green sea turtles (Coberley *et al.,* 2002). To propagate FP-associated herpesvirus, Coberley *et al.,* have experimented with cell culture techniques such as co-culture and cell transfection. Co-culture target cells were green turtle, loggerhead turtle and gopher tortoise whole embryos cell line, a green turtle embryo kidney cell line and the TH-1 line. All attempts (more than 300) to cultivate the FP-associated herpesvirus were unsuccessful. The positive control for these experiments was LETV which was successfully isolated in all cell culture experiments (Coberley *et al.,* 2002). ChHV5 has been recently propagated *in vivo* using green turtle skin grafts (Work *et al.,* 2017). This was a valuable step towards isolating ChHV5 in vitro after years of trial and error.

Cultivating LETV in cell culture led to antigen preparation for immunogenicity studies and detecting antibodies in green sea turtles (Coberley *et al.,* 2001). Consequently, the antibodies of immune green turtles have facilitated identification of LETD-associated immunogenic proteins: glycoprotein B and a scaffolding protein (Coberley *et al.,* 2002).

2.4.4. Molecular techniques

Since the early 2000s, nucleic acid based viral detection has emerged as a molecular technique in clinical virology. The key advantages of this method are the non-invasive nature and there being no need for specific preservation of viable cells. The emergence of new pathogens and infectious disease outbreaks has increased the demand for rapid and accurate diagnostic methods. Therefore, improving nucleic acid amplification tests such as polymerase chain reaction provides a powerful tool to attain virological diagnostic goals (Domiati-Saad and Scheuermann, 2006; Espy *et al.,* 2006).

Molecular methods have a number of advantages over other viral diagnostic methods. Cell cultures are not available for all viruses and rely on viable viruses. Serology techniques can directly detect a viral infection but is generally less sensitive than molecular techniques. Nucleic acids are easily

purified, amplified and detected using molecular techniques (although, the word "*easy*" is subject to levels of skills, specimen type, and sampling and extraction methods) (Leland and Ginocchio, 2007). Molecular techniques generally comprise two different stages of wet laboratory and bioinformatics analyses. Each stage requires expertise and specific skills. Normally, the experts at each stage are expected to have the basic knowledge of the other technique processes to make clear statements regarding their results and to be able to troubleshoot.

2.4.4.1. PCR amplification

Polymerase chain reaction (PCR) is the most common molecular method to detect the nucleic acid of viruses. A pair of complementary oligonucleotide sequences (primers) is assigned to target specific sequences of DNA or RNA in the sample. Conventional PCR is combined with gel electrophoresis or hybridization techniques to identify the amplified sequences. Real-time PCR allows detection and quantitation of PCR amplicons after each cycle (Domiati-Saad and Scheuermann, 2006).

2.4.4.2. Cloning in vectors and sequencing

The cloning step is normally used for complex or unknown sequence to identify the genes and the amino acids they encode (Alberts *et al.,* 2002). In reptilian virology, cloning and sequencing the products have been used to characterise new viruses (Duncan *et al*., 2004; Doszpoly *et al*., 2013), and this technique is becoming less labour-intensive and more affordable.

2.4.4.3. Viral metagenomics

The viral metagenomic approach is known as an unbiased innovation in molecular methods to detect unknown viruses directly from various samples for instance seawater, faeces and blood. This method does not require a specific primer design for known sequences of viruses (Delwart, 2007). However, the sensitivity of viral metagenomics to detect low amounts of viruses emphasises the possibility of false positive results. PCR-based protocols are suitable backups to validate aetiological agents (Rosseel *et al.,* 2014). The first and most important step in viral metagenomics analysis is selective enrichment by viral DNA/RNA purification and removal of non-viral fragments. Therefore, peripheral blood

mononuclear cells and skin samples, in which separating viral DNA and host is difficult, are the first challenges in viral metagenomics trials (Delwart, 2007).

The second step in metagenomics is sequencing the extracted DNA. In early studies sequencing was followed by cloning and generation of shotgun libraries (Random breakage of a DNA molecule into small pieces to create multiple starting points for sequencing.). Subsequently, the whole DNA was sequenced using the Sanger enzymatic method. Different sequencing platforms have been developed and linked with the advantages of the Sanger method described in 1977 (Delwart, 2007). One such sequencing method is done via next-generation sequencing (NGS) platform which share the advantage of high-throughput, fast and automated systems. High-throughput sequencing does not necessarily require the cloning step. These technical advances have improved screening and allows analysis of dsDNA, ssDNA and RNA (Mokili *et al.,* 2012). The last step is to identify the generated sequences of viruses. This is where viral sequences are compared with databases of viral nucleotides or translated proteins. However, bioinformatic analysis is the most challenging step in metagenomics (Delwart, 2007; Mokili *et al.,* 2012).

Metagenomics requires accurate and sensitive programs. The assembly programs ignore single base mismatches. Since differences in single bases may represent unique sequences from novel viruses, this is a drawback for metagenomics. In addition, the large number of viral genomes, repeated sequences and background sequences of host species emphasises the need to choose the right algorithm and fast computation (Edwards and Rohwer, 2005).

2.4.4.4. Molecular methods and sea turtles virology

FP and LETD associated viruses were first detected by pathology and TEM and then identified using conventional molecular methods (Herbst *et al.,* 1999a; Jacobson *et al.,* 1986; Lackovich *et al.,* 1999; Quackenbush *et al.,* 1998). PCR products were amplified from lesion or tumour samples and have been subjected to sequencing and cloning in vectors. Generally the PCR primers were designed based on the predicted homology of the turtle herpesvirus DNA polymerase gene with other animals' herpesviruses. The experiments were successful and the general results indicated that FP and LETD associated viruses are two different herpesviruses (Quackenbush *et al.,* 1998).

The only report of retrovirus discovery in green sea turtles was conducted by molecular techniques and electron microscopy. Polymerase enhanced reverse transcriptase and protein analysis confirmed reverse transcriptase activity in samples (Casey *et al.,* 1997), although the sequence references were not present for further investigations.

FP has been of most concern for sea turtle virology. Molecular based epidemiology has been performed to unravel the FP disease causation and has rejected the hypothesis of incidental presence. (Lackovich *et al.,* 1999). Further studies to classify the virus, designated chelonian herpesviruses in a monophyletic clade of alphaherpesviruses. McGeoch *et al.* conducted phylogenetic analysis using neighbor-joining and Bayesian methods. Their results showed that ChHV5 forms an outgroup in alphaherpesviruses. Therefore, a new genus was suggested for chelonian herpes viruses: *Scutavirus* (McGeoch and& Gatherer, 2005; McGeoch and Davison, 2010). The documented sequences are public references for further studies and taxonomic comparisons (Ackermann *et al.,* 2012);

The first reports of LGRV, LOCV (Stacy *et al.,* 2008), CmPV-1 and CcPV-1 (Herbst *et al.,* 2009) in sea turtles were carried out by PCR and sequencing analysis. LGRV, LOCV are also assigned to alpha-herpesviruses after partially sequencing DNA-dependent-DNA polymerase genes. 6 moribund turtles were investigated in this study and the sequences are present in public databases: EU004541 (case 1), EU004539 (case 2), EU004540 (case 3), EU004544 (case 4), EU004542 (case 5), and EU004543 (case 6) (Stacy *et al.,* 2008).

CmPV-1 and CcPV-1 were the first Papillomaviruses reported in free ranging turtles. These viruses have been completely sequenced and studied in terms of molecular virology: phylogenetic analysis, evolutionary rates, homology rates and ORFs models. The genome nucleotides are documented as follows: *C. mydas* papillomavirus 1, complete genome: GenBank: EU493091.1; *C. caretta* papillomavirus 1, complete genome: GenBank: EU493092.1 (Herbst *et al.,* 2009).

Viral metagenomics approaches led to the discovery of STTV1. The methods were based on building a clone library and shotgun sequencing. STTV1 has been completely sequenced and ORFs, simple nucleotide repeats and poly (A) signals of this genome have been identified (GenBank: EU867816 to EU867824). Yet the evidence is not sufficient to infer the role of STTV1 in FP or report STTV1 as a commensal virus in sea turtles (Ng *et al.,* 2009).

2.4.5. Serology

Serological investigations have been beneficial in diagnostic and epidemic virology. Plasma, serum or other body fluids are examined to detect either the presence of antibodies or antigens (Storch, 2000).

If coupled with other virological tests, antibody tracking is useful to attribute the presence of the virus with infection. Although, there are some issues associated with sero-diagnostics while detecting a virus: 1) Humoral immune system requires a length of time to produce antibodies. 2)

Immunocompromised individuals may give false negative responses and patients receiving blood may give false positive. 3) Cross-reactivity between antibodies and close viruses may give false positives. 4) Some viruses infect the respiratory system and produce clinical signs before humoral indications. 5) Some viruses infect the skin only and the infections will not induce humoral reactions. 6) Some viruses such as HIV have a "*window period*" before sero-conversion (Read *et al.,* 2000; Stacy *et al.,* 2008; Storch, 2000).

Serology has been combined with molecular techniques to specifically identify viruses. Recombinant proteins or synthetic peptides are manufactured to target specific antibodies directly from the patient or isolated viruses in cell culture (Read *et al.,* 2000). In order to survey humoral immunity or particular disease causing agents, a preliminary knowledge of the antigen is required.

2.4.5.1. Serology and sea turtles virology

Serological tests have been used to unravel the uncertainties around fibropapillomatosis. In 1998, Herbst, *et al.,* performed an enzyme-linked immunosorbent assay (ELISA) test to detect antibody titers against spirorchid (*Learedius learedi*) and FP-associated herpesvirus in green sea turtles. The test proved that spirorchids do not have a role in the pathogenesis of green turtle fibropapillomatosis (Herbst *et al.,* 1998).

In most cases, LETV is culturable and its antigens were propagated in TH-1. Inactivated LETV was used to produce anti-LETV antibodies in captive reared turtles. Plasma samples of test turtles were examined by ELISA, immunoblotting and immunohistochemistry techniques (Coberley *et al.,* 2001). Presence of LETV was detected by antigen ELISA after 120hrs of exposure to artificial seawater indicating that LETV is persistent in the environment for a long time (Curry *et al.,* 2000).

Recombinant glycoproteins have been expressed and used in ELISA to detect seropositivity of turtles against ChHV5. The test is 100% specific, but sensitivity is low and the probability of detecting antigenically similar herpesviruses in free-ranging turtles cannot be excluded (Herbst *et al.,* 2008).

2.5. Conclusion

Fibropapillomatosis is the only viral infection described in sea turtles in the Australian region but this cannot exclude the presence of other viruses in this region. Except for FP with worldwide incidences and reports of mortality (Alfaro-Núñez and Gilbert, 2014; Chaloupka *et al.,* 2008), the aforementioned viral infections are generally described in Florida, USA (Coberley *et al., 2002*; Herbst *et al.,* 1998; Stacy *et al.,* 2008). The early research on sea turtle viruses started in Florida and is actively continuing: FP

was first documented in 1938 in green turtles in a New York aquarium. The prevalence in wild population rose in 1980s and since then the disease has been documented worldwide (Lackovich *et al.,* 1999; Quackenbush *et al.,* 1998; Jones *et al.,* 2016). Harold Haines from the University of Miami, described GPD, its herpesvirus origin and mortality in green sea turtles in captivity (Haines, 1978). Curry *et al.,* from the University of Florida, have investigated an infectious disease in captivity (LETD) which was caused by another herpesvirus and was shown to trigger higher rates of mortality (Curry *et al.,* 2000).

As most published research on sea turtle viruses originated in Florida and there is a great need for identifying and characterising viruses in sea turtles in the rest of the world, as sea turtles are endangered and infectious diseases are threats to biodiversity (Jones, 2004). New viral discoveries will expand the limited knowledge of sea turtle viruses. Newly described isolates and strains will improve phylogenetical analysis of known viruses and assist in explaining the aetiology of sea turtle viral diseases. In this project, green turtles of the Great Barrier Reef, Australia, were screened for presence and impacts of viruses.

3. SEA TURTLE DISEASE RISK ANALYSIS[2]

The aim of the chapter:

To do a structured sea turtle Disease Risk Analysis (DRA) in collaboration with the experts in the field

3.1. Introduction

The International Union for Conservation of Nature (IUCN) has listed six of the seven sea turtle species on the IUCN Red List of Threatened Species while the seventh species, the flatback turtle (*Natator depressus*), is reported as "Data Deficient" (IUCN, 2015). Over the past 100 years, the world population of sea turtles has declined due to direct and indirect human interventions (Troëng and Rankin, 2005). Disease is likely a contributing or primary factor in sea turtle deaths and poses challenges to conservation programs (Herbst and Jacobson, 2002), but due to a number of factors including the challenges of sampling wild marine animals in remote areas, incidences are generally under-reported (Daszak *et al.,* 2000).

It is particularly difficult to capture a sea turtle with clinical signs in the wild as sea turtles are often hard to locate and difficult to access in remote areas (Jensen *et al.,* 2010). Postmortem examination provides the most common opportunity to identify diseases and their aetiology. Unfortunately, in the

[2] This chapter does not follow the methodology and the layouts of normal experimental chapters, but has a specific methodology that is explained in the chapter and referenced accordingly.

wild, the process of retrieving carcasses as well as postmortem changes, interfere with disease presentation and the possibility of making a reliable diagnosis (Pacioni *et al.,* 2015); decomposition is relatively fast for aquatic animals and the temperature in tropical and sub-tropical waters is another contributing factor for sea turtles. An alternative way to investigate wildlife disease is to conduct controlled experimental studies, but due to their endangered status, such studies are curbed for marine turtles (Alfaro *et al.,* 2008).

The presence of disease cannot always be determined purely on absence or presence of clinical signs during a routine physical examination (Pacioni *et al.,* 2015). Even asymptomatic diseases may have an impact on health and the immunological and physiological effects of a nonlethal disease may trigger higher predation risks or reduced reproduction potential (Pacioni *et al.,* 2015). Methods and procedures used in evaluating other animals are not broadly available or validated for sea turtles (Herbst and Jacobson, 2002), which makes it challenging to diagnose the cause of disease or death in sick animals (Lewbart *et al.,* 2014). In addition, there is the possibility of encountering diseases that have not been previously reported in sea turtles and therefore no diagnostic capacity exists (Herbst *et al.,* 1999a). It is therefore of little surprise that there are only a limited number of researchers specialising in the investigation of wildlife disease, especially in remote areas (Mörner, 2002) and the field of sea turtle health and management are fraught with ambiguities.

The limitations and uncertainties of wildlife disease assessment call for structured, evidence based approaches to inform management and reduce the risk of diseases, where disease drivers and their contribution to other threats can be defined. Disease Risk Analysis (DRA) is a multidisciplinary approach involving researchers, clinicians and various decision makers to encompass rational, effective and unbiased conclusions for wildlife health surveillance in support of conservation strategies.

The latest DRA manual was published by the World Organisation for Animal Health (OIE) and IUCN in 2014. The manual addresses different scenarios for endangered species and translocating them for conservation purposes and enables the pros and cons of these actions to be thoroughly investigated (Jakob-Hoff *et al.,* 2014). In order to accommodate the unique biology of sea turtles, the DRA process, as described in this manual, requires certain modifications to realistically articulate with situations such as translocating animals or investigating the risks of disease for a population in its normal habitat.

In 2015, Pacioni *et al.* published an article titled "*Disease Hazard Identification and Assessment Associated with Wildlife Population Decline*". This is a systematic approach to study disease-related population decline without confining the assessment to a particular scenario or location. Pacioni's method is a modified version of a DRA based on epidemiological principles (Pacioni *et al.,* 2015) for any declining wildlife population such as sea turtles. A successful DRA considers the study population

in the context of the environment. Veterinary epidemiologists have coined the term "*One Health*" that takes into account the inter-dependent health of humans, livestock and wildlife (American Veterinary Medical Association 2008).

There are currently no published reports on DRA for sea turtles and this gap compromise strategies presently implemented to address sea turtle sustainability such as disease control, clutch translocations and hatchery establishment. In this study, both DRA models described by Jakob-Hoff *et al.,* (2014) and Pacioni *et al.,* (2015) were integrated to identify and assess the risk of disease to sea turtle conservation plans globally. The interrelated health of sea turtles, marine and terrestrial animals, humans and the environment were also addressed to define One Health parameters.

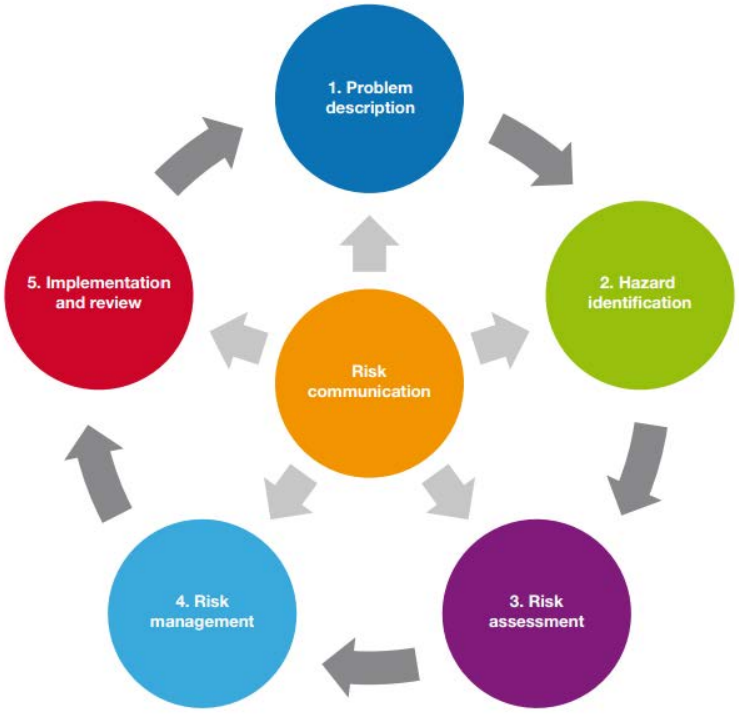


Figure 3-1 Steps in the disease risk analysis process, reproduced from the DRA manual published by OIE and IUCN in 2014

3.2. Methods

The process of a DRA is outlined in figure 3-1. Briefly, DRA organisers define a specific scenario for a wildlife population, for example translocating a clutch of sea turtles eggs from A to B (problem description). Then, published literature and unpublished reports about the hazards are collected and a group of experts are invited to review the information. This collection of comprehensive knowledge enables identification of hazards to the population under consideration (hazard identification). Assessing the knowledge for each hazard will help to prioritise the need for research or surveillance strategies (risk assessment). Following a structured risk assessment, which is ideally conducted as a workshop with invited experts, the prioritized health hazards or risks will be presented to a group of stakeholders who will decide on management options and the use of these options based on feasibility and effectiveness (risk management) (Jakob-Hoff *et al*., 2014).

3.2.1. Problem description

The sea turtle population decline was explained in "section 3.1." along with the difficulties in disease diagnosis. The Problem can be described as: "Disease is a likely contributing factor to sea turtle population decline." This description emphasises the need for a sea turtle DRA but is not specific enough to make the risk management achievable. The larger the spatial scale of the area of interest, the harder it is to describe the risks and apply management; for this reason "Management units" are to be defined alongside the problem description, or localised scenarios such as translocating a clutch of eggs from A to B, or establishing a turtle hatchery in location X. This DRA is a guideline for future conservation plans to facilitate realistic risk management.

3.2.2. Hazard Identification

In addition to an extensive review of the published literature, efforts were made to access unpublished information with the help of collaborators from various disciplines and parts of the world (e.g. veterinarians and researchers from rehabilitation centres and universities). The location of contributors are shown on the map in figure 3-2.

Figure 3-2 Origin of contributors to the hazard identification and assessment of sea turtle diseases.

For clarification, the disease hazards are divided into infectious and non-infectious and each of those further sub-divided to facilitate the risk assessment of each disease hazards.

3.2. 2.1. Infectious Hazards

Infectious disease is among the top five reasons for terrestrial species extinction (Smith *et al.,* 2006) and although the status of marine animals have not been assessed, infectious diseases are likely to have an equivalent impact. In addition to directly threatening the biodiversity of free-living animals, wildlife diseases can also pose a threat to domestic animals and humans if wildlife act as a reservoir for pathogens (Jones, 2004).

The infectious hazards for sea turtles were categorised into four groups: bacteria, fungi, parasites and viruses (appendices 1-4). In each category, pathogens were listed alphabetically and available information summarised for each pathogen. Table 3-1 is an example of a bacterial pathogen with available information. As sea turtles are migratory species and inhabit different marine environments at different life stages, the geographical distribution of pathogens and host age were included, if known. Likewise the presence of pathogens in wild and captive populations. The spectrum of infected or potential hosts were defined for each pathogen including related species, in order to address One Health considerations. Where possible, the correlation with climatic influence and/or anthropogenic events were also included.

Table 3-1 Example of an infectious bacterial health hazard "Lactococcus garviae" with the information about the region reported, the host species, the outcome of infection, the possibility of transmission to humans and cohabiting animals and the possible correlation to climatic influence and anthropogenic events

Infectious health hazard	**Region reported**	**Presence in sea turtle**		**Outcome of infection***	**Zoonotic/ transmissible to cohabiting animals**	**Correlation with climatic influence/ anthropogenic events**	**Key reference**
		Captive populations	Wild populations				
Lactococcus garviae	Tuscany, Italy		Loggerhead turtle *(Caretta caretta)* and Green turtle (*Chelonia mydas)*	Detected using PCR. No further studies were carried out	Fish, Molluscs and Crustaceans, identified in a bacterial epidemic in aquatic invertebrates, such as the giant freshwater prawn	Climate change may influence the threat levels associated with such exotic pathogens	(Buller, 2014, Fichi, *et al.,* 2016)

*(lesion, clinical sign and/or disease) symptom in individuals; ease of spread, rate of spread; is a diagnostic test or treatment available for the disease?

3.2.2.2. Non-infectious Hazards

Non-infectious diseases of sea turtles have been reported both in captivity and the wild (George, 1997), but little is known about the cause and extent of these diseases and their impact on the population (Flint *et al.,* 2010a). In this study, a broad range of health problems were described to form a platform for discussing their possible effects on the population. The groupings were adapted from the method used by George (1997) and consisted of four main groups, namely physical, nutritional, anthropogenic and medical problems. Table 3-2 shows an example of a physical problem and associated information. The regions where the hazards were reported are listed along with the species that were affected either in captivity or in the wild. For each health problem, the following information was collected (if available): Is the aetiology clear? What is the effect on individuals/population? Are there any treatments? Is mortality/ morbidity reported?

Table 3-2 Example of a non-infectious health hazard in the group of physical problems/injuries, with the information about regions it is reported from, species affected and the aetiology, effects on the population and treatment availability

Non-infectious health hazard	Health Problem	Region Reported	Species Affected		Explanation: aetiology? the effect on individuals / population? treatments? mortality/morbidity?	Key References
			Captive Population	Wild Population		
Physical problems	Injuries	All regions	All species	All species	Due to predator bites, by-catch or accidents. Can happen quite often and lead to infection, minor scars and/or deep wounds. Mortality may occur if the injury is traumatic. Appropriate modifications to vessel operation and configuration can reduce the threats.	Gilman *et al.,* 2006; Work *et al.,* 2010; Crane, 2013

					Aggressive males may bite females during mating Captive turtles are prone to injuries in overcrowded facilities. Existence of rehabilitation centers in the area to surrender injured or caught turtles for healing period followed by releasing may help the population	

A summary of infectious and non-infectious hazards are provided in the results section (3.3). These pathogens and diseases are important in the context of sea turtle conservation as described in the literature.

3.2.3. Risk assessment

Two workshops involving experts with a broad range of expertise were convened to systematically execute the risk assessment step. The consultation process was conducted in a formal and structured manner following an established protocol for a DRA, (See appendix 6 for workshop workbook and questionnaire) (Armstrong *et al.,* 2003; Jakob-Hoff *et al.,* 2014). The questionnaire was approved by James Cook University Human Ethics Committee, permit number H6834. The two international workshops were: ONE) the Turtle Health & Rehabilitation Workshop, September 2017, Townsville, Australia, that was attended by 25 participants mainly from South Africa and the Australasia region and TWO) the Medicine Workshop at the International Sea Turtle Symposium 2018, Kobe, Japan, where 35 originated from much broader regions and both hemispheres. The participants were veterinarians, microbiologists, members of the International Sea Turtle Society (ISTS) and IUCN Marine Turtle Specialist Group (MTSG) who are working on marine turtle research and conservation. Discussions among participants centered on the relevance, significance and prioritisation of infectious and non-infectious hazards.

The list of hazards compiled in the review of the literature were presented to the groups of specialists in sea turtle health. The "*Paired Ranking Tool*" was used to prioritise the top three hazards from each group according to a conservation, surveillance and research perspective (Table 3-3) (Jakob-Hoff *et al.,* 2014; Pacioni *et al.,* 2015). The criteria used to compare the diseases were defined as: current knowledge of the pathogen in sea turtles, the likelihood of exposure/susceptibility, the pathogenic potential, the severity for populations and the correlation with climatic/anthropogenic events (Jakob-Hoff *et al.,* 2014; Pacioni *et al.,* 2015).

3.2.4. One Health and DRA

"*One Health*" is an all-inclusive collaboration between public health, animal health and environmental specialists as well as communities and social scientists, through a transdisciplinary approach, to sustain the world's health (Zinsstag *et al.,* 2015). The founding belief behind promoting One Health is the interconnected health between humans, animals and the environment. Approximately 75% of human infectious diseases are zoonotic or in other words are caused by multi-host pathogens initiated in animals (American Veterinary Medical Association, 2008). On the other hand, unsustainable degradation of the environment by humans, toxins and chemical contaminants are known to enhance the rate of emerging diseases in human, wildlife and livestock (Keller *et al.,* 2004b; Webb, 2014). Humans are also putting pressure on animals by the increasing demands for meat protein and subsequent habitat degradation (American Veterinary Medical Association, 2008). In addition to providing traditional food and cultural importance to many indigenous people, sea turtles are iconic role players in marine ecotourism, which enhances the sense of control over our lives and improves human health (Tisdell and Wilson, 2002).

Disease affects not only a population, but also the habitat, the other animals and humans and *vice versa*. In the context of One Health, green turtles are particularly important due to their longevity and fidelity to a near-shore foraging site. Their continuous and long term residency in a given location make them good sentinels for local environmental health (Aguirre and Lutz, 2004) and thereby marine ambassadors for One Health. "*One Health*" and "*DRA*" share common goals, which are addressing complex health issues and reducing disease risks through multidisciplinary collaborations (Steveson and Firestone, 2015).

To address One Health considerations in this DRA, zoonotic pathogens of sea turtles and the possibility of disease transmission to/from sea turtles were documented. The information about socio-economic consequences of interaction with sea turtles and conservation were also collected and is described in the form of a review (3.3.2. Sea turtle and One Health consideration in the literature).

Two sections were dedicated to One Health in the expert workshops. One addressed infectious disease transmission and the other queried the expert opinion about socio-economic values of interaction with sea turtles and the contributions to conservation. Results are shown in table 3-4.

3.2.5. Risk management

Appropriate management interventions such as bycatch reduction, restrictions on commercial use and trade and creation of protected habitats, can allow recovery of a depleted population (Balazs and Chaloupka, 2004; Valdivia *et al.,* 2019) which emphasizes the importance of "*designing management*

with SMART (specific, measurable, achievable, realistic and time based) goals" (Hamann *et al.,* 2010). Disease risk management is the process of risk evaluation and identifying the measures that can be applied to reduce or eliminate the risk posed to the population of concern (Hartley and Sainsbury, 2017). To effectively reduce or eliminate the risks, the scale at which the management plans are evaluated and executed should be defined. Regional management units (RMUs) were developed for sea turtles to organise units of protection. These are functionally independent and provides a framework to evaluate conservation status and to address management challenges (Wallace *et al*., 2010).

After defining the management unit, the risk management step suggests **management option**s to reduce the risks that have been assessed and ranked in previous steps. These options are then evaluated according to their **feasibility** and **affordability** (Jakob-Hoff *et al.,* 2014). Reducing the risk is not implemented under a "*single correct answer*" achieved from risk assessment, it is rather a step-by-step procedure that needs modification through communication and cross governmental support as animals and their pathogens are not confined by political barriers but are distributed by topographic and ecological barriers (Jakob-Hoff *et al.,* 2014; Hartley and Sainsbury, 2017). This is especially true for migratory animals such as sea turtles (Hamann *et al.,* 2010).

In most cases the risk assessment process is separate from the risk management, merely because the scientists and veterinarians behind the risk assessment process are not policy or decision makers at government level (Hamann *et al.,* 2010). However, a scientifically based, clear DRA can help the decision makers to prioritise the actions to reduce the disease risk (Jakob-Hoff *et al.,* 2014). An understanding of the identified and assessed risk can facilitate practical and realistic interventions in the form of risk management of the most significant hazards (Hartley and Sainsbury, 2017).

3.2.5.1. International workshops

The DRA protocols were used to structure discussions around the current risk management, its difficulties and defects for the highest ranked hazards (table 3-5) based on globally identified challenges for risk management initiatives.

3.2.5.2. Local workshops

Executing risk management for a specific scenario and in a defined region is more realistic than a global disease risk management for sea turtle populations and further discussions were therefore conducted with appropriate representatives from the Australian government to identify possible pathways for

local disease risk management. The risk management workshop took place in February 2019 at James Cook University, Townsville, Australia. The attendees were provided with the DRA materials including the risk assessment results, a week prior to the meeting. The workshop workbook is provided in appendix 7. The workshop was divided into two sections, the first part was discussing management options for previously assessed risks and the second part was brainstorming to define critical control points for a mock clutch translocation.

Management options for previously assessed risks

From the highest ranked hazards, the "*Enterobacteriacae and multi-resistant bacteria"* was selected from the infectious hazard group and the "*Macro plastic pollution*" from the non-infectious hazards as these were considered to be most relevant to local conditions. Management options were suggested for these two hazards by the attendees and based on the discussions, effectiveness and feasibility was scored.

Critical control points for a mock clutch translocation.

The translocation of animals for conservation purposes was the original and primary aim of establishing DRA (Jakob-Hoff *et al.,* 2014). The problem description, scope of the risk, goals of risk analysis and the source of information will vary for each individual scenario. The hazard identification step is not as detailed as it is for a global population decline and is confined to the regions that "*animals are sourced from*" and the destination that "*the animals are going to be introduced to*". The list of the hazards are mainly focusing on the "*disease causing*" infectious and non-infectious agents. The risk assessment can be done through expert-involved paired ranking and also scenario trees for the specific translocation situation. Risk mitigation and contingency plans can be created with reference to the risk assessment. Finally, the stakeholders can plan for scientifically based, feasible and economic risk managements.

The "*Manual of Procedures for Wildlife Disease Risk Analysis*" has adapted a checklist for conducting a wildlife translocation disease risk analysis from Brückner *et al*. (2010). The checklist was employed here and modified for a scenario of sea turtle clutch translocation as an example – (See appendix 8). Such procedures are relevant for headstart programs[3] and rehabilitation and release of turtles, though individual and local considerations must be taken into account for each scenario.

[3] Head-starting programs are designed to increase hatching rate by captive rearing the sea turtles hatchlings and releasing them to the ocean when they are assumed to have higher survivorship (Heppell *et al*., 1996).

Risk management for a mock clutch translocation from an island to the mainland was assessed for the second part of the local workshop. After discussing the modes of transportation of the eggs, the potential transmission pathways for infectious organisms were agreed on. The potential transmission pathways and the critical control points were listed in a schematic representation on a whiteboard. Predation risk was also considered in the destination area and the potential hazards for a hatchery establishment were discussed.

3.3. Results

3.3.1. Hazard identification

Both "*infectious*" and "*non-infectious*" hazards were addressed and a comprehensive list is available as supplementary materials (Appendices 1-5). Here we consider only those pathogens and diseases that are important in the context of sea turtle conservation and have left out a large number of potential pathogens that would make the DRA unrealistic and unachievable (Hartley and Sainsbury, 2017).

3.3.1.1. Infectious disease

Previously undetected bacteria, viruses, parasites and fungi are frequently described in sea turtles and in new regions, but the health implications to sea turtles are not commonly addressed in the literature (Manire *et al.,* 2017). The manifestation of infections in sea turtles are often seen in immunocompromised individuals (Castellá *et al*., 1999; Work *et al*. 2004; Ritchie, 2006; Alfaro *et al.,* 2008). One of the causes of immunosuppression is suggested to be the result of environmental contaminants, which is an increasing concern with slow resolutions from management actions (Keller *et al.,* 2004a).

Bacteria

Most bacterial species are opportunistic pathogens in sea turtles and have been reported as natural flora in fish, crustaceans and other marine animals (Alfaro *et al*., 2008). Nevertheless, bacterial pathogens form the longest list of infectious hazards for sea turtles contributing to disease in captive, farmed and free-living sea turtles in many parts of the world (Glazebrook and Campbell, 1990; Campbell and Glazebrook, 1990; Raidal *et al.,* 1998).

Vibrio spp. Pseudomonas spp., Enterococcus spp., Aeromonas, C. freundii, E. coli, Edwarsiella spp., Proteus spp., Lactococcus garviae, and *Providencia* have been recorded in sick sea turtles as either potential pathogens or opportunistic bacteria (Zavala-Norzagaray *et al.,* 2015; Fichi *et al.,* 2016). *Vibrio spp.* are the most frequently studied bacterial isolates in sea turtles (especially *Vibrio alginolyticus*) and are repeatedly isolated from skin lesions, digestive organs and respiratory tract causing ulcerative stomatitis, obstructive rhinitis, and pneumonia along with *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Flavobacterium spp.*, and *Bacillus spp.* (Glazebrook and Campbell, 1990; Campbell and Glazebrook, 1990; Orós *et al.,* 2005). Infection with these bacteria can also cause mortality in oceanarium-reared and wild juvenile green and loggerhead turtles (Glazebrook and Campbell, 1990; Campbell and Glazebrook, 1990). Bacteria isolated in clinically healthy and wild-living turtles near urbanized areas show high levels of multidrug-resistance, indicating an accumulation of resistance in marine bacteria caused by exposure to anthropogenic factors. Of particular concern are the Enterobacteriaceae that are of One Health importance as potential zoonotic pathogens (Ahasan *et al.,* 2017).

Fungi

Fungal pathogens of sea turtles are usually opportunistic saprophytes causing infection under favorable circumstances (Phillot *et al*., 2001). Sea turtles in captivity or rehabilitation centres are prone to mycotic infections possibly due to other underlying health issues or immunosuppressive conditions (Alfaro *et al.,* 2008). *Fusarium* species have been isolated from cutaneous abscesses (Williams *et al.,* 2012), cutaneous/pneumonic lesions and bronchopneumonia (Sarmiento-Ramírez *et al.,* 2014). *Fusarium solani* is the most frequently identified fungus in sea turtle mycotic diseases, and is normally isolated and referred to as a 'species complex' including more than 60 phylogenetic species (Sarmiento-Ramírez *et al.,* 2014). *Fusarium* is widely distributed in soil and waste; it tends to enter the body through lesions, causing mycosis in humans and animals (Short *et al.,* 2011; Sarmiento-Ramírez *et al.,* 2014). *Fusarium* infections are a common pathological finding in sea turtle eggs. *Fusarium oxysporum*, *F. solani* and *Pseudallescheria boydii* were isolated from failed eggs found in eastern Australian loggerhead, green, hawksbill and flatback nests (Phillott *et al.,* 2004). *Fusarium falciforme* and *Fusarium keratoplasticum* were believed to reduce the hatching success to 10% in an infected clutch (Sarmiento-Ramírez *et al.,* 2014). Environmental stressors such as inundation (flooding of nest) and oxygen depletion seem to enhance the incidence of fungal infection and mortality of embryos (Sarmiento-Ramírez *et al.,* 2014). However, Phillott and Parmenter, (2014) determined that the fitness of the hatched green turtles were not affected by fungal colonisation of the nest. Sporadic opportunistic fungal infections are reported in sea turtles. These fungi are not true pathogens of

reptiles and are usually not associated with systemic infection or mortality unless the immune system is compromised (Donnelly *et al*., 2015).

Parasites

A variety of parasites infect sea turtles, primarily digenetic trematodes and nematodes (Greiner, 2013). Different factors influence the extent of damage a parasite may cause, such as the species of parasite and the general fitness of the host, habitat and availability of intermediate host. Therefore, the severity of infection, which is defined by the load of parasites in infected animals, is not consistent between individual turtles and between regions (Santoro and Mattiucci, 2009; Greiner, 2013).

The gastrointestinal flukes (Digeneans of the family *Pronocephalidae*) and cardiovascular flukes (Spirorchidae) are the most prevalent trematodes in sea turtles (Santoro *et al.,* 2006a; Greiner, 2013). Gastrointestinal flukes are widely distributed throughout the tract without any apparent ill effect; cardiovascular flukes on the other hand cause pathological effects in the circulatory system and multiple internal organs (Greiner 2013). The first definitive life cycle for a species of sea turtle blood fluke was recently described with vermetid snails as the intermediate hosts for *Amphiorchis sp* (Cribb *et al*., 2017).

In the nematode group, Anisakidae and Kathlanidae have been reported to infect sea turtles and are mainly found in the gastrointestinal tract of loggerhead turtles (Lester *et al.,* 1980; Santoro and Mattiucci, 2009). In Australia, the coccidian parasite *Caryospora cheloniae* and Spirorchids are reported to be the parasites of highest concern as they are associated with disease and high mortality rates under certain conditions (Flint, 2013).

Sea turtles are the definitive host for some of these parasites, but how host-specific or harmful these parasites are to the host is not known. *Lophotaspis valley*, *Learedius learedi* and *Styphlotrema solitaria* are some species-specific trematodes in marine turtles, while *Plesiochorus cymformis, Rhytidodes gelatinosus, Enodiotrema carettae* and *Pleurogonius trigonocephalus* show a wider host range (Greiner, 2013).

Viruses

Reptile virology is a relatively new field (Marschang, 2011); however, increased awareness and advances in molecular technology will undoubtedly bring about an increase in the knowledge and identification of new species (Ariel, 2011). The link between the presence of herpesvirus or ranavirus and clinical disease in chelonians are well established, whereas the link between disease and causative pathogen is still being explored for other viruses (Marschang, 2011). To date, members of *Herpesviridae* are the only causative agents of viral diseases investigated in marine turtles. The

presence of other viruses in marine turtles are sporadically reported: with one published report for each of tornovirus, retrovirus and betanodavirus (Casey *et al.,* 1997; Manire *et al.,* 2008, Ng *et al.,* 2009; Fichi *et al.,* 2016), and two reports of papillomaviruses (Manire *et al.,* 2008, Mashkour *et al.,* 2018).

Herpesviruses cause severe diseases in chelonians, especially in animals in stressful situations with associated lower immune function (Kirchgessner and Mitvhell, 2009). Gray-patch disease (GPD), lung, eye, trachea disease (LETD) and fibropapillomatosis (FP) are herpesvirus-associated diseases frequently described in marine turtles (Lackovich *et al.,* 1999; Herbst *et al.,* 1999b; Aguirre and Lutz, 2004; de Deus Santos *et al.,* 2015).

GPD was reported in captive reared green turtles (<1 year old) causing gray skin lesions. Overcrowded hatcheries and higher water temperatures appears to worsen the symptoms (Haines *et al.,* 1978). LETD, another disease of green turtles (>1 year old) was first described in captivity and then found in free ranging green turtles (Klein *et al.,* 2001; Coberley *et al.,* 2002; Marschang and Divers, 2014).

Fibropapillomatosis is a neoplastic disease affecting all species of sea turtles (Quackenbush *et al.,* 1998; Work *et al.,* 2004; Chaloupka *et al.,* 2008; Jones *et al.,* 2016). Tumour growth can be both external and internal, with juvenile turtles appearing to be most susceptible. Moreover, infected turtles are vulnerable to secondary infections and opportunistic pathogens due to immunosuppression (Work *et al*., 2004; Jones *et al.,* 2016). Environmental factors may contribute to the expression and the severity of the disease (Curry *et al.,* 2000; Work *et al.,* 2004; Van Houtan *et al.,* 2010). The disease was first reported in an aquarium in New York (Smith and Coates, 1938), but is now reported globally in tropical waters (Zavala-Norzagaray *et al.,* 2015; Jones *et al.,* 2016; Cárdenas *et al.,* 2018; Kumar *et al.,* 2018; Villanueva *et al.,* 2018).

3.3.1.2. Non-infectious diseases

Turtles are affected by a variety of non-infectious diseases occurring either as a direct result of natural or man-made threats (George, 1997), or they may act as multifactorial influences on disease outcome. In some cases, it is not easy to determine if clinical signs are caused by an infectious or non-infectious agent. Infection with coccidia can elicit neurological diseases, but neurological symptoms can also be caused by head injury or natural causes such as toxins and algal bloom (Jacobson *et al.,* 2006).

Serious alterations in the balance between the environment, the host and the pathogens can trigger or spread disease in a population (Smith *et al.,* 2009; Jakob-Hoff *et al.,* 2014; Ward and Lafferty, 2005). For example, loss of seagrass habitat due to human disturbances or severe weather events can influence water quality and lead to immunosuppression due to starvation (Dobbs, 2001; Hamann *et*

al., 2013). Anthropogenic effects such as habitat degradation, coastal light disturbance, pollution, by-catch and etc. are known threats posed to sea turtles and are ranked highest in terms of adverse effects they may have for sea turtle populations (Casale *et al.,* 2010; Cosgrove and Roe, 2012), but the flow-on effect of habitat disturbance for turtles, are likely to facilitate the emergence of infectious diseases at increasing incidences and exacerbate the risk of local population extirpation (Dobbs, 2001).

Trauma and injuries

Traumatic injuries are a major cause for stranding and may be caused by a range of factors from boat strikes and entanglement to shark bite or mating injuries (Campbell and Glazebrook, 1990; Cosgrove and Roe, 2012). In addition to direct lethal effects on individual turtles, open wounds are a portal of entry for pathogenic microorganisms into the turtle (Cosgrove and Roe, 2012). Perforating fishing hooks, plastics and fish spines can cause injuries in the gastrointestinal tract and respiratory system (Cosgrove and Roe, 2012; Manire *et al.,* 2017) after ingestion. Decompression sickness (DCS) was recently diagnosed by Garcia-Parraga *et al.* in loggerhead turtles captured in trawl and gill nets in the Mediterranean Sea (Garcia-Parraga *et al.,* 2014).

Debilitated Turtle Syndrome (DTS) and cold stunning

Debilitated Turtle Syndrome is used to describe the condition of a turtle with several of the following symptoms: emaciation, lethargy, hypoglycemia, anemia, and heavy coverage with epibiota (Stacy *et al*., 2018). Secondary infections are common and turtles may be immunosuppressed (Norton, 2014). A wide range of morphometric and metabolic variables is documented for chronically debilitated loggerhead turtles in the southeastern United States (Stacy *et al*., 2018). The main cause of DTS is not clear but cold stunning in some cases is an initial trigger (Davenport, 1997; Shaver *et al.,* 2017). Occasionally, large numbers of strandings are reported due to cold stunning (personal interview with rehabilitation centres from Dubai, UAE; Kish Island, Iran; New York, USA; Lampedusa, Italy). Epibiota can increase rapidly in numbers when turtles are floating or immobilised and some species of epibionts are detrimental to health due to their invasive nature. A high load of epibionts can lead to erosion in the carapace and plastron creating a portal of entry for secondary invaders (Manire *et al.,* 2017).

Gastrointestinal disorders

Gastrointestinal disorders are one of the main concerns for sea turtles in rehabilitation centres (Ahasan *et al.,* 2017). Gastrointestinal obstruction by debris such as plastic and other pollutants such

as agricultural run-off including pesticides and herbicides are a clear risk for turtles (Carr, 1987; Camedda *et al.,* 2014). However, gut impaction and faecoliths are also observed in stranded sea turtles with no obvious or physical cause (Manire *et al.,* 2017). Climatic events may alter the foraging grounds for turtles and thereby affect their nutritional choices (Hawkes *et al.,* 2009), but physical trauma, high parasitic load or chronic diseases can lead to loss of appetite, nutritional deficiencies and cachexia (George, 1997). Nutritional disorders can in turn affect the hepatobiliary system (Manire *et al.,* 2017).

Diseases caused by chemical and organic pollutants

Pollution can cause immune suppression and thereby increase vulnerability to pathogens (Smith *et al.,* 2009). Organic agricultural waste can elevate the nutrient level in the ocean and stimulate harmful algal and cyanobacterial blooms which can directly or indirectly harm turtles or exacerbate the effects of other diseases such as FP (Deem *et al.,* 2009; Fauquier *et al.,* 2013; Brodie *et al.,* 2014). In addition, long living animals, such as sea turtles, face the risk of accumulating these pollutants in their tissues over time and as a result the impact of toxicity will intensify (Cosgrove and Roe, 2012).

Chemical debris and organic pollutants can block the gastrointestinal tract and cause different problems such as accumulation of intestinal gas, local ulcerations, interference with metabolism and immune function and intoxication of body (Carr, 1987; Brodie *et al.,* 2014; Camedda *et al.,* 2014). Plastic is an example of an accumulating pollutant and sea turtles tend to ingest plastic debris (Orós *et al.,* 2016) which may block the gastrointestinal tract, accumulate intestinal gas, cause local ulcerations and interfere with metabolism (Torrent *et al.,* 2002; Carr, 1987). Gastrointestinal obstruction may lead to chronic debilitation and eventually death (Stahelin *et al.,* 2012). Cases of secondary infection and mortality are frequently reported due to plastic ingestion (Torrent *et al.,* 2002; Stamper *et al.,* 2009).

In summary, anthropogenic non-infectious diseases are the biggest challenge to sea turtle conservation (Manire *et al.,* 2017; Wallace 2011).

3.3.2. Risk assessment

To assess the disease hazards using expert opinion, group and forum discussions were facilitated and encouraged in the workshops. The discussion sessions, which formed the basis for the rankings, were an opportunity for the participants to explain their personal experiences with disease encounters and to improve the general knowledge of the participants about regional differences in disease manifestation. One point that was repeatedly mentioned was the "*quality of information available*"

and how this affected the ranking. Such level of confidence by experts is referred to in Pacioni's ranking criteria as "*levels of knowledge"* (Pacioni *et al.,* 2015).

The top three hazards from each group of infectious and non-infectious hazards were ranked according to a conservation, surveillance and research perspective (Table 3-3).

Table 3-3 The three highest ranked hazards of each infectious and non-infectious groups as determined by panels of experts in two international workshops. A) Turtle Health & Rehabilitation Workshop, September 2017, Townsville, Australia.

Hazard		Reasoning
Infectious health hazards		
Parasite	*Spirochiidae*	widespread, virulent and prevalent
	Caryospora cheloniae	virulent and episodic
	Ozobranchus branchiatus	possible vector for fibropapillomatosis associated herpesvirus
Virus	Chelonid α-herpes virus 5	associated with fibropapillomatosis: reported in all species, can cause debilitating syndrome and be life threatening
	Cm-PV1 and Cc-PV1:	skin lesions, data deficient
Gram negative bacteria	*Vibrio spp.*	Associated with ulcerative dermatitis, mortality reported; associated with hatching failure; possibly zoonotic for turtle meat and egg consumers.
	Pseudomonas spp.	Ulcerative stomatitis and dermatitis along with *vibrio alginolyticus*; associate with hatching failure; possibly zoonotic for meat and egg consumers
	Escherichia coli	Antibiotic resistant; opportunistic pathogen; zoonotic
Gram positive bacteria	Unfortunately there was not enough time to go through this list.	
Fungal infection	*Fusarium spp.* (mostly *Fusarium solani*)	Contributing to hatching failure, pneumonia, necrotic skin lesions mostly in captivity; potentially zoonotic.
	Aspergillus spp.	Hatching failure, mycotic infections in hatchlings; mycotic infections in captivity
	Cladosporium spp.	hatching failure, infections in captivity
Non-infectious health hazards		
	Anthropogenic: Habitat degradation	malnutrition, by-catch and accidents
	Environmental: Climate change	malnutrition, fibropapillomatosis and cold stunning or Debilitated Turtle Syndrome
	Anthropogenic: Pollution/plastic	entanglement, external and internal injuries, debris ingestion and neurological diseases

B) Medicine workshop at the International Sea Turtle Symposium 2018, Kobe, Japan

Hazard		Reasoning
Infectious health hazards		
Parasite	*Spirochiidae*	Geographical wide distribution, various species, high prevalence, different effect in different life stages, adult, juvenile, eggs, severe lesions, causes stranding and mortality.
	Annelids	Wide geographical distribution, various species, Loggerhead, Olive ridley and green turtles are affected, cutaneous ulcerations, *Ozobranchus* possible vector for FP
	Arthropods	Needs justification, worse in some regions, correlated to hatching failure anrd egg damage, causing mortality, regional reports
Virus	Herpesvirus	Tumours have been reported in new areas, ChHV5 is reported in clinically health turtles
	Papillomavirus	Only a few reports so far, not fully understood

Bacteria	*methicillin resistant staphylococcus aureus (MRSA), Ecoli and margonella*	Multi-resistant strains, public health concern
	Streptococcus iniae, Salmonella typhimurium, Ecoli.	Pathogenic and zoonotic
	Pseudomonas spp. Klebsiella	Mass mortalities, regional
Fungal infection	*Fusarium solani*	Problem for captive rearing, eggs and hatchling
	Penicillium spp.	Recorded in several areas, multi species infection recorded, different stages of life can be affected
	Cladosporium spp.	Recorded in several areas, may affect several life stages
Non-infectious health hazards		
	Anthropogenic	Human interactions are increasing, plastic ingestions are increasing
	Environmental	Climate change effects and also cold stunning
	Medical	Aftermath of anthropogenic and environmental incidences

Although the outcome from the two workshops are very similar, there were a few differences, which could reflect the broader geographical origins of participants in workshop TWO compared with workshop ONE. In workshop TWO the experts working on parasites ranked the hazards based on overarching classification, while participants in workshop ONE gave species names to the parasites. In both workshops, spirorchids were considered important due to their widespread presence and potential virulence. Ozobranchid leeches were also mentioned by both groups due to their possible role in FP. Viral pathogens were considered to be data-deficient by participants in both workshops, but both groups listed herpesvirus and papillomavirus as the highest ranking pathogens.

Antibacterial resistance and the associated public health concern were also consistently mentioned in the two workshops for the bacterial category. In Workshop ONE, the participants chose to focus on Gram negative bacteria only. *Fusarium* and *Cladosporium* spp were selected by both groups as the most important fungal pathogens, mainly for eggs on nesting beaches and hatchlings in captive situations. Climate change and anthropogenic impacts scored highest in non-infectious health hazards in both workshops and there was consensus, that anthropogenic influences on turtle health need the highest attention of all groups, both in terms of research and conservation management.

3.3.3. One Health and DRA

3.3.3.1. Sea turtle and One Health consideration in the literature

Sea turtles mostly encounter humans during harvest, on nesting beaches and in rehabilitation centres. Figure 3-3 shows the main sources of interaction between humans, sea turtles and the environment. These interactions can positively or negatively impact the players.

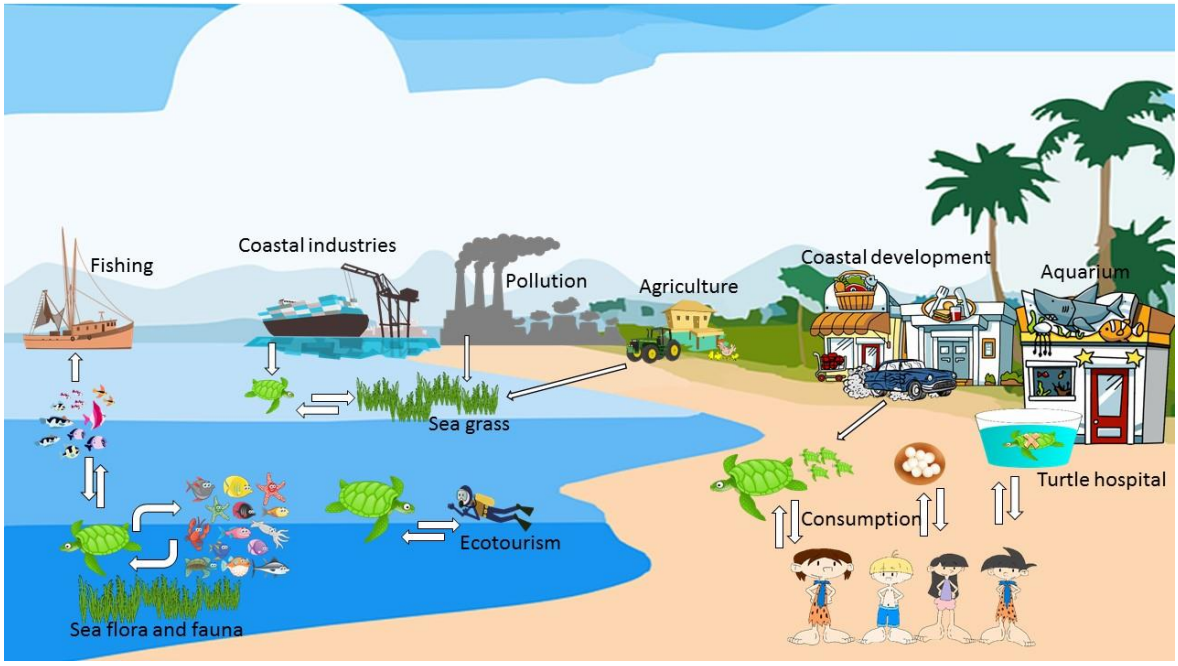


Figure 3-3 The schematic interactions between sea turtles, humans, co-habiting animals and the environment

Zoonosis

As an example of zoonotic infections, vibriosis in humans may develop due to consumption of contaminated meat and eggs (Warwick *et al.,* 2013). Field workers should consider disinfecting any wound received while handling sea turtles as there is the risk of infection with *Mycobacterium*, *Salmonella*, *Vibrio*, and *Chlamydia* species due to contact with infected animals (Herbst *et al.,* 1999b). There are also reports of fish pathogens in sea turtle which are of concern to aquaculture and the sea food industry (Fichi *et al.,* 2016). Sea turtles are exposed to toxins of either anthropogenic or natural origins, which may accumulate in their tissues and cause problems for meat and egg consumers (Warwick *et al.,* 2013). There are multiple reports of death, mass poisoning or sickness in a community after feasting on turtle meat (Ranaivoson *et al.,* 1994; Pavlin *et al.,* 2010; Ventura *et al.,* 2015). The condition is termed *chelonitoxication* and appears to be caused by the consumption of certain marine turtles (green, hawksbill and leatherback turtles). Children are more prone to intoxication and its lethal effects (Ranaivoson *et al.,* 1994; Pavlin *et al.,* 2010; Ventura *et al.,* 2015).

Fusarium solani can infect egg clutches, and high mortality rates are reported due to infection with this species of fungi. Being zoonotic, this pathogen poses a threat to the person handling the infected eggs as well. Such activities may take place while eggs are collected for consumption or in the hatcheries or on nesting beaches when the nests are cleaned out after the eggs are hatched. Dead/decomposing embryos are sources of nutrients for bacterial and/or fungal growth (Swingland *et al.,* 1978).

Humans can be the source of infection for sea turtles too. Examples of *Salmonella* and *Vibrio alginolyticus* transmission in captivity have been reported several times (Raidal *et al.,* 1998; Orós *et al.,* 2004b; Orós *et al.,* 2005; Alfaro *et al.,* 2008; Buller, 2014; Zavala-Norzagaray *et al.,* 2015). Humans are also posing an indirect threat to sea turtle health, via habitat destruction, distribution of pollutants, plastic and toxins (Keller *et al.,* 2004b; Webb *et al.,* 2014).

Cultural significance and sustainable conservation measures

Sea turtles are of great cultural value for indigenous communities (Tisdell and Wilson, 2002). Humans and their environment co-evolve and local culture and traditions reflects these relations. The legally recognised rights of indigenous communities to interact with sea turtles in line with their traditions is the foundation for a community-based conservation management where alternatives to hunting is introduced in consultation with the local communities (eg Caribbean Coast of Nicaragua). Such policies reduce the fear of arrest or reprisals while participating in local customs (Campbell, 2010) which in turn enhances the feeling of control over their lives and improves community health (MacKenzie, 2004).

Market-based solutions towards conservation and providing alternatives for consumption of sea turtle products have been successful in several projects such as the TAMAR (**Ta**rtarugas **Mar**inhas, the Sea Turtles) project sites in Brazil, and at Tortugeuro, Costa Rica (Campbell, 2010), where the hunting has decreased, while ecotourism based activities have been organized for local communities. Another example of working toward sustainable harvest of the ocean with the help of traditional owners is TUMRA (Traditional Use of Marine Resources Agreements) in Australia. TUMRA is a formal agreement between Traditional Owner groups and the Australian government about how Traditional Owner groups wish to manage their take of natural resources (including protected species), their role in compliance and their role in monitoring the condition of plants and animals, and human activities, in the Great Barrier Reef Marine Park (GBRMPA, 2018). Non-governmental organisations (NGOs) have also formed and evolved in various regions of the world to promote conservation with the help of local communities. One such example is ***New Idea*** in Hormozhgan, Iran (moassese ide no doostdare hormozgan) which was successful in eliminating egg harvest for overseas markets. The turtle nesting site is now an ecotourism destination with a financial return for the local community (personal interview with Maryam Eghbali the co-founder). A pro-environment establishment "Grupo Tortuguero" was formed in the Pacific Ocean in response to poaching and retaining the turtles after accidental catch by fishermen. The establishment is active in terms of education, funding and empowerment in response to loss of sea turtles, especially loggerheads (Senko *et al*., 2011).

Sea turtle conservation has a great impact on human communities, which is not limited to their abilities to use or interact with sea turtles but has an effect on the entire social-ecological system in which they are embedded (Campbell, 2010). Pro-environmental topics are categorised under human relationships and as such sea turtles conservation plans must articulate with diverse cultural, political and socio-economic needs (Barrios-Garrido *et al*., 2019). This poses a challenge to management policies and raise important questions about the purpose of research and conservation endeavours (Hamann *et al.,* 2010). As an example, in a recent publication by Barrios-Garrido *et al*. (2019), the conflicts related to sea turtle conservation programs in the Caribbean basin were identified. Dissimilar conservation objectives between local communities, non-governmental and governmental organisations were identified, along with lack of resources such as trained individuals for monitoring and enforcement roles, and scarce funding (Barrios-Garrido *et al*., 2019). The suggested solutions for the conflicts were rationalising the problem and promoting a mutual agreement based on common beliefs. Such multi-scale solutions would be achievable by co-management through bottom up (community based) actions and top-down changes (government policy) (Barrios-Garrido *et al*., 2019).

3.3.3.2. Sea turtle health and One Health according to expert opinion

The expert opinion on diseases transmission was consistent with the literature. The results are summarised in table 3-4.

Table 3-4 One Health consideration in disease risk analysis workshop. A) Transmission of pathogens from and to sea turtles in wild and captivity.

Pathogens	Main zoonotic pathogen of concern from turtles to humans	Pathogens being naturally transferred from humans to sea turtles	Main problematic pathogen in captivity for turtles	Pathogens to be considered as a risk for aquaculture and fisheries
Bacteria	*Salmonella* *Vibrio spp.* *Pseudomonas spp.* *Escherichia coli*	Very unlikely	Opportunistic bacteria	Data deficient
Fungi	Fusarium (*especially F. solani*) Aspergillus	Data deficient	Fusarium (*esp. F. solani*)	*Trichophytea spp.*
Parasites	Not a concern to date	Not a concern to date	Cryospora	Data deficient
Viruses	Not a concern to date	Not a concern to date	Herpesvirus	Herpesvirus

B) Non-infectious disease transmission between human and sea turtle

Human to turtles	Turtles to humans
Biotoxin pollution Plastic pollution Boat strike, by-catch	Toxins in egg and meat

C) Cultural values of sea turtles and socio-economic aspects of sea turtle conservation

Cultural dimensions of interacting with sea turtles have recently been brought to the attention of conservationists: • Rescue plans are rewarding for volunteers, rangers and people who are involved. • In the Caribbeans, the conservationists' goal is to interact with the locals and to allow traditional harvest in sustainable manners • In the Maldives, sea turtles can be kept as pes". The expert emphasised the special bond between the turtles and humans. • In the French Mediterranean, the aim is to involve fishermen in conservation initiatives to reduce the threat of bycatch. • In Australia, sea turtles are significant elements of indigenous culture and any conservation plans is considering their traditional expertise
Socio-economic advantage of sea turtle conservation need more attention: • Tourism value of healthy turtle population has not been evaluated. • Turtle watching tours are alternatives for fishing and has been successfully established in some regions. • Job generation through alternative projects may reduce poaching but needs more research. • Outreach opportunities to groups that are interested but not normally involved in sea turtle conservation • Sea turtles are charismatic species and on third highest ranked animal for conservation initiatives. • Turtles are indicators of environmental health, but the association between their health and the environmental health need more research and potentially funding.

It was agreed that *Fusarium solani* is the main concern for turtles in captivity and a threat to egg and meat consumers. In the non-infectious category, chelonitoxication and the mass poisoning it causes was considered of great importance. The pathogen transmission routes need further research to better understand the mechanisms at play. New hatcheries are being established in some areas to take economic advantage of tourism, without necessarily considering hygiene (e.g. wearing gloves while handling the eggs and the hatchling, digging the nest in new locations in consecutive seasons to avoid disease spread from previous dead-decayed eggs etc.) and the biological needs for the eggs to hatch (eg. right temperature, proper depth of the nest, how to handle the eggs etc.).

The discussion about the cultural dimensions of interacting with sea turtles or the importance for indigenous groups concluded that there was a lack of knowledge in this field among the workshop participants and a need for more social science studies. Social science experts work directly with the communities that interact with sea turtles. According to their experience, sea turtle conservation brings the communities together, gives them a common cause and sense of belonging to the environment.

3.3.4. Risk Management

3.3.4.1 International workshops

The current global management for the highest ranked hazards in risk assessment step were reported in table 3-5 along with the difficulties and defects for each strategy. One Health considerations were also reported, however data deficiency about zoonosis and biotoxicity limited the current management to "expand the knowledge and awareness of the egg and meat consumers". Several management options were suggested for socio-economic aspects of interacting with sea turtles, however this list included here is not exhaustive.

Table 3-5 Current risk management for sea turtle disease hazards with notes on difficulties and defects.

A) Infectious diseases

Hazard	**Current management**	**Difficulties and defects**
Parasites: *Spirochiidae,* *Caryospora cheloniae,* *Ozobranchus branchiatus* Arthropod *spp.*	Sporadic and opportunistic rehabilitation	Data deficient, limited number of experts in this area, the diagnostic tests are not performed in many regional management units
Bacteria: *Vibrio spp., Pseudomonas spp., Escherichia coli, MRSA, Klebsiella*	Sporadic and opportunistic rehabilitation More recent research on antibiotic resistant bacteria	Data deficient, limited number of experts in this area, the diagnostic tests are not performed in many regional management units
Fungi*:* *Fusarium solani, Aspergillus spp., Cladosporium spp., Penicillium spp*	Sporadic and opportunistic rehabilitation Quarantine and hygiene in captivity	Data deficient, limited number of experts in this area, the diagnostic tests are not performed in many regional management units
Viruses: Chelonid α-herpes virus 5 (FP) Cm-PV1 and CC-PV1:	Surgery in some regions, continuous research on epidemiology and aetiology N/A for papillomaviruses	Data deficient, limited number of experts in this area, the diagnostic tests are not performed in many regional management units

B) Non-infectious diseases

Hazard	**Current management**	**Difficulties and defects**
Anthropogenic: Habitat degradation, Pollution/plastic	By-catch, accidents and entanglement: Marine park and governmental policies to use TED, and avoid stainless steel fishing hooks, avoid trawling. Defining protected areas to avoid accidents. Debris ingestion: Public involving workshops and programs to reduce plastic usage and littering near the ocean, and cleaning the beaches, rehabilitation	Region based, incompatible ethical and legal approaches across borders.
Environmental: Climate change	Debilitated Turtle Syndrome and cold stunning: Rehabilitation, training, educations	The capacity of rehabilitation is not enough in some regions with mass stranding; more research is required in terms of treatment
Medical	Malnutrition: Rehabilitation Neurological diseases: managing toxin emissions in some areas	Neurological diseases: data deficiency. Lack of health baseline data

C) One Health

One Health consideration	**Current management**	**Difficulties and defects**
Zoonosis	Expanding the knowledge and awareness of meat and egg consumers	Sporadic reports

Bio-toxins	Expanding the knowledge and awareness of meat and egg consumers	Data deficient, mass death of humans, but no test to rule out contamination. Often in remote areas
Socio-economic and cultural aspects of interacting with sea turtles	Expanding ecotourism and turtle watching activities. Implementing alternative jobs to avoid overfishing and poaching. Defining and modifying "*sustainable*" hunting for cultural purposes. Spiritual and cultural wellbeing of communities with close relationships to environment. Involving the communities in conservation programs.	Needs more social science involved

3.3.4.2 Local workshop

In the local risk management workshop, the overarching concern was inadequate communication between different sectors working on sea turtle surveillance and conservation. The attendees referred to the lack of comparable and accessible data for researchers, conservationists and government sections. The reason behind "data protection" or limited information sharing can be confidentiality, or variations in legislation for different organisations collecting such information, nonetheless it impacts on the success of conservation initiatives.

Management options for previously assessed risks

The management options to reduce the risk of "Macro plastic pollution" and "*Enterobacteriacae* and multi-resistant bacteria" were summarised in table 3-6.

Table 3-6 Risk management options and scoring the effectiveness and feasibility in the Townsville management workshop.

A) Risk management options for Macro plastic pollution

Management options	Effectiveness	Feasibility	Decision
eliminating the impacts of the macro plastic that has already been released			
Initiatives to alter the disposing methods	7	5	Beyond the scope of this group
Initiatives to clean beaches	7	8	Beyond the scope of this group
Installing storm drain filters	9	7	Beyond the scope of this group
Research on engineering structures to remove macro plastics from the ocean	7	3 (due to cost)	Beyond the scope of this group
Governmental policies	8	3 (political decision)	Beyond the scope of this group
Reducing further input of macro plastic in the environment			

Research on providing affordable biodegradable items	7	3	Beyond the scope of this group
Education and awareness to reduce littering and purchasing of plastics	8	9	This forms part of existing university subject curriculum, but needs to be addressed in primary and secondary schools as well. Not known to this group. GBRMPA ReefHQ is an education facility and can educate on this topic as well.
Governmental policies	8	3	Beyond the scope of this group

B) Risk management options for Enterobacteriacae and multi-resistant bacteria

Management options	Effectiveness	Feasibility	Decision
Education and awareness including personal and protective equipment when working with sea turtles	9	8	DEHP and GBRMPA have staff that would be involved in such a relocation and would require PPE as part of their risk assessment
Education and awareness to reduce the prescription and consumption of antibiotics	6	8	Beyond the scope of this group
Sewage treatment and extracting the antibiotics from sewage water	7	3	Beyond the scope of this group

The management scale for "Macro plastic pollution" can be as small as a school or as big as the Queensland state. The group suggested that it was divided to two categories: 1) eliminating the impacts of the macro plastic that has already been released and 2) to reduce further input. For the first category, promotion of beach clean-up initiatives and rubbish collection; installing storm drain filters, which requires local and external donors and long-term monitoring; promotion of funding for large scale ocean clean-up projects. For the first category, the options to reduce the production and/or input included, but were not limited to **education and awareness** to reduce littering and use of disposable plastics, **research** on providing affordable biodegradable items and **governmental policies** to eliminate the use of single use plastics as initiated in Queensland in 2018[4].

For "*Enterobacteriacae* and multi-resistant bacteria" again, a preventive cause was to promote education and awareness including personal and protective equipment when working with sea turtles, and to reduce the prescription and consumption of antibiotics. The post-release management options included extracting the antibiotics from sewage water and promote funding for research into solutions for this procedure. The feasibility and effectiveness of these options were scored in table 3-6.

[4] https://www.qld.gov.au/environment/pollution/management/waste/recovery/reduction/plastic-bags

Critical control points for a mock clutch translocation

For a full explanation of thethe procedures and steps in a disease risk analysis for clutch translocation see appendix 8. The clutch translocation scenario and critical control point allocated by experts in the local management workshop are shown in figure 3-4.

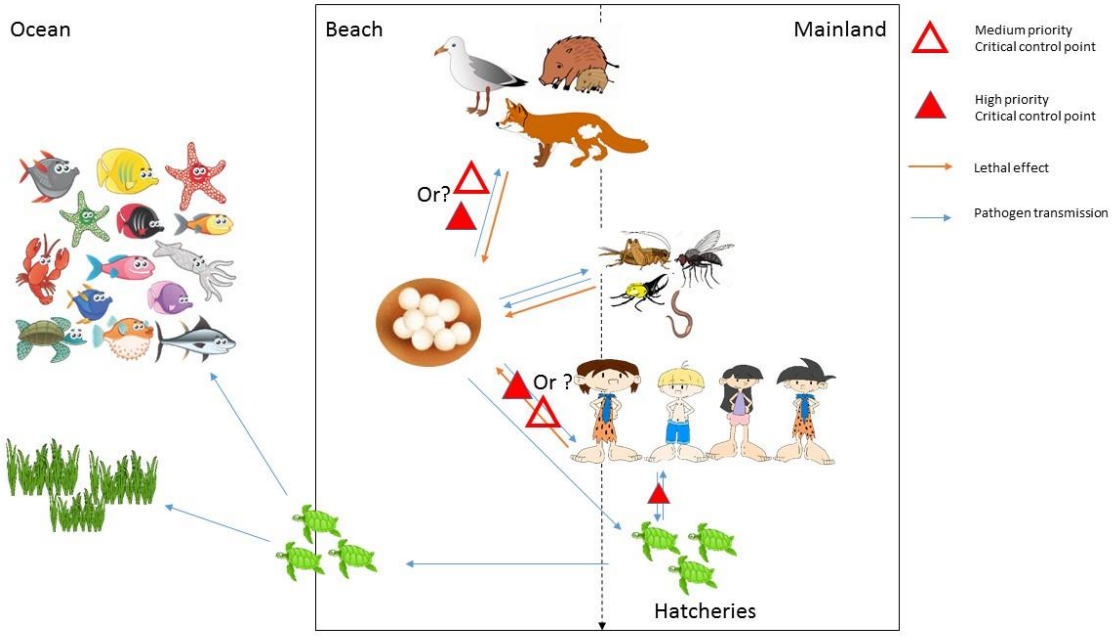


Figure 3-4 The clutch translocation scenario, pathogen transmission pathways, lethal effects of predators and critical control points

Time management and temperature control were suggested to be critical for transporting the eggs. Personal protective equipment and hygiene were proposed as the effective and feasible options to avoid the risk of contamination. Screening the destination for potential pathogens was suggested, however, the feasibility was ranked low. Nest protection and monitoring to reduce the risk of predation was critical to justify the time and cost spent for translocation. The group suggested development of protocols and surveillances for hatchery establishment.

3.4. Discussion

There is no standardised and unified method to perform a DRA (Hartley and Sainsbury, 2017). Workbooks, paired-ranking, expert workshops and scenario trees have been successfully used in previous analyses (Armstrong *et al.,* 2003; Jacob-Hoff *et al.,* 2014; Pacioni *et al.,* 2015) and were therefore adapted in this study. The comprehensive explanation of each method is provided in the "*The manual of procedure for wildlife disease risk analysis*" (Jacob-Hoff *et al.,* 2014).

The current study was an endeavour to update the information about health hazards of sea turtles in a structured way. Wildlife DRA as a decision making tool is gaining recognition and DRA procedures and manuals have recently been published (Jacob-Hoff *et al.,* 2014; Pacioni *et al.,* 2015). Although, it is more practical to use a DRA for a specific scenario or case such as clutch translocation or hatchery establishment, the current manuscript provides the up-to-date baseline information on a global scale and a guide to carry out such practices on a local scale.

The hazard identification was more exhaustive than a standard review for DRA, containing the collective information of disease causing hazards (appendices 1-5). The health hazards were assessed via a literature-based review and with input from experts in the field (section 3.3). One of the considerable uncertainties revealed in this process was the data deficiency in the link between the presence of pathogens and infectious diseases of sea turtles. Additionally, viruses were identified as the least studied pathogens, although FP is suggested to have a viral aetiology.

A higher rate of disease in immunocompromised individuals was repeatedly reported and a possible link between immunosuppression and environmental contaminants as a result of anthropogenic influence was suggested. One Health and the social aspect of interacting with sea turtles and desirably society based conservation appeared to need more attention and research.

In this manuscript, the risk management consisted of a global review of the current policies, possible management options and the difficulties of taking actions and was reviewed by members of IUCN SSC[5] Marine Turtle Specialist Group who are influential in making the policies and executing them.

This DRA is mainly a guide to support future risk assessments/management based on specific risk mitigating questions for which the management section should be done by regional policy makers. Such discussions were initiated with appropriate local Australian government representatives to clarify appropriate steps in risk management for specific scenarios.

Conducting a DRA is an iterative process and risk analysis should continuously be reviewed and modified to represent the most recent information for policy and management decisions (Hartley and Sainsbury, 2017). Disease surveillance and data collection to determine the contributing factors in population health is one practical approach to create informed risk management for wildlife; and sea turtles are no exception. The future DRAs can benefit from this comprehensive review, but with no doubt the baseline information will expand as more pathogens are discovered, disease manifestations are reported and diagnostic tools are introduced which will call for a review and modification of health and disease determinants included here.

The anthropogenic threats affecting sea turtles are increasing and so are the conservation initiatives to help these charismatic animals. Disease and health of sea turtles are not easily measured and

[5] Species Survival Commission (SSC)

management agencies are going to look for structured approaches to inform their decisions. The work presented here will hopefully form a platform for disease risk management of sea turtles.

How I achieved the aim of the chapter:

1. I collaborated with IUCN representatives in this field and acquired the appropriate guidelines to carry out the DRA
2. I performed hazard Identification using two approaches
   - A comprehensive literature review of the published information
   - Contact with the experts in the field to acquire unpublished information
3. I performed a risk assessment by
   - Conducting two international workshops with relevant experts
   - International collaboration using emails
   - Travelling to several rehabilitation centres for face to face interviews
4. I performed a collegial risk management exercise by
   - Collaboration with international experts using emails
   - A domestic workshop with local experts
5. I continued to communicate with experts throughout the process to get feedback on every stage

4. CELL LINE ESTABLISHMENT AND QPCR DEVELOPMENT TO SURVEY THE PRESENCE AND IMPACTS OF VIRUSES IN GREEN TURTLES[6]

The aim of the chapter:

To develop and assess virological methods to study the presence and impacts of viruses in green turtles from the northern Great Barrier Reef

4.1. Introduction

The **Sea Turtle Disease Risk Analysis** in Chapter three, revealed that viruses are the least studied health hazards of sea turtles. Only five families of viruses have been described in sea turtles: herpesviridae, retroviridae, unclassified tornovirus, papillomaviridae and nodaviridae. (Quackenbush *et al.,* 1997; Lackovich *et al.,* 1999; Manire *et al.,* 2008; Ng *et al.,* 2009; Fichi *et al.,* 2016). Gray patch disease, lung eye trachea disease and fibropapillomatosis are closely associated with herpesviruses (Lackovich *et al.,* 1999; Herbst *et al.,* 1999b). Papillomaviruses were detected in cutaneous lesions of a green and a loggerhead turtle (Manire *et al.,* 2008). The sporadic cases of retrovirus, tornovirus, and betanodavirus infections were identified via molecular analysis and have not been associated with any

[6] This chapter is adapted from a published manuscript: Mashkour, N., Maclaine, A., Burgess, G. W., & Ariel, E. (2018). Discovery of an Australian *Chelonia mydas* papillomavirus via green turtle primary cell culture and qPCR. *Journal of Virological Methods*, 258, 13-23. https://doi.org/10.1016/j.jviromet.2018.04.004. The co-author consent to include the paper in this thesis is provided in appendix 12.

diseases to date (Quackenbush *et al.,* 1997; Ng *et al.,* 2009; Fichi *et al.,* 2016). Other chelonian viruses such as adenovirus and iridovirus are of great importance in reptilian virology (Ariel, 2011) and sea turtles have never been tested for these viruses. Although, herpes and papilloma-viral infections have caused diseases in sea turtles and have the potential to be transmitted between stranded turtles in rehabilitation centres (Manire *et al.,* 2008), there are no established protocols to screen the patients for the presence of these viruses upon admission to turtle hospitals. It is important to be aware of these viral diseases and establish methods for their accurate and fast diagnosis.

Virological methods are diverse and rapidly evolving. Many of the conventional diagnostic methods have been complemented or even replaced by specific modern techniques (La'Toya and Wellehan, 2013). However, the study of viruses has always been a multidisciplinary science (Campbell, 1995) and no single method is sufficient for detection, identification and isolation of viruses. To optimise diagnosis, both culture and non-culture methods should be used in combination (Leland and Ginocchio, 2007).

Histopathological features, in combination with clinical information, can explain the progress of an infectious disease and help to identify a pathogen (Gupta *et al.,* 2009). Pathological findings guide the choice of virological methods used, particularly where the viral infections are influenced by variables such as: the infection period, the immune status of the individual and the population, as well as potential concurrent infections. Such concurrent infectious agents may be asymptomatic or the cause of the clinical and pathological signs (Campbell, 1995). Without pathological evidence, the role of isolated or detected viruses in sick reptiles can be challenged. Histopathological investigation can aid in interpreting the interaction between reptilian hosts and viruses that may be normal flora (La'Toya and Wellehan, 2013). Histological analysis helped the diagnosis of infectious diseases in sea turtles such as fibropapillomatosis (Jacobson, 1989). To benefit from the advantages of pathology, samples from turtles in this study were preserved for histopathological investigation.

Historically, cell culture was the golden standard for virological studies (Leland and Ginocchio, 2007; Ogilvie 2001). In recent years, the appeal of using cell culture in virology has decreased due to the rapid evolution of molecular methods and the skill level required in cell culture maintenance (Ogilvie, 2001).

Cell culture can facilitate the study of viruses which are obligate intracellular parasites. This method is more ethical and less expensive than propagating viruses in embryonated eggs or live animals (Zurlo *et al.,* 1994, Leland and Ginocchio, 2007). An immortal cell line can also be used for diagnostic purposes and vaccine production (Lee *et al.,* 2013). However, established cell lines with frequent sub-cultivation will undergo selection where after they will no longer reflect the diversity of cells in the

original source material (Hughes *et al.,* 2007). Primary cell cultures provide a broader spectrum of host cell types to propagate viruses thereby increasing viral susceptibility and the chance to isolate viruses compared to established homogenous cell lines. Primary cell cultures of the same host origin as the samples tested are also known to be more susceptible to viral isolation than those from a different host (Lu *et al.,* 2000, Leland and Ginocchio, 2007, Swaminathan *et al.,* 2015).

The only commercially available chelonian cell line originated from a terrestrial turtle (*Terrapene carolina*) (Clark and Karzon, 1967). Establishments of green turtle cell cultures have been reported in the literature (Koment *et al.,* 1982; Lu *et al.,* 1999; Moore *et al.,* 1997; Work *et al.,* 2009) but these are not commercially available. In addition, the Convention on International Trade in Endangered Species (CITES) has put limitations and controls on the international transfer of green turtle samples. The conditions are explained in Appendix I of the Checklist of CITES species (UNEP-WCMC, 2014). To provide a local repository of sea turtle primary cell culture and facilitate the isolation and study of sea turtle viruses, primary cell lines were established and characterised from local green turtle embryos in this study.

Specific and sensitive identification of viruses is made possible by PCR detection and subsequent sequencing. Reptilian virology is not an exception; intact nucleic acid from an infectious agent can be detected by PCR even if the agent is non-viable and non-culturable (Ariel, 2011). However, this can be problematic as the high sensitivity may lead to false positive results. To avoid errors, care must be taken during sample collection and analysis and the PCR products need to be identified after amplification. The use of probe in quantitative PCR followed by sequencing helps product validation and makes the results more reliable (La'Toya and Wellehan, 2013). To facilitate the study of viruses known to infect chelonians: herpesviruses, adenoviruses, papillomaviruses and iridoviruses, molecular analyses were applied. Initially four specific PCR primers were designed to detect these four DNA viruses. The idea was to design more PCR primers if the pathological reports or viral isolation in cell culture suggested infections with other DNA or RNA viruses that were not detectable with the first suite of PCR primers.

In this project, methods were developed for viral discovery in green turtles. Samples from dead or sick animals were screened by histopathology, primary cell culture and qPCR and this resulted in the discovery of the first Australian *C. mydas* papillomavirus.

4.2. Material and Methods

4.2.1. Animals and sampling protocols

Nine green turtle eggs from three different clutches were collected from Heron Island, Queensland to establish primary cell lines. Live (healthy and sick) green turtles were sampled for viral screening from Cockle Bay, Edgecumbe Bay, Ollera Beach and Toolakea Beach in Queensland, Australia. Turtles were considered to be sick if they were emaciated, floating, stranded, wounded or afflicted with skin tumours. Dead turtles from the same locations as well as from Cairns and Airlie Beach regions were sampled during necropsy. Some of these turtles were euthanased after unsuccessful rehabilitation while others were dead when collected. Egg and sample collections were carried out with approval from the James Cook University Animal Ethics Committee (Ethics Approval No. A2219), Department of Environment and Heritage Protection (DEHP) (Permit No. WITK15765815 and WISP16625115) and the Great Barrier Reef Marine Park Authority (GBRMPA) (Permit No. G382911). Skin samples were collected from the trailing edge of the front flippers after wiping the skin with alcohol swabs and using sterile scalpel blades, approximately 1cm×1cm×1cm. If the animals had abnormal skin growth a second up to three tumour tissues were collected after cleaning the surface with alcohol swabs. Blood samples were collected up to maximum of 5ml which were precipitated in the virology laboratory. The serum was shared with other group members for serology and the pellets were used for viral isolation and molecular analysis. Parallel samples were collected and either preserved in 95% molecular grade ethanol for molecular analysis or frozen at -20°C for cell culture viral isolation. From lesions, an additional sample was collected and preserved in 10% phosphate buffered formaldehyde for histological analysis.

4.2.2. Primary cell establishment and viral isolation

4.2.2.1. Primary cell establishment

The eggs were incubated at 29°C for up to 45 days to obtain culture explants from different embryonic stages. Embryonic development was ascertained via candling twice weekly in the first 35 days and daily thereafter. Seven eggs developed embryos and were suitable for primary cell line establishment. Prior to cultivation, the surface of the eggs were disinfected with 70% ethanol and the whole embryo (four eggs at day 35) or specific organs (three eggs at days 40 or 45) were collected for culture explants under sterile conditions (table 4-1).

Table 4-1 Culture explants from Chelonia mydas embryo and the nomenclature

Days post incubation	Culture explant	Primary cell lines nomenclature*
35	The whole embryo	CMEW (*Chelonia mydas* embryo whole)
40 and 45	Muscles	CMEM (*Chelonia mydas* embryo muscles)
40 and 45	Head	CMEH (*Chelonia mydas* embryo head)
40 and 45	Heart, liver, kidney, lung	CMEI (*Chelonia mydas* embryo internal organs)

*The primary cells with similar origin but from different eggs were given successive numbers, i.e. CMEM1, CMEM2 and CMEM3.

Tissues were washed three times with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) containing 4X Antibiotic-Antimycotic solution (Thermo Fisher Scientific: 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B), then finely chopped under sterile conditions. The enzymatic disaggregation was performed using 0.25% trypsin-EDTA (Thermo Fisher Scientific) on a magnetic stirrer at low speed for 30 minutes (min). The cell suspension was collected and centrifuged for 5 min at 492 × *g*. The pellet (~1ml) was then re-suspended in 5ml DMEM with 10% fetal bovine serum (FBS) (Bovogen biologicals) and 4X Antibiotic-Antimycotic solution. The cell suspension was added to 25cm^2 tissue culture flasks (SARSTEDT) and incubated at 25°C. The culture was maintained in non-vented flasks and the media contained 1.5 to 2.2 g/L sodium bicarbonate (5% CO_2). The following day, cells were washed with phosphate-buffered saline (PBS) and the media was changed.

4.2.2.2. Sub-culture and cryopreservation

After full confluency of the primary cell line, they were trypsinised using 0.25% trypsin-EDTA solution and transferred to new culture flasks. In total 13 primary cell lines were established, four whole embryos, three each of muscles, heads and internal organs. CMEM1, CMEM2 and CMEH were observed to have the highest growth rate: The passage ratio was 1:2 and on average was performed once a week until the 20th passage when the doubling time was reduced to ~5 days. The plating efficiency at this stage was calculated according to Mather and Roberts (1998). Medium was changed at sub-cultivation or in case the cells were not confluent but the colour of the medium indicated a decrease in pH. The antibiotic supplement in the media was reduced to normal 1X concentration after 4 passages. The cells were cryopreserved every passage up to the 20th in FBS supplemented with 10% (v/v) Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and were kept at -80°C for short term storage (up to 6 months) and submerged in liquid nitrogen for long term.

4.2.2.3. Temperature and serum optimisation

The optimal growth conditions were determined by measuring cell proliferation at different incubation temperatures and FBS concentration in the media. The experimental temperatures were chosen to reflect the suitable range for proliferation of ectothermic viruses (20°C 25°C and 28°C). Temperatures below 20°C and above 30°C were not conducive for growth of sea turtle cell lines according to Fukuda *et al.,* (2012) and Lu *et al.,* (1999). Foetal bovine serum supplementation was tested at three different concentrations: 5%, 8% and 10%. Individual cell viability was determined by trypan blue staining on the seeding day. Cells were seeded into 6-well cell culture plates at ~10^4 cells/ml with approximately 100% viability. Cultures were incubated for 7 days, with cells harvested from replicate wells each day and the number of cells in suspension was determined in a Neubauer counting chamber (The results from CMEM1 are shown in this chapter).

4.2.2.4. Authentication tests

Karyotyping

Cytological analysis to count chromosomes was conducted at passage 20 and 25 according to the method described by MacLeod and Drexler (2005). Cells were sub-cultured and incubated for 24 hours (h) to reach the logarithmic phase, then the complete medium was replaced with colcemid solution (final concentration of 40ng/ml) (Sigma-Aldrich). After 24 h incubation at 25°C, the cells were harvested and treated with 5 ml of hypotonic solution (KCl and Na-citrate) at room temperature for 7 min and then fixed for 5 min in 10 ml fresh, ice-cold fixative solution (1:3 acetic acid: methanol). A drop of fixed cells was spread onto clean pre-cooled glass slides and were aged by baking overnight at 60°C. The slides were stained with 5% Giemsa solution in PBS (pH 6.8) for 15 min. The slides were observed under light microscope and 54 random metaphase spreads were used for each chromosomal count.

Molecular analysis

Molecular analysis of mitochondrial DNA D-loop was done to provide further documentation of the origin of the primary cell line. DNA extraction was carried out on harvested cells and also blood or tissue samples from three green turtles, human, eastern water dragon

(*Intellagama lesueurii lesueurii*), giant tiger prawn (*Penaeus monodon*) and krefft's river turtle (*Emydura macquarii krefftii*) using Wizard® Genomic DNA Purification Kit (Promega) according to manufacturer's protocol. Two green turtle samples were from two of the three female turtles that laid the eggs (mother turtles) on Heron Island and one was from a green turtle from Cockle Bay. To amplify a 400bp region of the mitochondrial DNA D-loop a set of PCR primers were used that were designed by Norman *et al.* and have been frequently described for ecological and population studies of sea turtles: TCR5 (5'-TTG TAC ATC TAC TTA TIT ACC AC-3') and TCR6 (5'-GTA CGT AC A AGT AAA ACT ACC GTA TGC C-3') (Norman *et al.,* 1994; Joseph *et al.,* 2014; Hayashi 2015). To carry out the PCR, 10µl of GoTaq Green Hot Start Mastermix (Promega), 0.8µM of each primers and ~80 ng of template DNA were used and nuclease free water was added to a final volume of 20µl. Thermocycling was performed with a Rotor-Gene 6000 machine and with following reaction conditions: 94°C for 2 min denaturation followed by 30 cycles of 94°C for 30s, 55 °C for 35s, and 72°C for 30s, with a final extension of 72°C for 2 min (Joseph *et al.,* 2014). The PCR products were visualised on 1.2% agarose gel to confirm the product size and green turtle and cell culture products were sent for sequencing (Macrogen, Korea). The results were analysed using Geneious 10.2.3.

4.2.2.5. Mycoplasma test

Mycoplasma can alter the cell biosynthetic and even the chromosomal properties of the cells. There are also reports of gene expression interference due to mycoplasma contamination (Chernov *et al.,* 2014). To test the primary cell lines for mycoplasma contamination, Mycoplasma Detection Kit-QuickTest (BioTools) was used according to the manufacturer's protocol. Ten µl of the cell culture supernatant was collected from cell culture after 36 h of incubation. Clean culture media (DMEM) was used as negative control. Positive control was the metabolites of mycoplasma provided in the kit.

4.2.2.6. Primary cell viral susceptibility test- case of Bohle iridovirus

Newly established primary cell lines were tested for their susceptibility to *Bohle iridovirus* (BIV) a ranavirus first described in north Queensland (Speare and Smith, 1992; Whittington *et al.,* 2010; Ariel *et al.,* 2015). Two cell lines were selected to represent host tissues originating from muscles and head: CMEM2 passage 21 and CMEH passage 19. The reason behind this selection is providing broader host range and also higher growth rate of these primary cells. The susceptibility to BIV was compared to two fish cell lines: fathead minnow (FHM) epithelial cell line and bluegill fry (BF-2) fibroblastic cell line (Granzow *et al.,* 2001). The fish cell lines were obtained from the Aqua-pathology teaching laboratory,

Department of Biomedicine, James Cook University and were tested for cross contamination with other cell lineages and mycoplasma contamination. The BIV isolate used in this project was isolated from an experimental inoculation in juvenile eastern water dragons (*Intellagama lesueurii lesueurii*) (Maclaine *et al.,* 2018). Cells were seeded in 24-well plates (~10^4 cells/ml) and incubated at 25°C. When a sub-confluent cell monolayer formed, these four cell lines were inoculated with a BIV infected kidney tissue homogenate after filtering through 0.45µm membrane syringe filters (Millex-GV). The viral titration of the infected tissue was $10^{5.33}$ $TCID_{50}$/ml. Ten-fold serial dilution of the viral isolate was prepared from 1 to 10^{-9}. One hundred µl of each dilution was added to three wells of each cell line in 24-wells plates. Following low speed centrifugation and viral adsorption for 1 h at 25°C, excess viral suspension was removed and 500 µl complete medium was added to each well of the 24-well tissue culture plates. Six wells of each cell line were reserved as negative controls and were inoculated with DMEM without virus. Cultures were monitored daily for viral-specific CPE and the viral titre was calculated after 6 days incubation.

4.2.2.7. Screening green turtle samples for presence of viruses using primary cell cultures

Blood, skin biopsy and cloacal swabs were taken from sick and healthy green turtles foraging on the east coast of Queensland. In addition to these samples, spleen, liver, lung, kidney, heart and muscle were sampled during necropsies of dead and euthanased turtles.

For viral isolation using primary cell lines, ~20grams of each sample were homogenised with 1 ml DMEM and freeze/thawed three times at -20°C. The homogenised samples were filtered through 0.45µm membrane syringe filters (Millex-GV). Ten-fold serial dilutions were prepared from each sample (as explained in section 4.2.2.6.) and a total of 50µl from each dilution was added to three wells of 80% confluent cell line monolayers. CMEM1 (passage 20) and CMEH (passage 20) are discussed here. Primary cells were grown in DMEM with 10% FBS at 25°C in 96 well plates. The plates were incubated for one week and checked daily under a microscope at x40 magnification for CPE. In ~30% of the cases the culture colour was orange or yellow indicating low pH due to cytotoxicity of the samples. To improve the screening accuracy, if cytotoxic effects were observed in all wells, the 10^{-9th} dilution of the samples were subjected to additional dilution of 1/5, 1/25, 1/125 and inoculated on fresh cell monolayers. To decrease the possibility of false negatives, blind passaging was performed after seven days of incubation as follows: 50 µl of cell culture supernatant was transferred from each of the inoculated wells to a corresponding well with freshly prepared, non-infected cell monolayers on a separate plate. Supernatant from wells with CPE or suspected cell changes were preserved at -20°C for further analysis.

4.2.3. Molecular analysis establishment and viral detection

4.2.3.1. DNA extraction and qPCR set up

For molecular screening, four DNA viruses were selected: herpesvirus, ranavirus, adenovirus and papillomavirus. DNA extraction directly from collected samples or from primary cell pellets was performed as explained (Section 4.2.2.4: Molecular analysis). Extracted DNA from non-infected primary cell lines were used as negative control. The dye based qPCR reactions (20µl) were made with 10µl of 5X GoTaq® qPCR Hot Start Master Mix (Promega), 1.6µl from each primers (0.8µM), ~2µl of template DNA (~80 ng) and 4.8µl nuclease free water. The dye based qPCR protocols for each viruses can be found in table 4-2. GoTaq® Probe qPCR reactions (20µl) were made with 10µl of GoTaq® Probe qPCR Master Mix (Promega), 1.6µl from each primers (0.8µM), 0.2 µl of probe, ~2µl of template DNA (~80 ng) and 4.6µl nuclease free water. PCR protocols for GoTaq® Probe qPCR assays are explained in table 4-2. Thermocycling was performed on a Rotor-Gene 6000 Real-Time PCR Machine.

4.2.3.2. PCR primer, probes and positive control design

For each virus the primers described in the literature were used, but more specific primers were also designed to detect green turtles viruses (only the primers designed for papillomaviruses are explained here). To test the samples for the presence of herpesviruses, PCR amplification of a partial sequence of the membrane associated phosphoprotein (MPP) gene was done using the primers described by Greenblatt *et al.,* (2005), modified by Ariel *et al.,* (2017). The sequences of the primers and the PCR protocols are listed in table 4-2. To test for ranavirus, PCR amplification targeting the major capsid protein (MCP) region of the EHNV genome was performed using primers and protocols previously described by Jaramillo *et al.,* (2012) (table 4-2). The adenovirus primers were adapted from a protocol that Wellehan and his colleagues developed in 2004 to detect reptilian adenoviruses (Wellehan *et al.,* 2004). A nested PCR was performed to amplify a partial sequence of DNA-dependent DNA polymerase (table 4-2).

To test for presence of papillomaviruses the primers designed by Rector *et al.,* (2005) were used to detect the E1 gene of raccoon (*Procyon lotor*) papillomavirus type 1 (Table 4-2). The primers and PCR protocol were also used by Manire *et al.,* (2008) to detect *C. mydas* papillomavirus 1 (CmPV-1) and *C. caretta* papillomavirus 1 (CcPV-1) for the first time (Rector *et al.,* 2005; Manire *et al.,* 2008). Knowing the full genome of CmPV-1 (Herbst *et al.,* 2009), different sets of specific primers and probes were

also designed in this study to amplify partial sequences of the E1 and L1 genes. To design the primers the CmPV-1 and CcPV-1 full genomes were downloaded from NCBI (GenBank accession no. EU257705 and EU257704 respectively). The sequences were imported to Geneious software, version 10.0.9. The desired regions of E1 and L1 gene were extracted from each virus and were exported to AlleleID® for primer design. The important design considerations were examined such as primer length, melting and annealing temperature. The designed primers were examined for specificity against targeted sequences in genbank using the Basic Local Alignment Search Tool (BLAST).

A synthetic positive control was developed to detect potential contaminations and functionality of designed primers (Cm-Pap-109 primer from table 4-2). This method was previously described by (Van den Hurk *et al.,* 2010). The same oligonucleotides designed for Cm-Pap-109 forward primer and the complementary sequence for the reverse primer (underlined) was used including a section of ChHV5 glycoprotein B sequence flanked by A residues (in bold):

5' -GCCGATGATGTCCACTTAT**A**AACAACACGCGAGCCAGAGC**A**CTACCTCTGTGGATTCAGC- 3'

ChHV5 glycoprotein B sequence was used in this synthesised control to have a product of known size that is different from papillomavirus to check the efficiency of the primers. In this case, if the positive control is amplified but there's no amplification in the test tube the negative result is not related to the PCR primer efficiency.

Table 4-2. PCR primers and protocols to detect herpesvirus, ranavirus, adenovirus or papillomavirus

Viruses	Target gene	Primers and probes names	Primer sequences	PCR protocols	References
Herpesvirus	MPP gene	UL-34A-F	5'CCT GAG CAA ATT TCT GGA CCTG3'	Dye basedPCR: -2 min at 95 °C -40 cycles (95 °C for 15s, 58 °C for 20s, 72°C for 90s) -72°C for 2 min	(Greenblatt *et al.,* 2005; Ariel, *et al.,* 2017)
		UL-34A-R	5'AAT TTT CGC GGC TTC TCG3'		
Ranavirus	94bp of major capsid protein (MCP)		5' GAC TGA CCA ACG CCA GCC TTA ACG3'	Dye basedPCR: -2 min at 95 °C -40 cycles (95 °C for 5s, 58 °C for 10s, 72°C for 15s) -95°C for 2 min	(Jaramillo *et al.,* 2012)
			5'GCG GTG GTG TAC CCA GAG TTG TCG3'		
Adenovirus	318-324bp of DNA	polFouter	5'TNM GNG GNG GNM GNT GYT AYC C3', where Y = C or T, N = A, C, G, or T, and M = A or C	Dye basedPCR: Nested PCR:	(Wellehan *et al.,* 2004)

	polymerase gene	polRouter	5'GTD GCR AAN SHN CCR TAB ARN GMR TT3' where R = A or G, M = A or C, d = A, G, or T, S = G or C, h = A, T, or C, and B = G, T, or C	-Nuclease free water 4.8 µl -Template 2 µl PCR protocol: -2 min at 95°C -45 cycles (95°C for 30s, 46°C for 60s, and 72°C for 60s) -7 min at 72°C *2 µl of product from above was used and amplified under the same conditions with* ***polFinner*** *and* ***polRinner*** *primers*	
		polFinner	5'GTNTWYGAYATHTGYGGHATGTAYGC3', where W = A or T		
		polRinner	5'CCA NCC BCD RTT RTG NAR NGT RA3'		
Papillomavirus	480bp of E1 gene	Degenerate primers AR-E1F2	(5'-ATGGTNCAGTGGGCNTATGA-3'),	-5 min at 94°C -45 cycles (94°C for 60s, 47 °C for 60s, and 72°C for 60s) -7 min at 72°C	(Manire *et al.,* 2008; Rector *et al.,* 2005)
		AR-E1R4 and 9	AR-E1R4 (5'ATTNCCATCHADDGCATTTCT-3') and AR-E1R9 (5'CATTWGTDGTDAYMAGSAKRGGVGGGCA-3')		
	109bp of E1 gene	Cm-Pap-109-F	5'GCC GAT GAT GTC CAC TTA T3'	Dye basedPCR: -2 min at 95°C -40 cycles (95°C for 10s, 60 °C for 20s, and 72°C for 30s) -2 min at 72°C	Current chapter
		Chm-Pap-109-R	5'GCT GAA TCC ACA GAG GTA G3'		
		Chm-Pap-109-P	5'FAM CGA CCC ATG AAG CCG CTG T BHQ13'	GoTaq® Probe qPCR: -2min at 95°C -40 cycles (95°C for 5s, 60°C for 15s)	
	183bp of E1 gene	Chm-Pap-E1-183-F	5'GCT CTG ATA TTA GCT TGC TG3'	Dye basedPCR: -2 min at 95°C -40 cycles (94°C for 10s min, 51 °C for 15s, and 72°C for 30s) -2 min at 72°C	
		Chm-Pap-E1-183-R	5'CGC ATA AGT GGA CAT CAT C3'		
	108bp of L1 gene	Chm-Pap-L1-108-F	5'GGG ATT AGG CAC TCT CAA3'		
		Chm-Pap-L1-108-R	5'GCG TTC ATT GTC AAC CAA3'		
		Chm-Pap-L1-108-P	5'FAM AAA CAA ATC CAC CCT GCC ATT AGA BHQ13'	GoTaq® Probe qPCR: -2min at 95°C	

				-40 cycles (95°C for 5s, 60°C for 15s)	

4.2.2.7. Viral detection via qPCR

Blood, skin biopsy and cloacal swabs were taken from sick and healthy green turtles foraging on the east coast of Queensland. In addition to these samples, spleen, liver, lung, kidney, heart and muscle were sampled during necropsies of dead and euthanased turtles. Molecular detection using qPCR were performed in parallel to cell culture and following the protocols mentioned in table 4-2.

4.2.4. Pathological analysis

Skin biopsies were taken from fibropapillomatosis skin tumours. During necropsies internal organs were also sampled for histopathological analysis if lesions were of an unknown aetiology or were indicative of infections. Samples were preserved in 10% phosphate buffered formaldehyde for histological follow-ups and as a back-up plan if the specific molecular analyses were not able to identify a virus. Histopathology was conducted by Veterinary Pathology and Biomedical Sciences, department of James Cook University, Townsville, Australia.

4.3. Results

4.3.1. Primary cell establishment

4.3.1.1. Morphology and growth characteristics

Initially a mixed morphology of epithelial and fibroblastic-shaped cells was observed (figure 4-1A) but after the 15th passage cells were all uniformly fibroblastic-shaped (figure 4-1B).

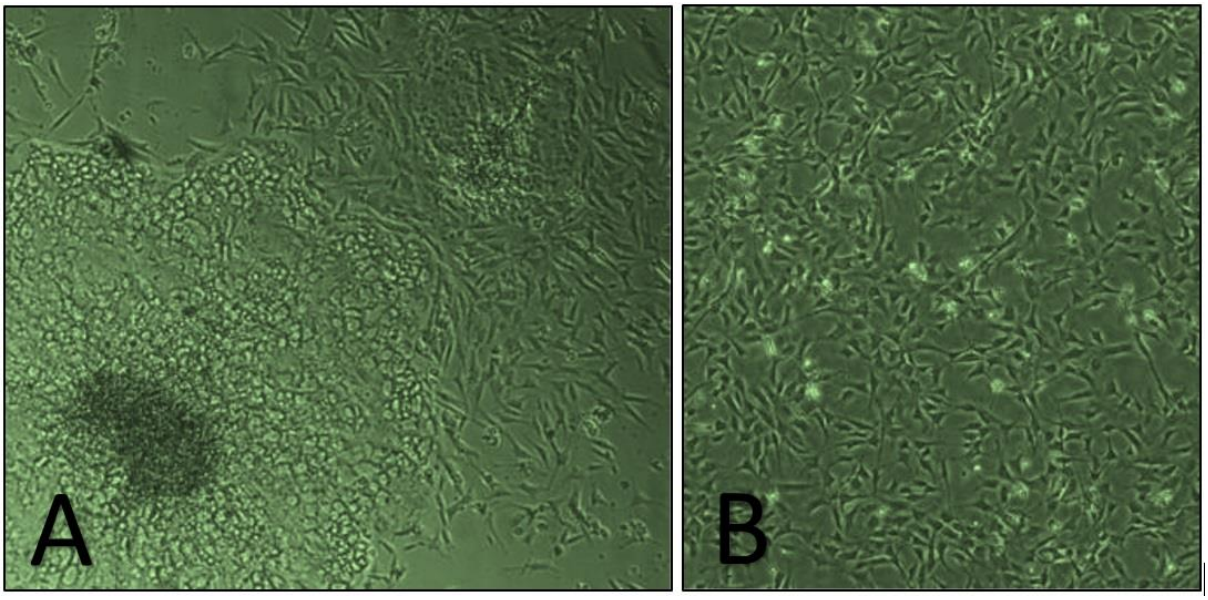


Figure 4-1 A) Mixed morphology of CMEM1 at passage three under phase-contrast microscope (40X). B) The morphology of CMEM1 remained consistently fibroblastic from passage fifteen (40X).

The plating efficiency was up to 50% and the average doubling time was ~5 days from the 20th passage on (figure 4-2).

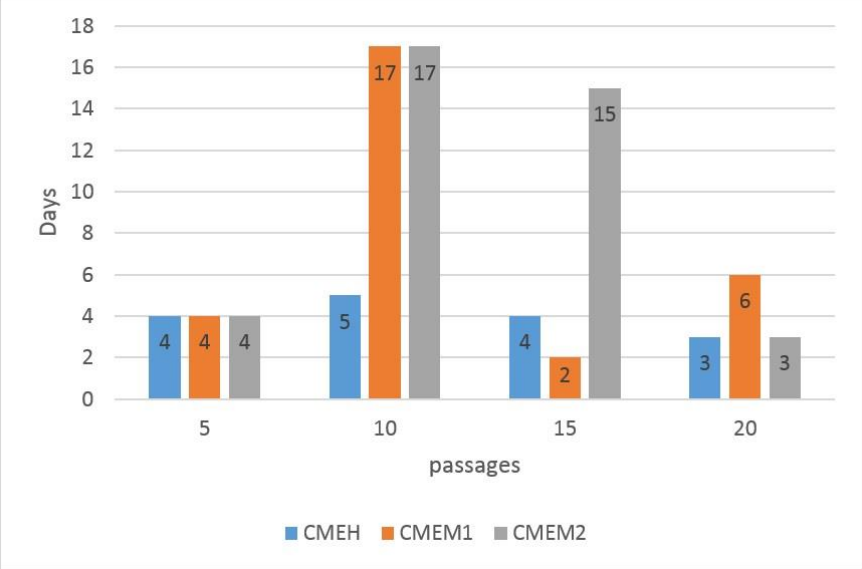


Figure 4-2 Doubling time of CMEH, CMEM1 and CMEM2 primary cell lines from passage 5 to 20.

4.3.1.2. Temperature and serum optimization

In two different experiments, primary cell lines were supplemented with 5%, 8% and 10% FBS and incubated at 20°C 25°C and 28°C. Figure 4-3 and 4-4 show the growth rate of CMEM1 under different FBS concentrations in the media and incubated at different temperatures. The viability of cells were

more than 95% in both experiments. The optimum FBS concentration and temperature were 10% and 28°C, respectively. But in order compare the viral susceptibility of the turtle primary cultures to that of FHM and BF-2, cells were inoculated at 25°C (Granzow *et al.,* 2001; Nishizawa *et al.,* 2008).

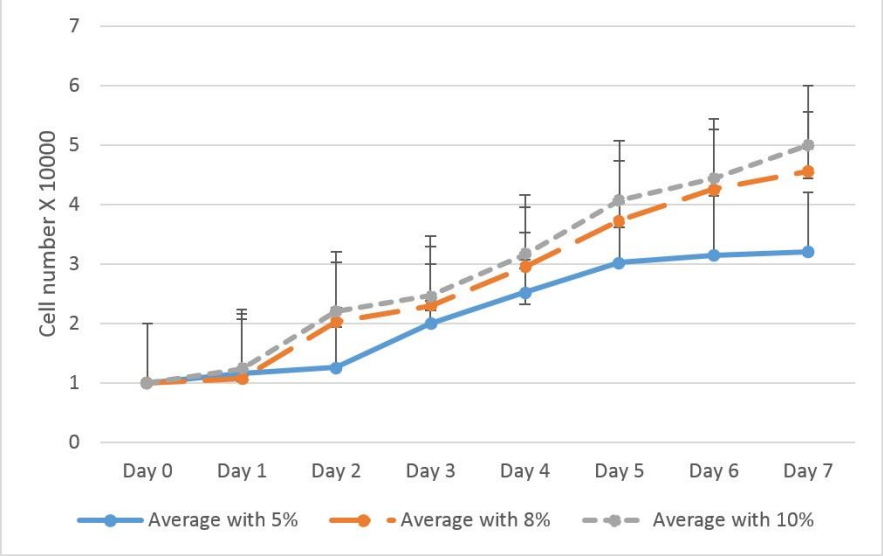


Figure 4-3 The growth rate of CMEM1 (passage 20) in DMEM supplemented with 5, 8 and 10% concentration of FBS at 25°C. Results for each day are the mean of triplicates.

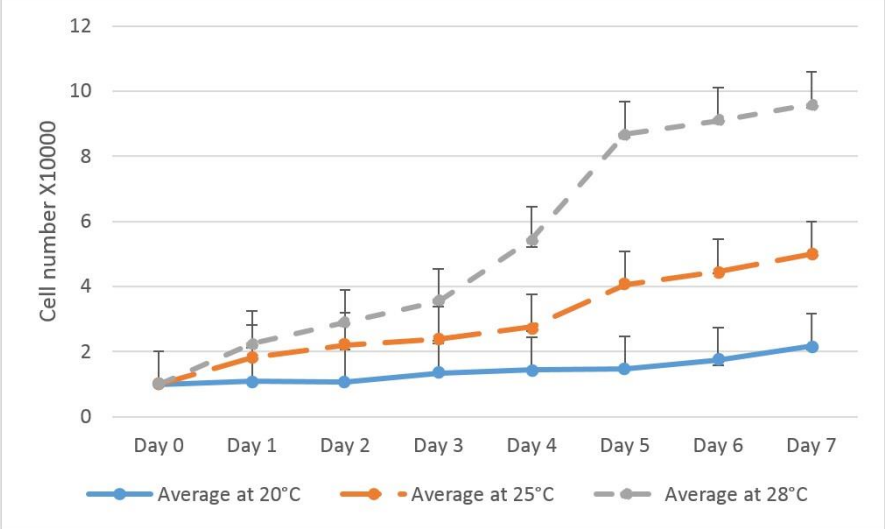


Figure 4-4 The growth rate of CMEM1 (passage 20) in DMEM and supplemented with 10% FBS at. 20 25 and 28°C. Results for each day are the mean of triplicates.

4.3.1.3. Authentication test

Karyotyping

The results from chromosome counting of CMEM1, CMEM2 and CMEH showed that the chromosome numbers were 56. In figure 4-5 chromosome analysis of CMEM1 is shown. The morphological features and chromosome counts remained stable in passages 20 and 25 of CMEM1, CMEM2 and CMEH.

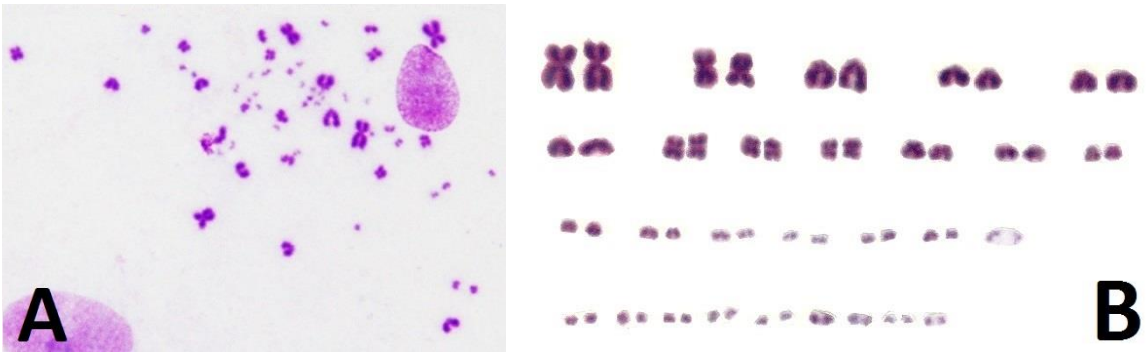
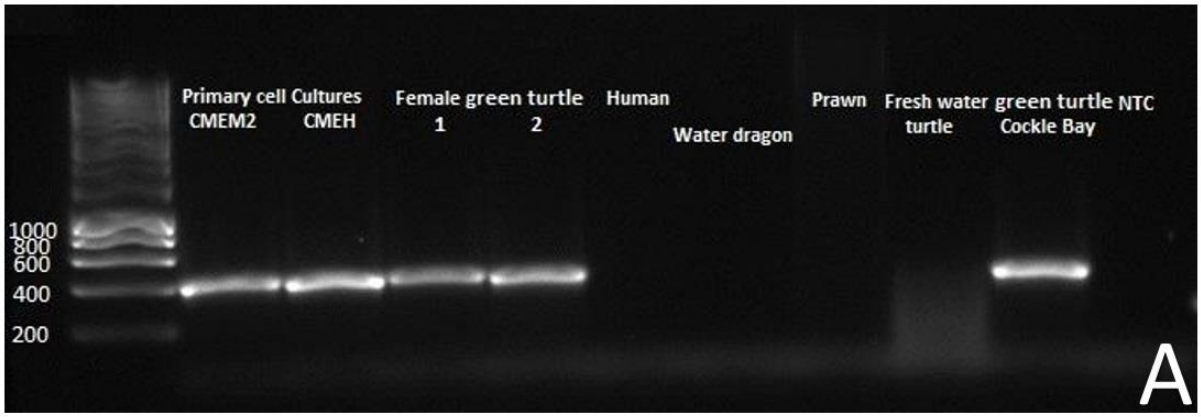


Figure 4-5 A) CMEM1 metaphase chromosome spread under light microscope. B) Karyogram of CMEM1. (Chelonia mydas 2N=56 in 54 chromosome spread)

Molecular analysis

The PCR products of mtDNA D-loop from CMEM2, CMEH, female green turtles from Heron Island, human, eastern water dragon, giant tiger prawn, Krefft's river turtle and a green turtle from Cockle Bay are shown in figure 4-6A. The amplified D-loop sequences of our primary cell lines were 100% identical to that of green turtles which confirms that these cells originated from green turtle culture explants.



5'TATTTACCACTAGCATATGACCAGTAATGTTAACAGTTGATTTGGCCCTAAACATGAAAATTATTGAATTTGCATAAACATTTTAAT
AACATGAATATTAAGCAGAGAATTAAAAGTGAAATGATATAGGACATAAAATTAAACCATTATACTCAACCATGAATATTGTCACA
GTAATTGGTTATTTCTTAAATAGCTATTCACGAGAAATAAGCAACCCTTGTTAGTAAGATACAACATTACCAGTTTCAAGCCCATTCA
ATTTGTGGCGTACATAATCTGATCTATTCTGGCCTCTGGTTGTTCCTTCAGGCACATATAAATAACGACGTTCATTCGTTCCTCTTTAA
AAGGCCTTTGGTTGAATGAGTTCTATACATTAGATTTATAACCTGGCATACG3'

B

Figure 4-6 A) The PCR products of mtDNA D-loop from CMEM2, CMEH, female green turtle 1 (Heron Island), female green turtle 2 (Heron Island), human, eastern water dragon, giant tiger prawn, fresh water turtle (Krefft's river turtle), green turtle from Cockle Bay, no temple control. B) 402bp of the amplified mtDNA D-loop. The sequences from female green turtles that laid the eggs, primary cell lines and a green turtle from Cockle Bay were 100% identical.

4.3.1.4. Mycoplasma test

Non-infected primary cell lines did not produce any PCR products using the primers to detect adenovirus, herpesvirus, ranavirus or papillomavirus, and were also mycoplasma free in passages 15 and 20.

4.3.1.5. Primary cell viral susceptibility test – case of Bohle iridovirus

Two of our primary cell lines, CMEH (passage 19) and CMEM2 (passage 21) and two fish cell lines, FHM (passage 90) and BF-2 (passage unknown) (Granzow *et al.,* 2001) were plated and inoculated with BIV. Four days after infection with BIV the cells in all four cultures started to shrink, aggregate and then separated from the plate surface (figure 4-7) showing that our primary cell lines were susceptible to this pathogen of lower vertebrates ($TCID_{50}=10^{5.5}$/ml). The $TCID_{50}$s were in similar ranges for all four cell lines: $10^{5.5}$ in CMEM2, CMEH and FHM and 10^{5} in BF-2.

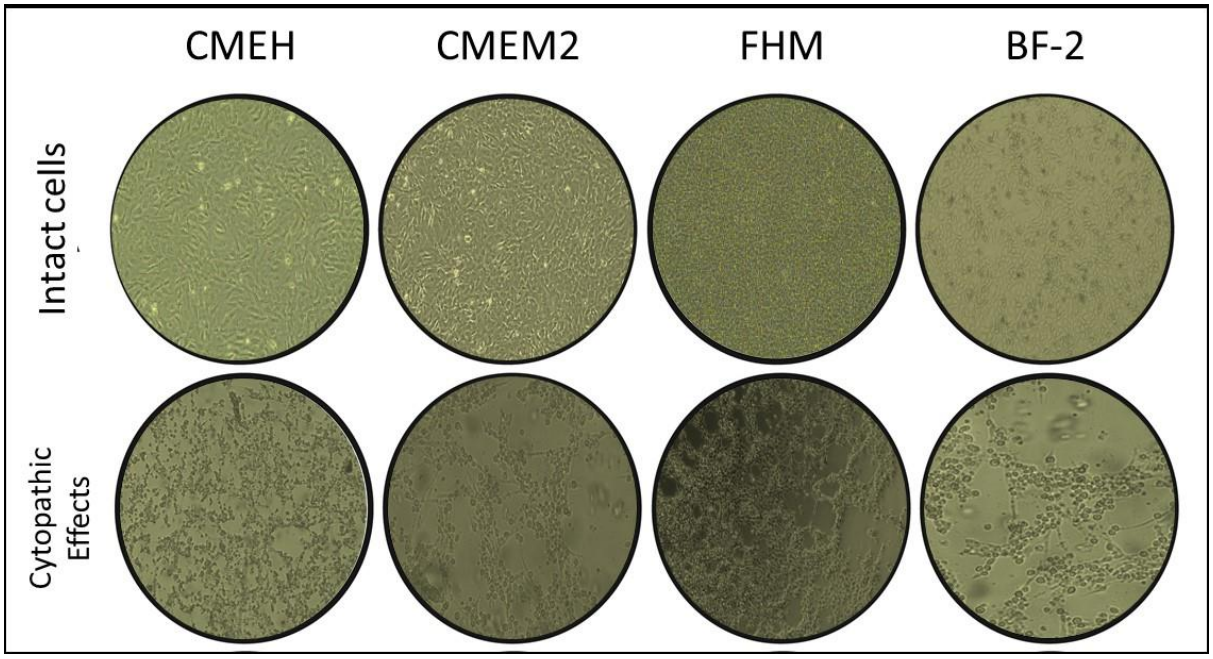


Figure 4-7 Morphology of CMEH, CMEM2, FHM and BF-2 cells before inoculation with BIV and after development of CPE

4.3.2. Screening green turtle samples for presence of viruses using cell culture

CMEM1 and CMEH were used to screen samples from green turtles for presence of viruses. The majority of samples tested did not induce morphological changes. However, eight skin tumour samples induced patches of cell aggregation after 4-5 days of first inoculation on CMEM1. The cells became round and dense in these areas (figure 4-8B, C). After 6 days the monolayers detached from the plate surfaces. No morphological changes were seen in CMEH during the experiment.

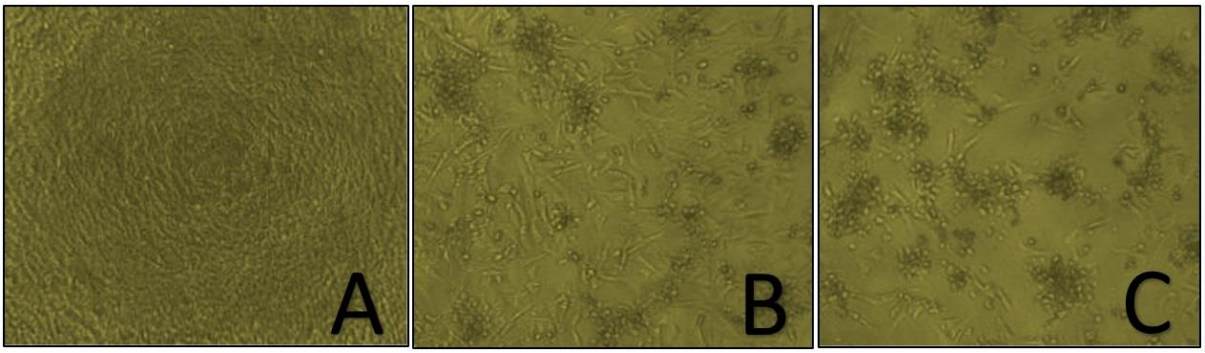


Figure 4-8 A) The morphology of the uninfected CMEM1 (passage 20), day 1. B) Infected CMEM1 (passage 20) four days post inoculation with tumour skin samples from green turtles. Several cell aggregations can be seen in the photo. C) Cells started to detach from the culture surface five days post infection.

The corresponding tumour tissue samples tested positive for herpesvirus (ChHV5) and papillomavirus but the extracted DNA from the primary cell pellets 6 days post-inoculation were only positive for papillomavirus.

4.3.3. Molecular analysis establishment and viral detection

4.3.3.1. Viral detection using designed qPCR primers, probes and positive control

The samples that tested positive are shown in figure 4-9. AR-E1-F2 and AR-E1-R9 primers produced a smear of products (figure 4-9 A) that needed excision and purification as described by Manire *et al.,* (2008), but Chm-Pap-109 primers specifically amplified 109bp of E1 (figure 4-9 B, C, and D).

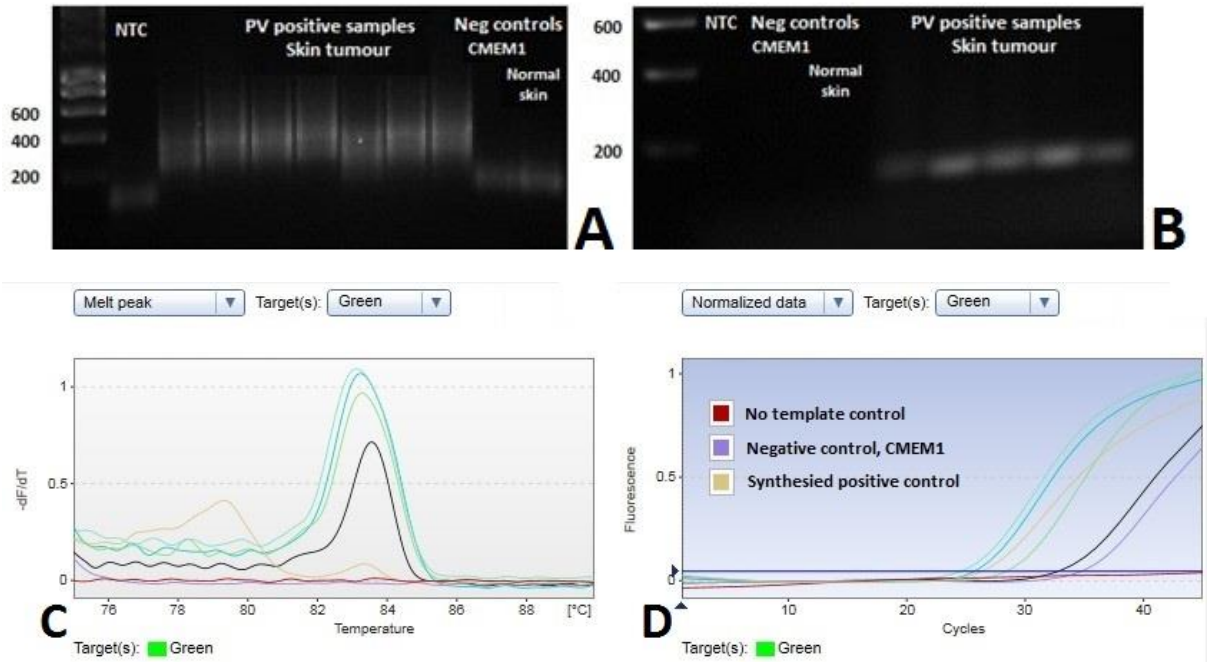


Figure 4-9 Skin tumour samples (homogenised tumour tissues) were positive for papillomavirus. 1.2% gel electrophoresis of PCR products using A) AR-E1-F2 and AR-E1-R9 primers and B) Chm-Pap-109 primers from table 4-2. C) The melt peak of Dye basedqPCR using Chm-Pap-109 primers. D) The amplification curves of Dye basedqPCR using Chm-Pap-109 primers. The colours of synthesised positive control, negative control (CMEM1) and no template control (NTC) are shown in the figure.

Quantitative PCR, using GoTaq® Probe assays showed that the Cq value decreased 6 days post inoculation but the ΔCq of day 6 (extracted DNA from cell culture pellets) and day 1 (extracted DNA from homogenised tumour tissues) are not considerable (The data from Chm-Pap-109 primers and probe (table 4-2) are shown in figure 4-10).

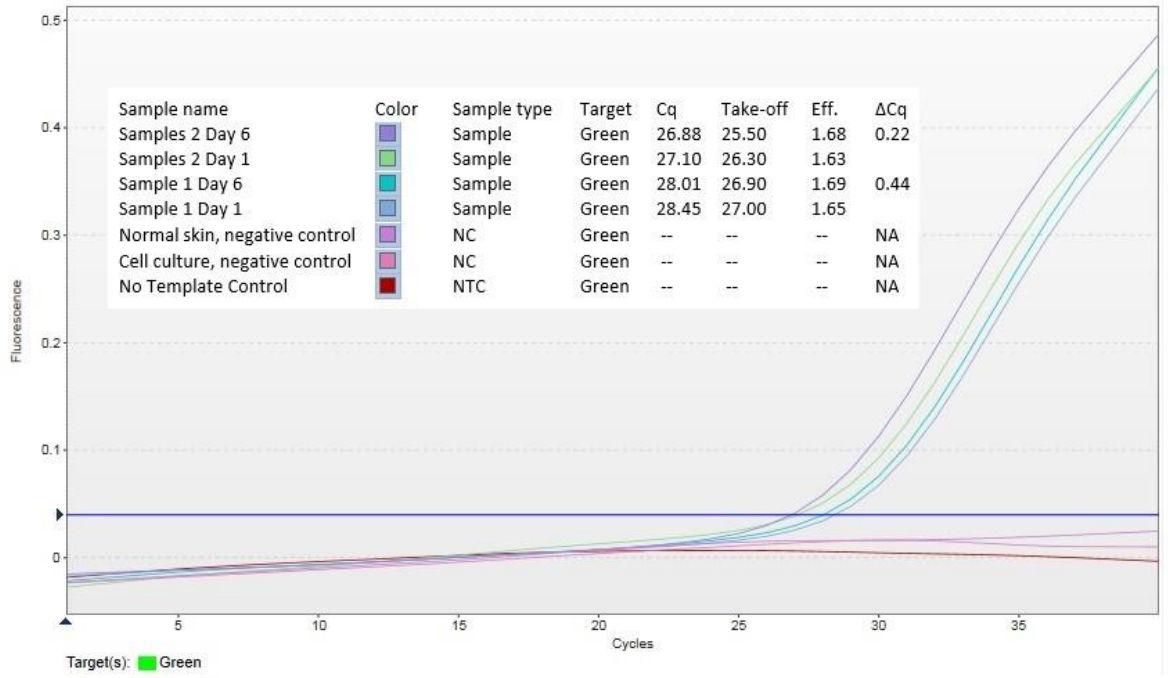


Figure 4-10 The results of the quantitative PCR assays on two PV positive samples 6 days post cell culture inoculation (extracted DNA from cell culture pellets) and on day 1 (extracted DNA from homogenised tumour tissues). The results from Chm-Pap-109 primers and probe from (table 4- 2) on two samples.

No PCR product was amplified using the primers described in the literature to detect adenovirus and ranavirus in either cell culture or tissue samples. Samples were also negative using the primers that were designed in this study to detect iridoviruses, adenoviruses and herpesviruses (table 4-2).

PV was detected in samples from skin tumours of turtles foraging in Cockle Bay, Townsville and Edgecumbe Bay, Bowen, Australia. Blood, cloacal swab and normal skin samples from these turtles tested negative for papillomaviruses.

4.3.4. Histopathological analysis

The papillomavirus positive tissue samples were investigated by histopathology and were typified as different stages of fibropapillomatosis ranging from papillomas to fibropapillomas. The tumours represented dermal and epidermal growth to neoplastic proliferation and basal cell degradation in line with papilloma-viral infections.

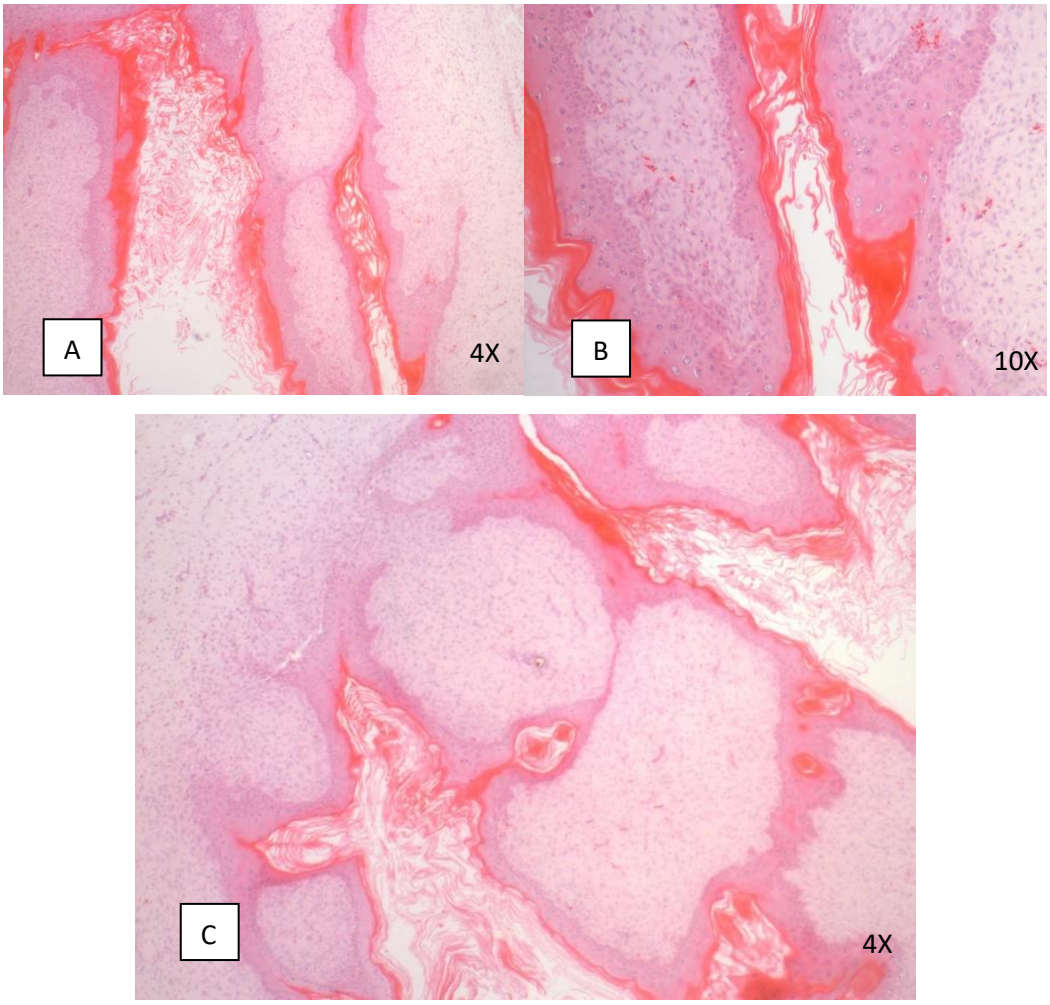


Figure 4-11 A, B and C) The histopathological analysis of FP tumour tissues of a green turtle that tested positive for ChHV5 and CmPV. The figures represent the typical fibropapillomatosis as seen in mammalian species which are caused by papillomaviruses. B is a magnified section of A.

4.4. Discussion

Conventional methods and modern biological techniques play complementary roles in virology (Mokili *et al.,* 2012), no single method can be sufficient for detection, identification and isolation of viruses. In this study histopathology, cell culture and molecular analysis were used to investigate the presence of viruses in green turtles from the northern Great Barrier Reef and successfully found PV.

Several primary cell lines were established and characterised from green turtle embryos. The cells were incubated at 25°C and the optimal growth rates were within the range reported for primary cell lines originating from other ectothermic animals (Lu *et al.,* 1999; Fukuda *et al.,* 2012; Li *et al.,* 2016). The cells were maintained at 25°C to facilitate a comparison with the established fish cell lines FHM and BF-2 in terms of susceptibility to BIV. The results from temperature and serum optimization were

consistent with previous cultures of green turtle primary cultures at different temperature and serum concentrations (Fukuda *et al.,* 2012; Koment *et al.,* 1982; Lu *et al.,* 1999).

The diploid chromosome number of male green turtles is 56 and 55 for females (male XX 2N = 56, female XO 2N = 55) (Koment *et al.,* 1982; Moore *et al.,* 1997). The modal chromosome number of CMEM1, CMEM2 and CMEH was 56, so the cell lines are assumed to originate from male green turtle embryos. Reptilian and avian chromosome complements are similar and consist of macro and micro-chromosomes (Lu *et al.,* 1999). These morphological components were clear in the cytogenetic analysis of our established primaries. The diploid chromosome numbers and morphologies were similar to the previous reports of green turtle chromosomes (Koment *et al.,* 1982; Moore *et al.,* 1997).

The American Type Culture Collection (ATCC) recommends providing additional identity verification along with chromosomal counts (ATCC 2010). Molecular analysis on D-loop region of mitochondrial DNA was done to further authenticate our primary cell lines. In addition to genetic diversity and origin of a species, D-loop region sequences have been successfully used to differentiate between closely related species such as domestic and wild ducks (Hao *et al.,* 2017; Jin *et al.,* 2014). Mitochondrial genes have also been used to verify cell line origins and cross-contaminations (Swaminathan *et al.,* 2015). The phylogenetic analysis of mtDNA D-loop partial amplification indicated that our primary cell lines have the highest identity with green turtles. It should be noted that these PCR primers did not differentiate the green turtles from Heron Island and Cockle Bay.

The newly established primary cell lines were successfully used to titrate BIV from infected organs and the viral titres were comparable to commercially available fish cell lines. BIV is a pathogen of poikilothermic animals with a broad host range from fresh water fish, amphibians, turtles and crocodiles to eastern water dragons (Whittington *et al.,* 2010; Cullen *et al.,* 2002; Ariel *et al.,* 2015; Maclaine *et al.,* 2018). BIV is in the genus *Ranavirus*, family *Iridoviridae* and was first isolated from an ornate burrowing frog (*Limnodynastes ornatus*) in North Queensland (Speare and Smith, 1992). Establishment of cell lines from various cold-blooded animals may improve the ability to study infections with ranaviruses at a cellular level.

Four suites of PCR primers were designed to detect herpesviruses, papillomaviruses, adenoviruses and iridoviruses in green turtles. The three PCR primers that were designed to detect iridoviruses, herpesviruses and adenoviruses did not react in any of the screened samples (data not shown here). These primers were validated and will be used in future projects and in searches for these three viruses in green turtles. The specific PCR primers successfully detected CmPV in tumour tissue samples.

Two-dimensional cultures normally do not support growth of PVs due to the strict link of PV replication with epithelial cell differentiation. *In vitro* studies of PVs are carried out in three-dimensional raft cell cultures to investigate propagation, infection and neutralization of PVs (He *et al.,* 2016) therefore it is unlikely that the complete virion is formed in our primary cell lines. In addition, integration is a key element in PV oncogenic progressions (McBride 2017) which establishes a baseline expression level for the viral oncogenic proteins of PV (E6/E7) that maintain the transformed state of the cell. The PVs were found in tumour tissues using PCR and detected CPE in cell culture. The aggregation of tumour-like changes in the morphology of the cells may represent the presence of E6 and E7. Such vigorous growths and compact colonies of small proliferating cells were previously described in cell cultures affected by E6 and E7 proteins (Halbert *et al.,* 1991; DeFilippis *et al.,* 2003). The quantitative PCR shows that 6 days post inoculation PV exists in cell culture and the Δcq has slightly decreased. Although, the doubling time of CMEM1 is 5 days, 6 days of cell growth is probably not providing enough host cells to support abundant viral proliferation. It is worthwhile mentioning that CPE only developed in CMEM1 indicating that these cells were more compatible target cells than CMEH which emphasize the very selective nature of PV.

Similar cell aggregations have previously been observed in green turtle primary cell line inoculated with cell-free medium from FP derived samples (Lu *et al.,* 2000). In that study Lu *et al.* noticed tumour-like growth in primary cultures from healthy hatchlings inoculated with supernatant of tumour-derived cell lines. Transmission electron microscopy of cell aggregations showed small, naked viral particles measuring 50±5 nm in diameter assumed to be "*papovavirus*"-this term is not used in taxonomy anymore, the family is divided into *papillomaviridae* and *polyomaviridae* (Van Regenmortel *et al.,* 2000). In that study, several PCR primers to detect human PV (HPV) were used to amplify partial sequences of the L1 gene. One of the primers designed to detect the L1 gene of HPV type 6 amplified 235bp sequences from eight out of ten different supernatants from cell lines with CPE and showed 36-44% similarity with known animal papillomaviruses (Lu *et al.,* 2000). Aligning the sequence they provided, with HPV-6 (KX514429.1), CmPV-1 (EU493091) and CcPV-1 (EU493092) showed that the partial sequence from their study[7] has 33.33, 53.33 and 53.33% similarity to HPV-6, CmPV-1 and CcPV-1, respectively. All of their samples tested negative for herpesvirus using generic PCR primers (Lu *et al.,* 2000). Some of these PCR primer combinations were also used by Brown *et al.,* (1999). They successfully detected human, bovine and equine PVs but failed to amplify a putative avian PV and to find PV in FP samples. Their test seems to be specific for mammalian PV and unable to detect non-

[7] The partial sequence of L1 gene published by Lu *et al.,* 2000: 5'GGGGTACGCC CAGGGACATAACAATGGTGG AGTGTTCCAC ACTATGTCAT CGGGGATCCG ATGTTCACGC AGAAACACAA CCTGACTATA TTTGGCTGTC CCTGGGCTAC TTCCTAGTCT CCCACTGTAA TGTACGTGTG GTGTGGGGTC ATCCCCTGTC TCTTCAGGGA ACAGGTTGAA TGGCAGTCCT GGTAGCTCTTCTCCCTGGAT CAGTTTCCCC TGGGACGTCT AGACC3'.

mammalian PVs as they argued in their article too (Brown *et al.,* 1999). There were no reports of subsequent investigations of a possible association between papillomavirus and FP, nor molecular analysis to identify a different viral agent in the samples, although papillomatous tumours and fibroblastic proliferations have been abundantly explained in the histology of the disease (Vogelnest, L. 2018).

In the current study too, corresponding samples were confirmed to be characteristic of fibropapillomas when histologically examined. The epidermes exhibited mild to severe papillomatous hyperplasia and hyperkeratosis typical of papilloma-viral infections. Also a similar type of CPE was observed in green turtle primary cell cultures that were inoculated with skin tumour homogenates. PV was detected in all cultures using qPCR primers specific for green turtle PV based on the full genome as published by Herbst *et al.,* (2009) and also using PCR primers that have been frequently used to detect PV in animals (Rector *et al.,* 2005). The latter primers reacted non-specifically as expected from degenerate PCR primers, therefore, in this study mostly green turtle specific primers that target E1 and L1 were used. E1 is the most conserved open reading frame among papillomaviruses reflecting the critical role of E1 protein in viral genomic replication (Manire *et al.,* 2008). L1 is also conserved but suitable to be used as a family taxonomic criterion (Chiesa *et al.,* 2016). In addition, these two genes are present in the episomes and also in integrated viral particles. In some studies the E1 gene is shown to be partially disrupted in the process of integration (McBride 2017). Hence, there was a possibility for the primers not to be able to amplify the target sequences or to find a part of host gene along with a part of the virus. But the diagnostic PCR primers successfully amplified 109 and 183 bp of the E1 gene.

The virological methods established in this chapter were successfully used to detect a virus in green turtles for the first time in Australia. This emphasises the idea that the quest for viral discovery is only in its infancy and more viruses in species with low commercial values such as sea turtles are yet to be revealed. The newly established methods will be used in future projects and they form the basis for the characterisation of the papillomavirus found in Australian green turtles.

How I achieved the aim of the chapter:

1. I collected samples from live and dead green turtles and obtained permissions to use archived samples
2. I established, characterised and authenticated primary cell cultures from green turtle embryos
3. I used the primary cultures for viral isolation
4. I developing PCR assays to detect viruses, the processes contained
    - Primer design and evaluation
    - PCR assay optimisation
5. I used the PCR assays to screen the samples for presence of viruses
6. I prepared samples for histopathological analysis

5. PARTIAL MOLECULAR CHARACTERISATION OF AUSTRALIAN *CHELONIA MYDAS* PAPILLOMAVIRUS

The aim of the chapter:

To characterise the Australian *Chelonia mydas* papillomavirus isolates

5.1. Introduction

Papillomaviruses are small, non-enveloped, double-stranded DNA viruses containing a 7-8 kb circular DNA molecule. The DNA genome usually encodes eight open-reading frames (ORFs) (Doorbar *et al.,* 2015). Six of these ORFs are the early genes (E1, E2, E4, E5, E6, E7), expressing functional proteins early in the infection. The other two are late genes (L1 and L2) encoding the structural proteins (Rector and Ranst, 2013; Doorbar *et al.,* 2015). E1 and E2 are well-conserved and are involved in replication. E4, E5, E6 and E7 have greater diversity; E4 and E5 are involved in different stages of the cell cycle while E6 and E7 target several regulators of the cell cycles and mediate immune evasion and cancer. The late genes L1 and L2 code for the icosahedral capsid protein and this constructs a small 50-55 nanometer viral particle (Doorbar *et al.,* 2015).

For classification of the virus, two conserved papilloma-viral proteins, E1 and L1 (Doorbar *et al.,* 2015) are used (Sverdrupa and Myers, 1997). The first 200 amino acids of E1 N-terminal are the least conserved parts of the E1 protein. However, short motifs such as the nuclear localization signal (NLS)

are variably conserved amongst different papillomaviruses (Bergvall *et al.,* 2013). Specific regions in the DNA binding domains (DBD) that support replication are also well conserved (Bergvall *et al.,* 2013). L1 is used routinely for papillomavirus classification and typing due to the hypervariable loops in the structure of this protein exposed on the virion surface (Egawa *et al.,* 2015). To use L1 for distinguishing between strains of viruses, the nucleotide sequences of two papillomaviral L1 genes must be at least 10% divergent from each other (Lange *et al.,* 2011; Egawa *et al.,* 2015; Doorbar *et al.,* 2015). In 2013, only 37 genera had been reported in the *Papillomaviridae* family (Van Doorslaer, 2013), but due to advances in sequencing methods, the complete genomic sequences of many new papillomaviruses have since been published (Van Mechelen *et al.,* 2017). Two subfamilies and more than 53 genera are now listed by the International Committee on Taxonomy of Viruses (ICTV) (Burk and Chen, 2017).

Papillomaviruses are host specific and it is thought that they have evolved or co-speciated with their hosts for the last 100 million years with little cross-over between the host and the virus (Herbst *et al.,* 2009; Van Doorslaer, 2013; Egawa *et al.,* 2015). The only exception reported was in Iberian bats with five distantly related lineages of papillomaviruses that lack congruence with bat hosts phylogenies. *Eptesicus serotinus* papillomavirus 2 and 3 can infect two different bat species (*Eptesicus serotinus and Eptesicus isabellinus*) which goes against the strict host specificity of papillomaviruses (García-Pérez *et al.,* 2014). Tissue specificity (tropism) is another characteristic of particular papillomaviruses. The epithelial niches are the preferred propagation sites for papillomaviruses. Specific anatomical sites are targeted by these papillomavirus types to cause lesions with distinctive pathologies, from benign proliferation such as warts to high-grade neoplasia and in some cases invasive malignant tumours (Egawa *et al.,* 2015).

Papillomavirus research has traditionally focused on mammalian hosts and especially humans, but characterisation of reptilian papillomaviruses is expanding the knowledge of papilloma-viral infections to all amniotes (mammals, birds and reptiles) (Manire *et al.,* 2008; Lange *et al.,* 2011; Rector and Ranst, 2013). In the phylogenetic tree, the reptilian papillomaviruses form a separate clade closer to avian papillomaviruses with one exception; the Australian diamond python papillomavirus is closer to mammalian papillomaviruses (Gull *et al.,* 2012). Possible reasons for snake papillomavirus not clustering with the five other sauropsid papillomaviruses may be that one ancestral papillomavirus crossed the species barriers, or this virus existed in early amniotes before the Sauropsida (birds and reptiles) and the Synapsida (mammals) diverged (Lange *et al.,* 2011).

The first incidence of papilloma-viral infection in sea turtles was described in 2008 (Manire *et al.,* 2008). In 2009, Herbs *et al.,* published the whole genomes of *C. mydas* papillomavirus 1 (CmPV1) and *C. caretta* papillomavirus 1 (CcPV1) and proposed a new genus of papillomaviruses:

Dyozetapapillomavirus (Herbst *et al.,* 2009). CcPV has been given the species name *Dyozetapapillomavirus 1* in the genus *Dyozetapapillomavirus* (Burk, 2017). To date, CmPV1 and CcPV1 are the smallest papillomaviruses described, 6953 and 7020bp respectively, compared with the largest papillomavirus, 8607bp infecting domestic dogs (Rector and Ranst, 2013).

Sea turtle papillomaviruses were only identified in two individual cases in captivity and there are no reports of sea turtle papilloma-viral infection in any regions other than Florida, USA. In this chapter the first Australian *C. mydas* papillomaviruses found in skin tumours of eight green turtles from two regions of Queensland are characterised.

5.2. Materials and Methods

5.2.1. Samples

Extracted DNA from eight skin tumour samples previously reacted in PCR assays for *C. mydas* papillomavirus (explained in section 4.3.2. and 4.3.3.) were used for viral characterisation. Extracted DNA from non-infected green turtle primary cell cultures were used as negative controls.

5.2.2. Molecular characterisation of the partial E1 gene and the full L1 gene

5.2.2.1. Primer design, qPCR and sequencing E1-NLS and DBD

A set of sequencing PCR primers were designed to cover the NLS, the phosphorylation unit and the DBD of the E1 gene as explained in "section 4.2.3.2. PCR primer, probes and positive control design". These three regions cover amino acids 25 to 300 of the E1 protein (Sverdrupa and Myers, 1997; Bergvall *et al.,* 2013) which is shown by a green arrow in figure 5-1 and was amplified by using the following primer set: Chm-Pap-798-F 5'AAG GAC CCT CAC ATA GCA3' and Chm-Pap-798-R: 5'CAT TAT TGT CTT CAG TTG CCA TT3'.

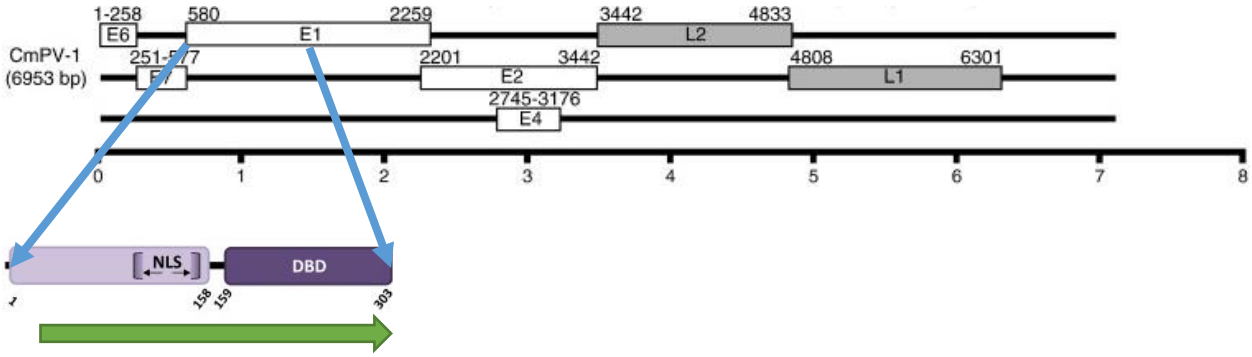


Figure 5-1 A schematic map of CmPV-1 adapted from Herbst et al., 2009 showing the eight early and late ORFs of this papillomavirus. The cutout region (dark and light purple) is adapted from Bergvall et al., 2013 showing the nuclear localization signal (NLS) and the DNA binding domains (DBD) of the E1 protein from bovine papillomavirus. The green arrow indicates the 798bp of the E1 gene amplified by our sequencing primers corresponding approximately to amino acids 25 to 300.

The Dye based qPCR reactions (20µl) were made with 10µl of 2X GoTaq® qPCR Hot Start Master Mix (Promega), 1.6µl from each primer (0.8µM), ~2µl of template DNA (~80 ng) and 4.8µl nuclease free water. Thermocycling was performed on a Rotor-Gene 6000 Real-Time PCR Machine. The PCR protocol initiated with a hot start at 95 °C for 2 min and was followed by 40 cycles of denaturation at 96 °C for 10s, annealing at 60 °C for 15s, extension at 72°C for 40s and a final extension at 72°C for 5 min. The PCR products were visualised on 1.2% agarose gel to confirm the product size, products were excised, gel purified with Wizard® SV Gel and PCR Clean-Up System (Promega) and sent to Macrogen, Korea for sequencing. The results were analysed using Geneious 10.2.3.

5.2.2.2. Overlapping primer design, qPCR and sequencing the L1 gene

Three pairs of PCR primers were designed based on the full genome of CmPV-1 (Herbst *et al.,* 2009) to cover three overlapping regions of the L1 genes (blue, red and green arrows in figure 5-2). The dye based qPCR reaction described in "section 5.2.2.1" was also used here. The PCR primers and protocols for each region are listed in table 5-1.

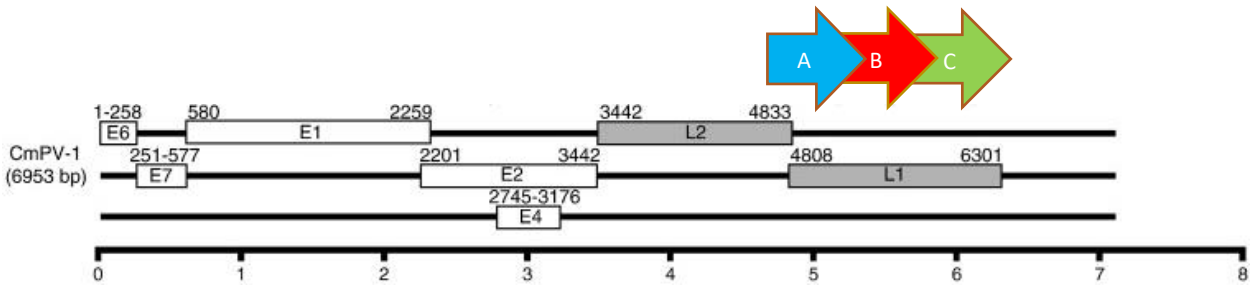


Figure 5-2 A schematic map of CmPV-1 adapted from Herbst et al., 2009 showing the eight early and late ORFs of this papillomavirus. The arrows indicate the approximate overlapping regions of the L1 gene amplified by three sets of PCR primers. The colours of the arrows correspond to the regions listed below in Table 5-1.

Table 5-1 Three different regions of the L1 gene amplified via three qPCR assays. The sequences of the PCR primers and the protocols for each region are listed.

Target genes	Regions	Primers and probes names	Primer sequences	PCR protocols
L1	A: 751bp of 5' end of L1 gene	Chm-pap-L1-751-F	ATGGAAGTCCGCTCATTC	Dye based PCR: -2 min at 95 °C -40 cycles (96 °C for 10s, 60 °C for 15s, 72°C for 40s) -72°C for 5 min
		Chm-pap-L1-751-R	GAGTGCCTAATCCCATATCA	
	B: 793bp in the middle of L1 gene	Chm-pap-L1-793-F	AAC AAG TTT CCA ATT CCT GAG T	
		Chm-pap-L1-793-R	TTG TAT TGC GTG TTG TGT CT	
	C: 772bp of 3' end of L1 gene	Chm-pap-L1-772-F	AACCCACTGATTATCACATTAAG	
		Chm-pap-L1-772-R	TAGACAAACACGGCACAA	

5.2.3. Inverted Primers and Sanger Sequencing

Knowing the partial sequences of E1 (section 4.3.3. and Herbst *et al.,* 2009), a pair of inverted primers were designed (figure 5-3). GoTaq® Long PCR Master Mix (Promega) was used to amplify the remaining circular genome. The primer sequences were as follows: forward primer (yellow triangle in figure 5-3): 5'AGA TTC ATT GCT AGG CTT GC3'; reverse primer (blue triangle in figure 5-3): 5'GAT TGT GGT CTG TAA GAA TTG ATG3'. Product length was anticipated to be 6903bp.

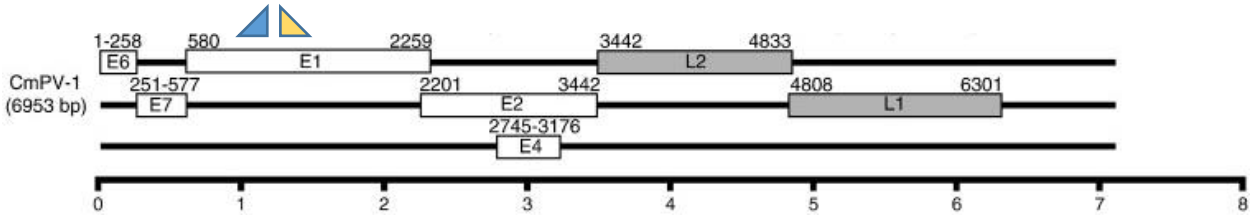


Figure 5-3 A schematic map of CmPV-1 adapted from Herbst et al., 2009 showing the eight early and late ORFs of this papillomavirus. The direction of inverted primers are shown in yellow and blue for forward and reverse primers, respectively.

The long PCR protocol consisted of a denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30s, 55 °C for 35s, and 72°C for 7min (1kb/min according to the GoTaq® Long PCR Master Mix (Promega) technical manual) with a final extension of 72°C for 2 min. The products were extracted from 1.2% agarose gel and sent to Macrogen, Korea for Sanger sequencing after gel purification with Wizard® SV Gel and PCR Clean-Up System (Promega).

5.2.4. Rolling Circle Amplification and Next Generation Sequencing

To sequence the whole genome, DNA was extracted from skin tumour samples and infected cell cultures (section 4.2.3.) and sent to the University College London, Department of Genetics, Environment and Evolution for Next Generation Sequencing (NGS) with the Illumina Miseq Genome Analyser System.

To enrich the viral DNA where viral titres were too low for NGS and to prepare the templates for DNA sequencing, rolling circle amplification (RCA) was done using Illustra TempliPhi™ 100 DNA Amplification kit (GE Healthcare Life Sciences, New Jersey, USA). The RCA technique favours propagation of the circular DNAs in samples. The kit uses the ϕ29 DNA polymerase and random primers (Van Mechelen *et al.,* 2017).

Total DNA extracted from eight tumour tissue samples were amplified according to the instructions in the kit. Briefly, the TempliPhi premix and denature buffer was thawed on ice, 10 µl aliquots of denature buffer was dispensed into 8 microfuge tubes and samples were added. Samples were denatured at 95˚C for 3 min and first cooled to room temperature and then to 4˚C, 10 µl of TempliPhi premix was added to the cooled samples and incubated at 30˚C for 16–18 h. After the incubation, the enzyme was heat-inactivated by incubating at 65˚C for 10 min, and subsequently cooled down to 4˚C. Amplified samples were visualised on a 0.9% agarose gel.

The kit provided the positive control, 50 µl of pUC19 DNA (2 ng/µl), the negative control was total DNA extracted from green turtle primary cell line. A second positive control which was a custom made circular plasmid (109bp of CmPVE1 gene was cloned in pGEM®-T vector described in section 6.3.1.) was also used. The same protocol was used for the control tubes with a reduced volume of control DNA (0.5 µl) added to a reaction tube containing 10 µl of denature buffer.

5.3. Results

5.3.1. Molecular characteristics of the partial E1 gene and the full L1 gene

5.3.1.1. Sequencing the E1-NLS and DBD

Chm-Pap-798 primers successfully amplified 798bp of E1 (figure 5-4) covering the NLS and DBD of the E1 protein.

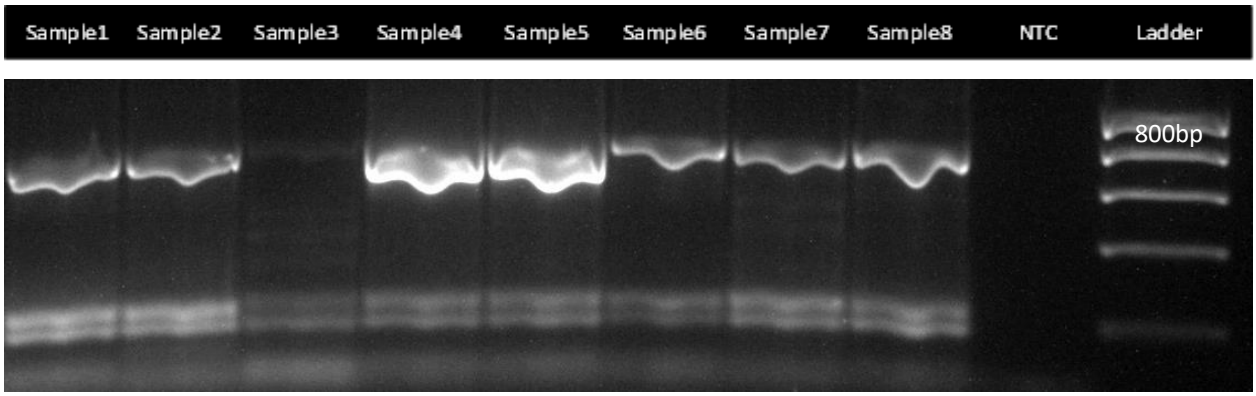


Figure 5-4 The gel electrophoresis results of 798bp of the E1 gene amplified via qPCR. Lane 1 to 8 contains the eight green turtle tumour samples reacted in qPCR assay. NTC=no template control.

Two single nucleotide polymorphisms (SNPs) were seen in sequences of the eight Australian CmPVE1-DBD when mapped to reference (CmPV-1, GenBank: EU493091.1). The change occurred in position 559 and 561 of the consensus sequence corresponding to position 1138 and 1140 of the CmPV-1-E1 gene (figure 5-5). These SNPs have altered a codon from CTC to TTG. These codons are degenerate and translate for leucine and therefore the substitution is a synonymous nucleotide substitution. The rest of the sequences and the consensus are 100% similar to CmPV-1.

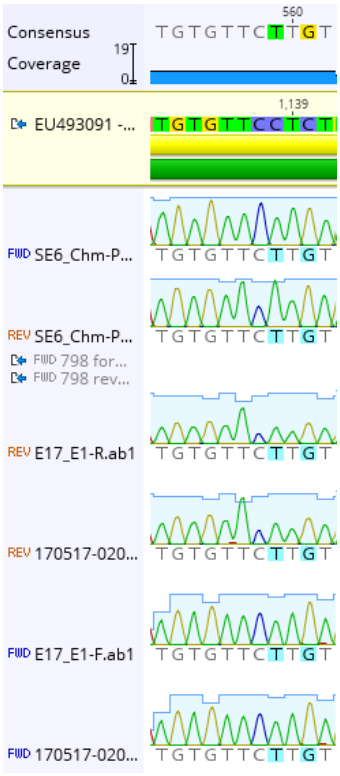


Figure 5-5 The alignment of DBD of E1 gene from Australian CmPV isolates and CmPV-1 (Genbank accession number: EU493091) in Geneious version 10.1. Two single nucleotide polymorphisms were observed in the consensus sequences of Australian CmPV which exist in all sequenced samples. The change from C to T in position 559 of consensus (1138 of CmPV-1 E1 gene) and C to G in position 561 of consensus (1140 of CmPV-1 E1 gene). CTC and TTG both translate for Leucine.

5.3.1.2. Sequencing L1 gene

The L1 gene was completely sequenced using the overlapping PCR primers discussed in section 5.2.2. The consensus sequences of the partial E2, complete L1 and the partial long control region (LCR) of Australian CmPV isolates are shown in figure 5-6. The sequences were compared to reference (CmPV-1, GenBank: EU493091.1) and indicated that the L1 genes of Australian CmPV isolates and CmPV-1 are congruent (100% similarity) both the Australian CmPV isolates and CmPV-1 have 73% similarity to CcPV-1 (E-value: 6e-130; GenBank: EU493092.1).

5’GATGGAAGTCCGCTCATTCCCCTTTTTCCTTCTTTTGATAAAGTGCATCCTGCATATTCTGTAACGTTTTCTATGTTATCTGAGTTAGATGACCCTTTTCTAA
CCAAAAAGCGTAAAAAATGTTTTGCAGATGGCTGTTTGGACACCTTCTACTAGTGCATTGTTTGTACCCCCTGTTAATGTACCTGCTTTGTATTCTACTAAAG
AATTTGTACAACGTACATCTTATGTTTTTCATGGAACAACTGAACGTTTGTTAACAATTGGCAACCCCTATTTTCCACTCATTGACCGTGATGTAGTTATAGT
CCCCAAAGTGTCTGCTTATCAATATCGCGTTTTTAGAATAAAGCTTCCAGATCCAAACAAGTTTCCAATTCCTGAGTCTGCACGGGGGGACAAAGATAGTA
CCCGGTTGGTGTGGGCTGTGCAGGGCATTCAAGTAAATAAAAGTCAACCACTGGGGGTGGGGCCCTCTGGAAACACAATGTCAAGCGGGACGCTTGATT
ATGCTGACTCTCACCATCCAGGCAATGAAAAACCACCGCCTCCGGATGATAGGCGCATAAACAGCGCTTCAGATTCAAAACAAAGCCAGGTGCTAATTGT
GGGGTGTATTCCCCCAATGGGCCAACATTGGGACGCGAGCATAAGGTGCACTGAGGATAATGACAAAGAGATGTGTCCTCCCTTAGAATTAAAACATACA
GTCATAGAAGATGGCGACATGGTTGATATGGGATTAGGCACTCTCAATTTTTCAACACTTTGCTCAAACAAATCCACCCTGCCATTAGAATTAATTAACTCA
ATTTCTAAATACCCCGATTGGTTGACAATGAACGCGGACCCGTTTGGAAACCACTGCTTCTTTATGCTGCGCAGAGAACAGGTGTACCTGAAATCTGTTGG
CATGCAATATGGCAACGTGGGTGAGGATGAACCCACTGATTATCACATTAAGGGTGGCACAGGCACGCTGTGGCAAACGCCAGGCCGGCACAGCTGGTT
TCCATTAGTATCTGGGTCCCTTGCGACTTCAGACAATCAGCTGTATAATCGTCCATATTGGATAGAAAACAGCACTGCCCCAAATGATGGCATTTGCTGGC
ATAATCAGCTGTTTGTCACTTGTGCAGACACAACACGCAATACAATATTTAATATATCCACGCTAAAAAAGAACGTACCAGCTACCTCAGACTATAAAGAG
AGTAATTTCACAAATTATTCCCGCCATGTTGAGGAGTATGAAATTTCGTTTATACTTAGGCTATGCATGGTAAACATGGATTTGCCTGTGCTCAATCATCTCC
ACAACATGGATCCCATGCTGCTAGAGGATTGGGGGTTTGGTACCACGCCCCCACCTAATTTAACGGTAGAAGACCAATATAGGTTTCTCCAGTCAAAAGCA
ACCAAGTGCCCACCCACACCGGCTACCCCCCCCGATGCAGACAAATGGGCAAAATACAAGTTCTGGGACGTAGACTGCACCGAGCAAATTTCTTCGGATC
TCAGCCCTTTTCCACTCGGTCGCCGGTTTTTGCAACTGTACCCGAAAGCTGCAACTGCTACACGCACTCTGAGCTCCAGTTCCAGAAAACGCCGTCGTGGCC
GATAATGTTATTATTTGAAGTGCTTTTAAATGTTCATCAACATGCTGTGTTTCTTTTTTTGGTTCTTTTTATTTACTTGTGC3’

Figure 5-6 The consensus sequences of the partial E2 (highlighted in green), complete L1 (brown text) and the partial LCR (highlighted in grey) of Australian CmPV isolates.

5.3.2. Inverted Primers and Sanger Sequencing

The inverted primers (section 5.2.3.) did not produce the anticipated results. Only one sample reacted and a band of approximately 7000bp size was observed (figure 5-7) (6903 anticipated). The quantity of the amplified product was low and was deemed inappropriate for cloning. However, the PCR product was sent for sequencing and further investigation. The sequencing chromatogram indicated that the quality of the Sanger sequencing results was not suitable for interpretation.

The other drawback to this set of primers was non-specific amplification. Non-specific bands at 3000bp appeared to be the result of reaction with host genome, as it was also seen in the lane of green turtle primary cell line (negative control; figure 5-7, lane 3).

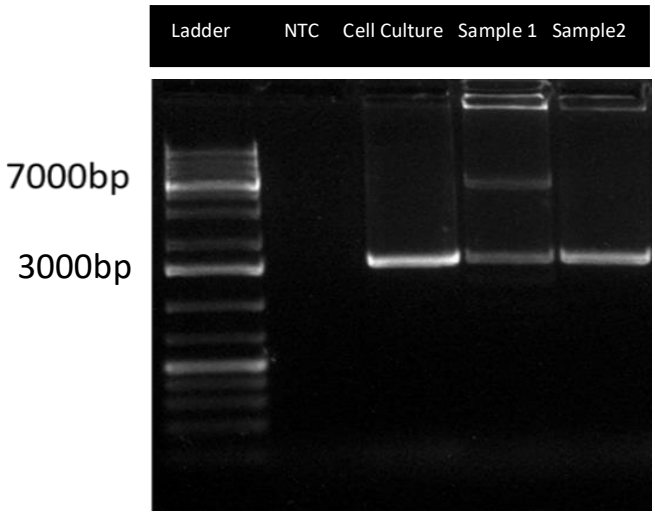


Figure 5-7 The gel electrophoresis results of the long run PCR products using inverted primers. Lane 1 is DNA marker, lane 2 is NTC=no template control, lane 3 is negative control (green turtle primary cell line), lane 4 and 5 are Sample 1 and 2 (tumour tissue of green turtles). Sample 1 shows a band of approximately 7000bp (close to 6903bp anticipated product). The three lanes of cell line, sample 1 and sample2 have a nonspecific band of 3000bp.

5.3.3. Rolling Circle Amplification and Next Generation Sequencing

The attempt to carry out NGS on the DNA extracted from tumour tissues and cell culture supernatant was not successful. Short reads were produced and host genome noise called for enrichment of the viral DNA. To achieve this, the RCA, the method frequently used to enrich viral DNA through amplification of circular episomal DNA and especially papillomavirus (de Oliveira *et al.,* 2017; Van Mechelen *et al.,* 2017) was chosen.

The eight samples used for this characterisation were DNA extracted from tumour tissues. No amplification was observed using the RCA kit (The predicted supercoiled amplified product appears as a sharp band of approximately 10kb size). The positive control was successfully amplified and visualised on gel. Two gels from two separate experiments are shown in figure 5-8.

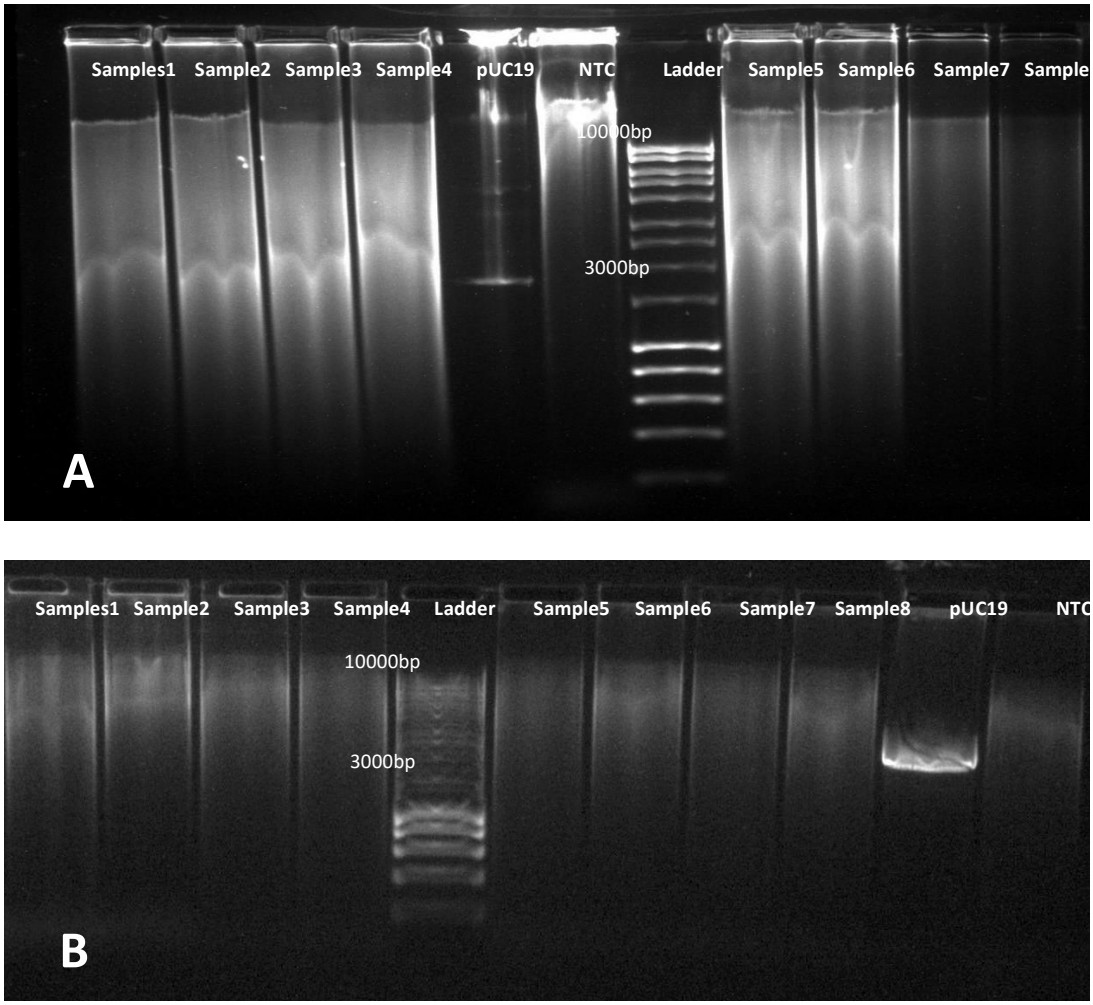


Figure 5-8 Gel electrophoresis results of rolling circle amplification on eight CmPV positive samples from skin tumours. Gel A) and B) show two separate experiments. The lanes are labelled on each gel which include eight positive samples, NTC=no template control and one positive sample: pUC19 (2.7kb).

To test the kit and assay, the kit was also used to amplify cloned CmPVE1 (109bp) in pGEM® vector (3015bp) (The making of the clone is explained in Section 6.3.1.). The cloned pCmPV-E1 was considered as a second positive control that is not provided in the kit. The RCA was successful in amplifying the plasmid and insert as shown in figure 5-9. Genomic DNA was used as a negative control and no contamination was seen in the negative control lane.

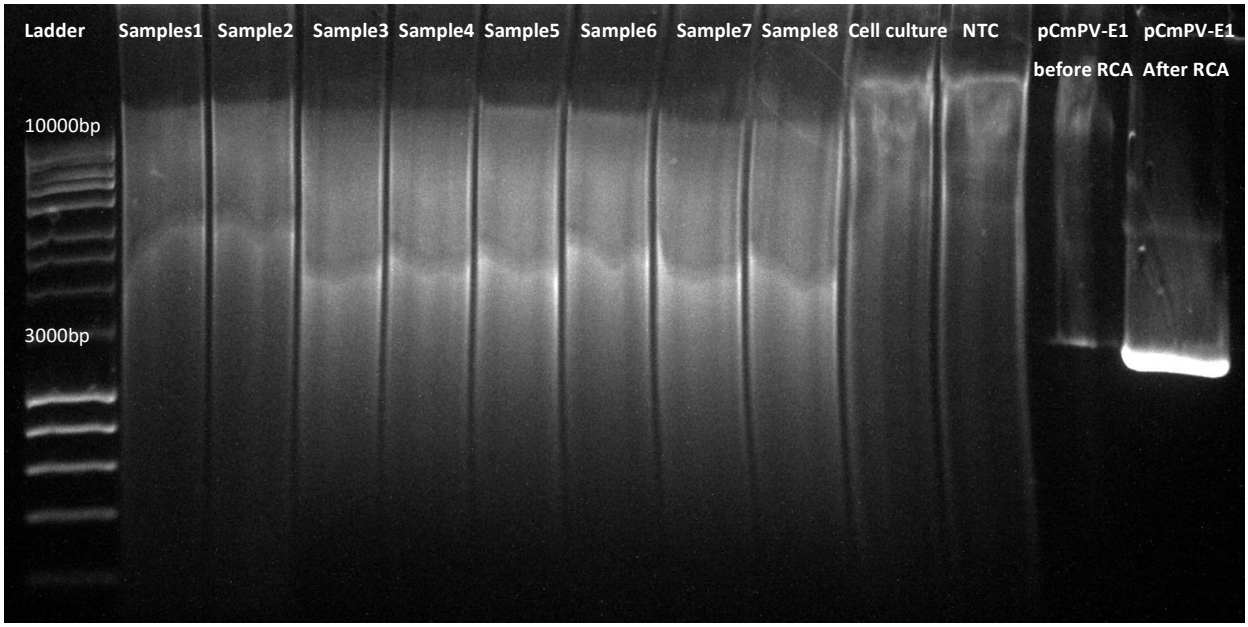


Figure 5-9 Gel electrophoresis results of rolling circle amplification on eight CmPV positive samples from skin tumours. The lanes include eight positive samples, extracted DNA from green turtle primary cell line (negative control), NTC=no template control and pCmPV-E1 before and after RCA. The pCmPV-E1 plasmid is 3124bp.

5.4. Discussion

Papillomaviruses are thought to have evolved over millions of years (Egawa *et al.,* 2015) and co-speciated in different hosts, including sea turtles (Herbst *et al.,* 2008). Two sea turtle papillomaviruses associated with benign skin proliferations in green (CmPV-1) and loggerhead turtles (CcPV-1), were described in 2008. Routine screening of animals admitted for rehabilitation was suggested, as the papilloma-viral infections can potentially be transmitted between individuals (Manire *et al.,* 2008). Regardless, no publications on papilloma-viral infections in sea turtles, nor records of screening them for the presence of papillomaviruses, have been published after 2008. In this project, green turtle primary cell lines were established and qPCR diagnostic primers were designed and evaluated, leading to the discovery of Australian *C. mydas* papillomaviruses (Mashkour *et al.,* 2018).

The first attempt to characterise the CmPV isolates were to analyse the sequences of E1 and L1 genes as these two genes are conserved among the papillomaviruses (Egawa *et al.,* 2015; Doorbar *et al.,* 2015). The NLS of the eight Australian CmPV isolates are 100% similar to CmPV-1 and the DBDs are 99% similar. The only differences are two SNPs in one codon of the DBD that is kept in this well-conserved gene. Both CTC and AUG codes translate for Leucine so this substitution is synonymous.

The L1 gene has been used as the standard for papillomavirus classification in other hosts (Doorbar *et al.,* 2015) and the Australian CmPV-L1 is 100% similar to CmPV-1. There must be at least 10%

divergence between two papillomaviruses for them to be classed as two different types (Lange *et al.,* 2011; Chen *et al.,* 2015; Doorbar *et al.,* 2015) and, there must be 60 to 70% similarity to put two papillomaviruses in one genus (Chen *et al.,* 2015). In our case, the Australian CmPV have 73% similarity to the loggerhead papillomavirus (CcPV-1) but is 100% identical to the green turtle papillomavirus (CmPV-1). The L1 based classification suggests that the Australian CmPV isolates and CmPV-1 may be of the same papilloma-viral type although they are from two different continents. The Australian CmPV isolates share 73% similarity with CcPV-1, the loggerhead papillomavirus and therefore appear to be from the same species of papillomavirus.

The *C. mydas* papillomavirus appears to not to have a link with the geographical distribution of the host. However from two distinct regions the papillomavirus typing gene (L1) is identical between all the sequenced isolates. Herbst *et al.,* suggested a low rate of evolution for non-mammalian PVs due to lower rates of metabolism (Herbst *et al.,* 2009). Such interactions with the hosts may suggest a vertical viral transfer for CmPV.

To be able to further characterise the eight Australian CmPV isolates, full genome sequencing was attempted for these viruses. The first trial with inverted primers was not successful in amplifying enough products for cloning and sequencing. Although the band was excised and purified before Sanger sequencing, the concentration was quite low and some DNA was probably lost during column purification and elution. Next generation sequencing was not effective either, probably due to the low copy number of the virus in samples. The next plan was to enrich the viral DNA and reduce the interference of the host genome before doing another round of NGS. The RCA was performed and although the positive controls were amplified, there was no amplification of circular DNA. To rule out possible faults with the kit or the assay, a second positive control was used and successfully amplified. No contamination was observed while checking the kit with whole DNA extracted from green turtle primary cell line. The experiment was done on 8 DNA samples extracted from skin tumour tissues and one possible explanation for failure in RCA-amplification of the viral genome could be the integration of the viral DNAs into the host genome. Therefore, the very low copy number of episomal circular DNA in sample vials was not favoured by RCA. This has been reported in previous RCA attempts to amplify human papillomavirus 33 (HPV-33) (de Oliveira *et al.,* 2017). In de Oliveira's experiment, HPV-33 was detected using Sanger sequencing but RCA failed to amplify the virus, therefore the follow-up NGS was unsuccessful. Previous studies on HPV-33 also indicated that after integration of this virus, the episomal viral load is low, which is considered to be a significant biological marker for HPV-33 (Khouadri *et al.,* 2007). This characteristic is interpreted as a key element in the carcinogenesis of HPV-16 and 18 as well, where HPV-associated lesions have a low copy number of episomal viruses and their incidence increases in cells with integrated viruses (Münger *et al.,* 2004). The frequency of integrated

HPV-16 is approximately 70% in associated cervical cancers, whereas for HPV-18, the viral genome is nearly always integrated (Doorbar *et al.,* 2015)

To repeat the RCA experiment, episomal viruses are needed, which may be present in non-tumour positive samples or keratinised cells with a high titer of the virus where multi-copy circular extra-chromosomal elements (episomes) are present (Egawa and Doorbar, 2017). This could have been the case for Herbst *et al.,* (2009), as they obtained the samples from epidermal lesions (not tumours) and probably with a higher copy number of the virus and were successful in amplifying the whole genome with a long PCR run and cloning. Additional overlapping primers could be used to utilise Sanger Sequencing and the resultant contigs assembled to produce a full genome sequence, as the virus is small and non-segmented.

The comprehensive analysis of the full genome of Australian CmPV isolates remains a challenge for future studies and is contingent on samples suitable for RCA and NGS. However, the Australian CmPV isolates are partially characterised and are likely to be from the same papilloma-viral type as CmPV-1 based on E1 and L1 sequences.

How I achieved the aim of the chapter:

1. I developed PCR assays to sequence specific parts of the E1 and L1 gene; the process included:
    - Choosing specific regions of these two genes suitable for amplification and sequencing
    - Designing specific PCR primers for each gene
    - Evaluating the protocols and amplifying DNA binding region of E1 gene and the full length of the L1 gene
    - Sequencing the PCR amplicons and bioinformatics analysis
2. I used inverted PCR primers to sequence the whole genome of Australian *C. mydas* papillomavirus isolates. The amplification was successfully performed but the titration was too low for conventional Sanger sequencing
3. I used rolling circle amplification and next generation sequencing to sequence the whole genome of Australian *C. mydas* papillomavirus isolates. The trials were not conclusive and possible alterations are contemplated for future attempts

6. THE CONCURRENT INFECTION OF CHELONID ALPHAHERPESVIRUS 5 AND *CHELONIA MYDAS* PAPILLOMAVIRUS IN GREEN TURTLES WITH FIBROPAPILLOMATOSIS

The aim of the chapter:

To determine the level of co-infection of ChHV5 and CmPV in fibropapillomas and normal skin biopsies of green turtles from the northern Great Barrier Reef

6.1. Introduction

Fibropapillomatosis (FP) is a disease chracterised by tumours in free ranging and captive sea turtles. The disease was first documented in 1938, at the New York Aquarium, in a green turtle originally from the Florida Keys (Smith and Coates, 1938). Observations of FP recorded in wild population increased in the 1980s and the disease has since been documented worldwide (Lackovich *et al.,* 1999; Quackenbush *et al.,* 1998) and all species of sea turtles (Jones *et al.,* 2016). Six of these sea turtles are listed on the IUCN[8] Red List of Threatened Species, unfortunately there is not enough information to evaluate the seventh species, flatback, which is reported as "*Data Deficient*" (IUCN 2015).

In a survey done in Hawaiian Islands, Work *et al.* reported that 67% of green turtles with tumour were juveniles and they were not able to find any patterns between sex, age and seasons when compared

[8] International Union for Conservation of Nature

to tumour scores of the green turtles sampled (Work *et al.,* 2004). Affected turtles are also vulnarable to secondary infections due to immunosuppression (Castellá *et al*., 1999; Work *et al*., 2004; Ritchie, 2006; Alfaro *et al*., 2008). Of the seven species of sea turtles, it appears that green turtle are most susceptible to the disease; in some regions (i.e. Indian River Lagoon in Florida and Kaneohe Bay in Hawaii) more than 50% of the green turtle population had clinical signs in form of tumours in the late 1990's (Lackovich *et al.,* 1999; Quackenbush *et al.,* 1998). Chaloupka *et al.* (2008) reported FP as the most common cause for stranding in Hawaii during 1982–2003 (Chaloupka *et al.,* 2008; Ng *et al.,* 2009; Quackenbush *et al.,* 1998). Environmental factors such as elevated temperature (Ward and Lafferty, 2004) and the presence of leeches (as mechanical vectors) on turtles have putative roles in outbreaks of the disease (Curry *et al.,* 2000).

The internal and external tumours that develop in affected turtles are categorised as papillomas, fibropapillomas and fibromas. The first stage of the disease is characterised by epidermal proliferations in the form of papillomas (Kang *et al.,* 2008) with large surface area. Fibromas (chronic tumours) affect the dermal surface and the collagenous matrix. Fibropapillomas are the intermediate stage between papillomas and fibromas (Kang *et al.,* 2008). Fibropapillomatosis is not just a cosmetic disease (Lu *et al*., 2000). Although the tumours are benign, their occurrence on conjunctivae can destruct vision and thereby compromise foraging and predation avoidance. When turtles are afflicted with numerous tumours, their locomotion can be restricted. Visceral tumours may interfere with normal organ function (Lu *et al.,* 2000; Ng *et al.,* 2009).

Research to date has suggested *Chelonid alphaherpesvirus 5* (ChHV5) as the aetiological agent for FP (Kang *et al.,* 2008; Lackovich *et al.,* 1999; Mansell *et al.,* 1989; Work *et al.,* 2017). The first study to successfully inoculate naïve turtles with tumour tissue filtrates and thereby transmit the disease, provided a clue for viruses as the causative agent of the disease (Herbst *et al.,* 1995). Molecular techniques have also provided substantial evidence for this, by consistently associating herpesviruses with FP. *Chelonid alphaherpesvirus 5* has been amplified from FP lesions and from turtles without lesions, which is consistent with the latent nature of herpesviral infections (Alfaro-Núñez *et al.,* 2014; Lackovich *et al.,* 1999; Lu *et al.,* 2000; McGeoch *et al.,* 2005).

Based on the morphology of the lesion, an alternative aetiological agent can be papillomavirus. The epidermal hyperplasia and mesenchymal proliferation in green turtle FP, is similar to equine sarcoids, a neoplastic disease of horses caused by bovine papillomavirus type 1 or type 2. The morphology of the dermal proliferation was also similar to those observed in cattle and deer FP (Jacobson *et al.,* 1989). The histopathological indicators triggered the study of FP tumours for the potential presence

of other neoplastic viruses. Papova-like[9] viruses and retroviruses were suggested as possible viral candidates (Lu *et al.,* 2000; Casey *et al.,* 1997), however these studies were sporadic and non-conclusive.

Other studies have attemoted to detect papillomavirus in FP. In 1999, Brown *et al*. tested for papillomaviruses in FP tumours with PCR, but did not amplify the sequences they were looking to detect. In the discussion Bowen *et al.* mentioned that the PCR primers they had used were successful in detecting mammalian papillomaviruses, but not a putative avian papillomavirus. They also suggested that the low sensitivity of their test may render it incapable of detecting a low titer of papillomavirus in the tumour tissues (Brown *et al.,* 1999). In 2000, Lu *et al.,* used transmission electron microscopy and found small naked viruses similar in morphology to papillomavirus in their primary cultures that were inoculated with cell free tumour tissue homogenates. But again, the degenerate PCR primers they used seemed to lack the specificity required to detect papillomavirus. These PCR primers were designed and used for human, bovine and equine papillomaviruses (Lu *et al.,* 2000).

Recently, the presence of *C. mydas* papillomavirus (CmPV) along with ChHV5 were reported in eight skin tumour tissue samples from green turtles with FP, using specific PCR primers for ChHV5 and CmPV in green turtles (explained in Chapter four) (Mashkour *et al.,* 2018).The PCR products were sent for sequencing and sonfirmed to have 100% similarity to CmPV-1.

In this study a larger number of tumour and healthy skin samples were screened by molecular techniques to further investigate the presence and co-occurance of CmPV and ChHV5 in green turtles foraging on the east coast of Queensland, Australia.

6.2. Materials and methods

6.2.1. Animals, sample collection and DNA extraction

The James Cook University Turtle Health Research Team conducts surveys on health and genetics of sea turtles in the coastal regions of Townsville and Bowen and archived samples dating back to 2011 were made available for his study. The sampling protocol is explained in section 4.2.1. In addition, tumour tissues were received frozen on ice from Australian collaborators: Moreton Bay research station, Gladstone, Cairns, Sea World Marine Park Gold Coast, and Airlie Beach.

[9] The term is not used in taxonomy anymore and two families of *Papillomaviridae* and the *Polyomaviridae* are proposed instead.

Table 6-1 The list of samples examined in this study[10]

Region	FP afflicted green turtles			Asymptomatic green turtles (clinically healthy)
	Number of green turtles with FP lesions	Number of tumour samples (multiple from some turtles)	Number of normal samples	
Cockle Bay, Townsville	24	52	13	21
Bowen	49	53	15	17
Sea World, Gold Coast	2	4	-	-
Cairns	2	3	-	-
Airlie Beach	1	4	-	-
Gladstone	5	4	4	9
Moreton Bay	6	11	4	-
Total	89	131	36	47

DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega) according to manufacturer's protocol. The extracted DNA that was used to quantitatively identify the presence and viral loads of ChHV5 and CmPV in tumour tissues and tumour-free tissues. (From here on the tissues unaffected by tumours will be referred to as 'normal' samples or tissues). In total, 131 skin tumour biopsies and 36 normal tissues from 89 green turtles with FP were analysed for the presence and viral loads of CmPV and ChHV5. A green turtle embryo fibroblast cell line, CMEM (Mashkour *et al.,* 2018), was used as a negative control. An additional 47 clinically healthy green turtles from Cockle Bay, Bowen and Gladstone were also screened for the presence of CmPV and ChHV5.

Collaborators from Western Australia and Bowen kindly provided six skin tumour tissue samples of loggerhead turtles; these samples were examined for the presence of CmPV, CcPV and ChHV5.

[10] Sample collections are with approval from the James Cook University Animal Ethics Committee, Department of Environment and Heritage Protection (DEHP) and the Great Barrier Reef Marine Park Authority (GBRMPA).

6.2.4. Cloning of CmPV-E1, ChHV5-DNApol and GAPDH amplicons into pGEM®-T Easy Vectors

Conserved regions of CmPV and ChHV5, the E1 gene and DNA polymerase respectively, were selected for cloning and to make quantification standards. To clone the genomic DNA, a region containing a section of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) intron and exon was also selected. The justification of this selection was to aim for DNA and not mRNA, in order to achieve a more accurate quantification based on genomic DNA. The PCR primers to target DNA polymerase (Dpol) from ChHV5, the E1 gene from CmPV and GAPDH from green turtles are shown in table 6-2. To design the primers the CmPV-1 and ChHV5 full genomes and the GAPDH portion of green turtle genome were downloaded from NCBI (GenBank accession no. EU493091.1, HQ878327.2 and FJ234450.1 respectively). The sequences were imported to Geneious software, version 10.0.9. The E1 gene of CmPV-1 and the DNA polymerase gene of ChHV5 were extracted and were exported to AlleleID® for primer design, along with GAPDH gene. For each target, the important design considerations were examined such as primer length, melting and annealing temperature (Apne and Singh, 2007). The designed primers were examined for specificity against targeted sequences in genbank using the Basic Local Alignment Search Tool (BLAST).

After successful polymerisation, the amplicons CmPV-E1, ChHV5-DNApol and GAPDH were cloned separately into pGEM®-T Easy Vectors using the TA cloning system according to pGEM®-T Easy Vector Systems protocol (Promega). Briefly, fragments of CmPV-E1 (109 bp) and ChHV5-DNApol gene (864 bp) were amplified from putative positive samples for CmPV and ChHV5, while a section of GAPDH (431 bp) was amplified from extracted DNA from normal skin samples. The PCR was carried out on a Rotor-Gene 6000 thermocycler machine and the reaction comprised 10µl of GoTaq Green Hot Start Mastermix (Promega), 0.8µM of each primers and ~80 ng of template DNA and nuclease free water to a final volume of 20µl. PCR primers and cycling conditions are listed in Table 6-2.

Table 6-2 Primers to amplify viral and genomic DNA for cloning

Primers and probes	Target selection	Sequences	Cycling condition	Reference
Chm-Pap-109-F	E1 gene of CmPV	5'GCC GAT GAT GTC CAC TTA T3'	Dye based qPCR: -2 min at 95°C -40 cycles (95°C for 10s, 60 °C for 20s, and 72°C for 30s) -2 min at 72°C	(Mashkour *et al.,* 2018)
Chm-Pap-109-R		5'GCT GAA TCC ACA GAG GTA G3'		
Cm-Dpol-864-F	DNA polymerase of ChHV5	5'ATG ACG GAC GGA CAA CAG3'	Dye based qPCR: -2 min at 95°C	Designed for this project

Cm-Dpol-864-R		5'GGA GAT GAC GGC TGC TAA3'	-35 cycles (95°C for 10s, 57 °C for 15s, and 72°C for 55s) -5 min at 72°C	explained in section 6.2.4
GAPDH-475-F	Glyceraldehyde 3-phosphate dehydrogenase intron 11 of green turtle	5'CCT TTA ATG CGG GTG CTG3'	Dye based qPCR: -2 min at 95°C -35 cycles (95°C for 10s, 57 °C for 15s, and 72°C for 30s) -5 min at 72°C	
GAPDH-475-R		5'CAC GGT TGC TGT ATC CAA3'		

Abbreviations: F, forward primer; R, reverse primer

The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). The column purified amplicons were quantified by spectrophotometry and 3:1 ratio of insert:plasmid was calculated. The amplicons were inserted into pGEM®-T Easy Vectors using T4 DNA Ligase and transferred to JM109 High Efficiency Competent Cells (Promega). The following day white colonies were selected, boiled at 100°C for 10 min to lyse the bacteria and screened for presence of desired amplicons using M13 PCR primers. The expected length of pGAPDH was 717 bp resulted from 431 bp of amplified GAPDH+ 286bp of M13 forward to reverse on pGEM®-T Easy Vector; The expected length of pChHV5-Dpol was 1150 bp resulted from 864 bp of amplified pChHV5-Dpol + 286bp of M13 forward to reverse on pGEM®-T Easy Vector. The expected length of pCmPV-E1 was 395 bp resulted from 109 bp of amplified pCmPV-E1 + 286bp of M13 forward to reverse on pGEM®-T Easy Vector.

The positive plasmids were purified using miniprep system (Promega) and kept in nuclease free water at -20°C for following steps.

6.2.5. Restriction Enzyme treatment

A supercoiled circular plasmid can suppress the PCR amplification and result in an error as big as seven copies of the target gene per cell (Hou *et al.,* 2010). To avoid misinterpretation of papilloma and herpes viral load the cloned plasmids were treated with restriction enzymes. To select an enzyme which only cut the pGEM®-T Easy Vector once without digesting the target genes, the vector and the cloned sequences were analysed via Geneious 10.1, subsequently SacI and PstI were chosen for treatment.

The enzymes SacI and PstI (Promega), were used according to the manufacturer: a mixture of 15.2 µl water, 2 µl optimum buffer (buffer J for SacI and buffer H for PstI), 0.3 µl BSA, 1 µl restriction enzyme, and 1.5 µl of the cloned vector was prepared and incubated at 37°C for 2-3 hours. The restriction enzymes were inactivated by putting the vials in heat blocks (65°C) for 15 minutes. The products were

visualised on agarose gel along with circular plasmids as controls for the restriction activity of SacI and PstI. The bands were excised and purified through a PCR clean up kit (Promega). The linear plasmids were eluted in DNAse free water and stored at -20°C.

6.2.6. GoTaq® Probe qPCR primer and Probes

Three sets of PCR primers and probes were designed and used to specifically amplify the target genes from cloned plasmids (standards), positive controls and test samples. The synthesised primers and probes are listed in table 6-3.

Table 6-3 The sequences of the PCR primers and probes along with the PCR cycling protocols used in the probe based qPCR to amplify the target genes from cloned plasmids (standards), positive controls and test samples.

Primers and probes	Target selection	Sequences	Cycling condition
Cm-Pap-109-F	E1 gene of CmPV	5'GCC GAT GAT GTC CAC TTA T3'	GoTaq® Probe qPCR: -2min at 95°C -50 cycles (95°C for 10s, 60°C for 10s)
Chm-Pap-109-R		5'GCT GAA TCC ACA GAG GTA G3'	
Chm-Pap-109-P		5'FAM CGA CCC ATG AAG CCG CTG T BHQ13'	
Dpol-ChHV5-82-F	DNA polymerase of ChHV5	5'CTA CCT TGT CTG GAG GTG GC3'	
Dpol-ChHV5-82-R		5'GGG TGT GAA TAA AAT CCC GCG3'	
Dpol-ChHV5-82-P		5'FAM TAG GGC GCG ACA TGC TTC BHQ13'	
GAPDH83-F	Glyceraldehyde 3-phosphate dehydrogenase of green turtle	T-GAPDH-83 F CTG GTC TCC TGG TAT GGA	
GAPDH83-R		T-GAPDH-83 R CAT GGA CTC CCA ACC TAT C	
GAPDH83-P		T-GAPDH-83 P 5'FAM AAA CCA CCC TCC AAA TCT GGC BHQ13'	

Abbreviations: BHQ1, black hole quencher-1; FAM, 6-carboxyfluorescein; F, forward primer; R, reverse primer; P, probe

6.2.7. Calibration curves and real-time quantitative PCR

For the setup of a dilution series the following calculation was used to obtain mass of one copy of the cloned PV DNA plasmid (g):

$$= \frac{\text{Molecular weight of the cloned PV DNA (vector plus insert)=the number of nucleotides} \times 660}{(6.023 \times 10^{23})}$$

660 is the average molecular weight of one mole base pair in grams

6.023×10^{23} is Avogadro constant (N_A)

The dsDNA concentration (ng/µl) was measured for each linear plasmid: pCmPV-E1, pChHV5-DNApol and pGAPDH and was used to determine the appropriate amount needed for the initial dilution step. The measurement was done by a Quantus™ Fluorometer using QuantiFluor® dsDNA Systems.

Tenfold serial dilutions were prepared in nuclease free water to produce a titration series from 10^8 to 10^1 of each plasmids in 2µl/reaction (as the GoTaq® Probe qPCR was undertaken using 2µl of template DNA in a 20µl PCR). From this, calibration curves for pCmPV-E1, pChHV5-DNApol and pGAPDH were produced. To make the standard curves, each dilution point was assayed in triplicate and the highest efficiencies were considered for the following calculations.

Care was taken to provide a wide range standard curve and cover the copy number range observed in "*unknown*" samples. Samples were considered positive if the emitted fluorescence of both duplicates exceeded the threshold limits and within 0.5 C_t of each other and negative otherwise.

All GoTaq® Probe qPCRs were carried out using a Magnetic Induction Cycler (MIC) qPCR machine (Bio Molecular Systems) with GoTaq® Probe qPCR Master Mix (Promega) as explained in table 6-3. Calibration curves for pCmPV-E1, pChHV5-DNApol and pGAPDH were constructed by plotting cycle numbers versus the log concentrations using the MIC software.

The viral copy number of each virus per cell was calculated according to CmPV-E1 and/or ChHV5-DNApol copies per host cell using the following equation:

$$= \frac{\text{CmPVE1 and/or ChHV5DNApol quantity}}{\frac{1}{2} GAPDH\ guantity}$$

Where, for all samples, viral load per diploid genome was determined by dividing CmPV and ChHV5 copy numbers by half of the GAPDH copy number.

6.2.8. Statistical analysis

The resulting copy numbers of CmPV and ChHV5 were compared using paired t-test with a 95% confidence interval. The two-tailed P value was also calculated. The viral loads of tumour tissues and normal skins of the green turtles with FP lesions were also compared using a t-test. Dealing with non-parametric groups of results, the Kruskal–Wallis test by ranks was done on samples from different regions to investigate the distribution of each virus in seven regions covered by this study. The correlation between the viral loads and the size/age of the turtle, calculated by curved carapace lengths (CCL) was also examined. Values with $P < 0.05$ were considered statistically significant. The statistical analyses were done with IBM SPSS Statistics 25.

6.2.9. Presence of CcPV and CmPV in loggerhead turtles

Two sets of primers were designed to screen the loggerhead turtles for presence of CmPV and/or CcPV. One set was designed to specifically target CcPV, and the other covered a more general target to be able to survey green and loggerhead turtles in one experiment. To design the latter the E1 genes of CmPV-1 GenBank: EU493091.1) and CcPV-1 (GenBank: EU493092.1) were aligned and the PCR primers were designed on the basis of the consensus sequence through Allele ID. The PCR primers and the optimised cycling protocol are described in table 6-4.

Table 6-4 The sequences of PCR primers along with PCR cycling condition used to detect Caretta caretta papillomavirus in samples from loggerhead turtle (Cc-Pap-99 primers) and generally detect papillomavirus in samples from loggerhead and green turtles (Turt-Pap-218 primers)

Primers and probes	Target selection	Sequences	Cycling condition
Cc-Pap-99-F	E2 gene	5'AAA GGG CAG TGG GAA ATC TC3'	Dye based qPCR: -2 min at 95°C -40 cycles (95°C for 10s, 58 °C for 20s, and 72°C for 30s) -2 min at 72°C
Cc-Pap-99-R		5'TGT GAT GGC GAC GAT GTG3'	
Turt-Pap-218-F	E1 gene	5'CTA CCT CTG TGG ATT CAG C3'	
Turt-Pap-218-R		5'ACC ATA ACT GCA CAA TCC TTC3'	

F, forward primer; R, reverse primer

6.3. Results

6.3.1. Cloning of CmPV-E1, ChHV5-DNApol and GAPDH amplicons into pGEM®-T Easy Vectors

Universal M13 PCR primers were used to check the accuracy of cloned plasmisd, pGAPDH, pChHV5-Dpol, and pCmPV-E1. The expected length of the bands, as explained in section 6.2.4, were seen in the gel picture (figure 6-1). For ChHV5-Dpol lane 5 was selected to proceed with standard curve prepararion as lane 6 and 10 had non-specific amplifications (~1000bp).

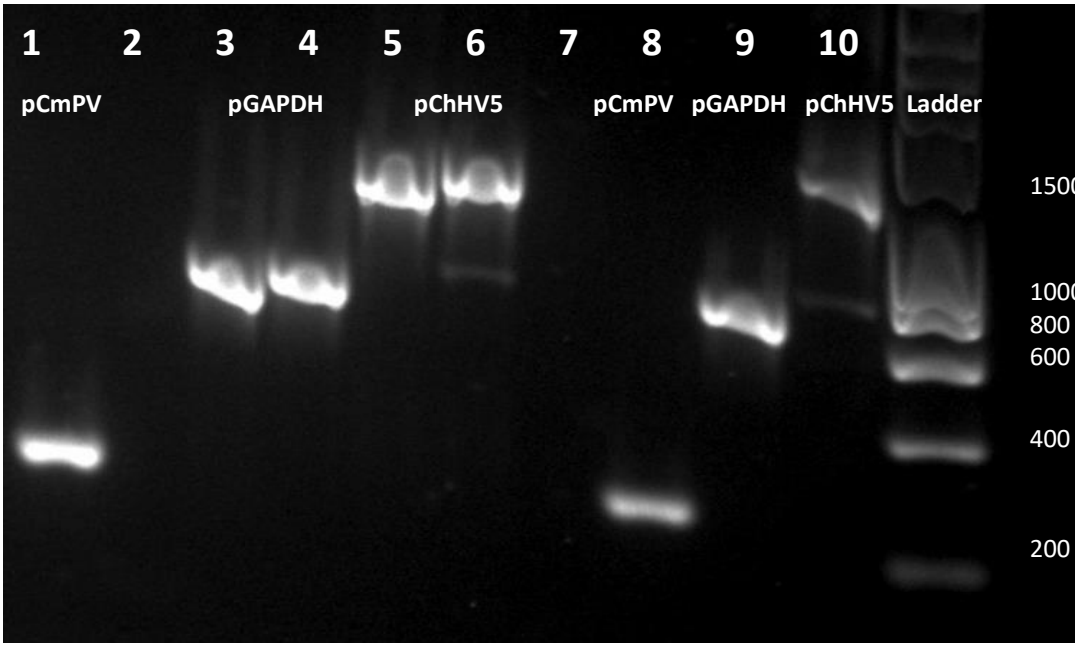


Figure 6-1 The expected length of PCR amplicons using M13 universal primers were observed: 395bp for pCmPV-E1 (lane 1), 717bp for pGAPDH (lane 2) and 1150bp for pChHV5-Dpol (lane 3).

The sequencing results of the amplified regions confirmed that the plasmids contained the desired sequences. The NCBI Basic Local Alignment Search Tool (BLAST) results are as follows:

pGAPDH cloned plasmid:

>FJ234450.1 *Chelonia mydas* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, intron 11 and partial cds
Length=490
Score = 769 bits (416), Expect = 0.0
Identities = 427/432 (99%), Gaps = 1/432 (0%)
Strand=Plus/Plus

pChHV5-Dpol cloned plasmid:

>HQ878327.2 *Chelonid alphaherpesvirus 5* , partial genome
Length=132233
Score = 1555 bits (842), Expect = 0.0
Identities = 859/867 (99%), Gaps = 2/867 (0%)
Strand=Plus/Plus

pCmPV-E1 cloned plasmid:

>EU493091.1 *Chelonia mydas* papillomavirus 1, complete genome
Length=6953
Score = 198 bits (107), Expect = 7e-47
Identities = 112/114 (98%), Gaps = 1/114 (1%)
Strand=Plus/Minus

6.3.2. Restriction Enzyme treatment

The cloned plasmids, pChHV5-Dpol, pGAPDG and pCmPV-E1 were treated by restriction enzymes, SacI and Pst I. Figure 6-2 is a gel from SacI digestion showing the expected size for linear pChHV5-Dpol: 3879bp, linear pGAPDH: 3446bp and linear pCmPV-E1: 3124bp.

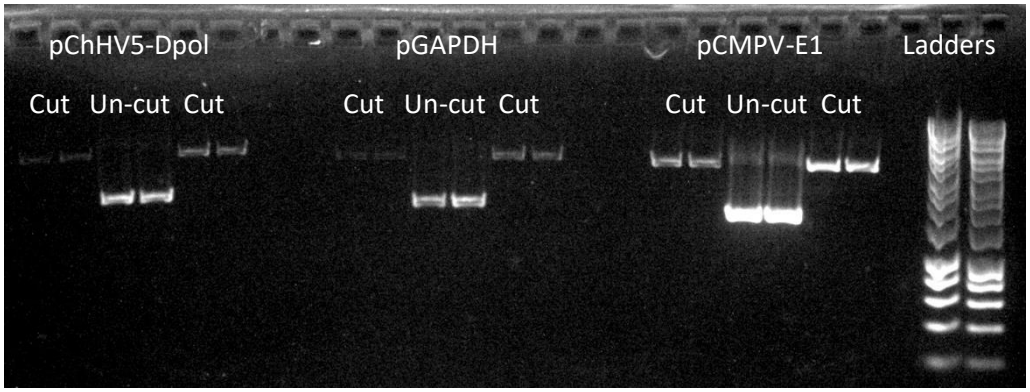


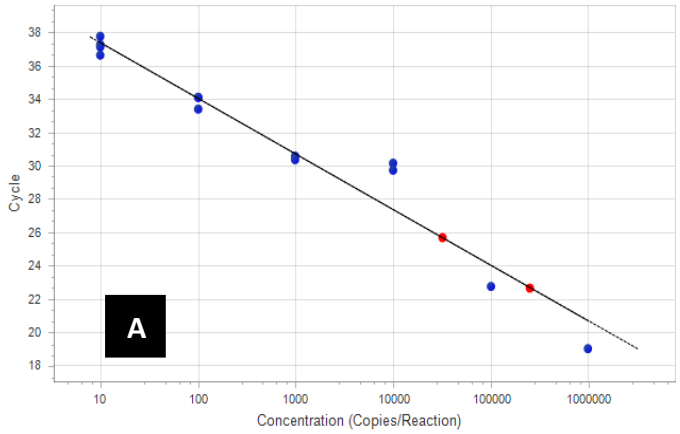
Figure 6-2 The cloned plasmis, pChHV5-Dpol, pGAPDH and pCmPV-E1 treated by restriction enzyme, SacI. The circular plasmids are labled as uncut and the linear plasmids as cut. Gel pattern is 2x cut, 2x uncut, 2x cut for each plasmid. The expected size for linear pChHV5-Dpol is 3879bp, for linear pGAPDG is 3446bp and for linear pCmPV-E1 is 3124bp.

6.3.3. The Calibration Curves

The standard curves were successfully constructed and used for the quantitative PCRs and subsequently the calculation of copy numbers per cells of each virus. The standards curve for GAPDH, CmPV and ChHV5 are shown in figure 6-3. Absolute quantification and the standard curves for three cloned plasmids were plotted based on the cycles (cq values) and the log of concentration (10 to 10^8 copies per reaction). The data points represent three replicates of each dilution. The R^2 values, the efficiency of each qPCR and the equation of the standard curves are as follows: pGAPDH R^2 0.950; Efficiency 0.99; Equation y = -3.35 x + 40.76; pChHV5 Dpol R^2 0.993; Efficiency 0.92; Equation y = -3.54 x + 40.49; pCmPV-E1 R^2 0.974; Efficiency 1.05; Equation y = -3.21 x + 40.48.

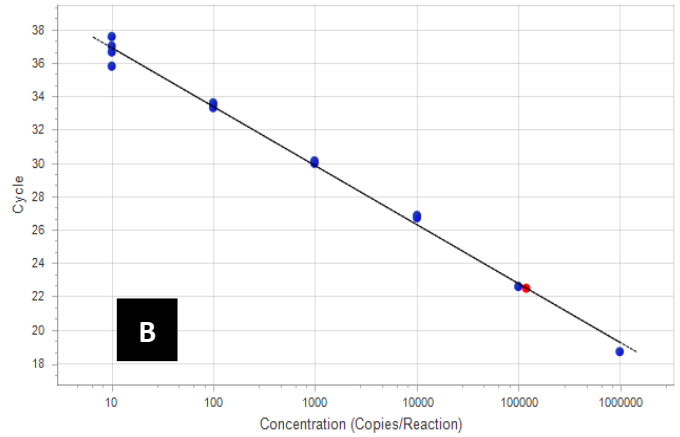
Absolute Quantification: GAPDH-83

Target	GAPDH-83 → GAPDH-83



Absolute Quantification: DPOL-83

Target	DPOL-83 → DPOL-83



Absolute Quantification: CHM-PAP-109

Target	CHM-PAP → CHM-PAP-109

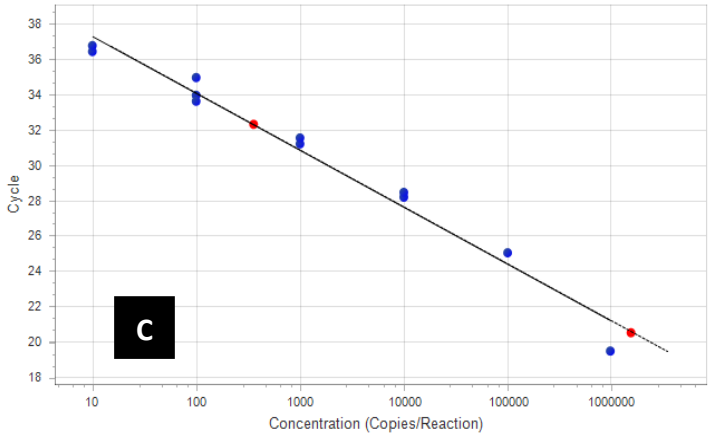
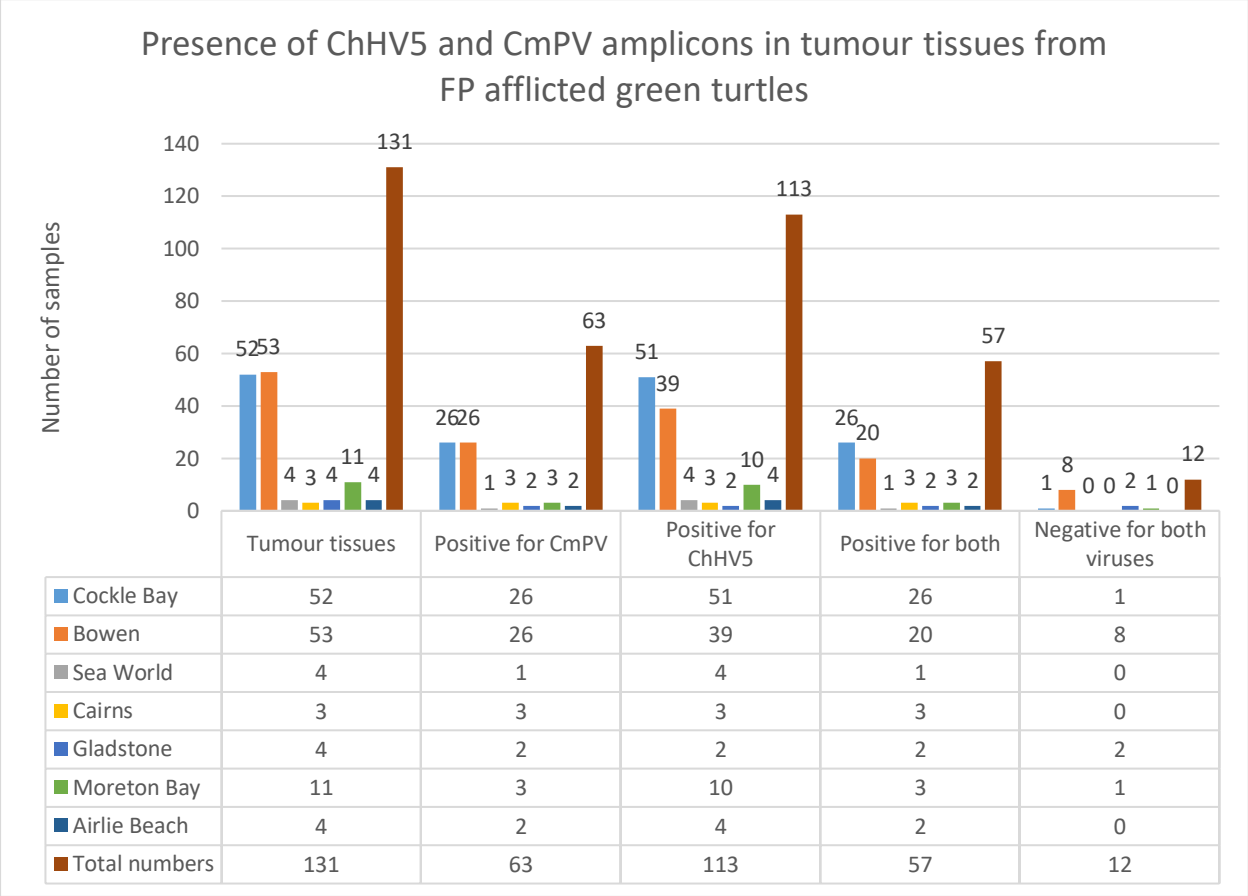


Figure 6-3 Absolute quantification and the standard curves for three cloned plasmids: A) GAPDH, B) ChHV5 Dpol and C) CmPV-E1 were plotted based on the cycles (cq values) and the log of concentration (10 to 10^8 copies per reaction).

6.3.4. The geographic distribution of positive samples for CmPV and ChHV5

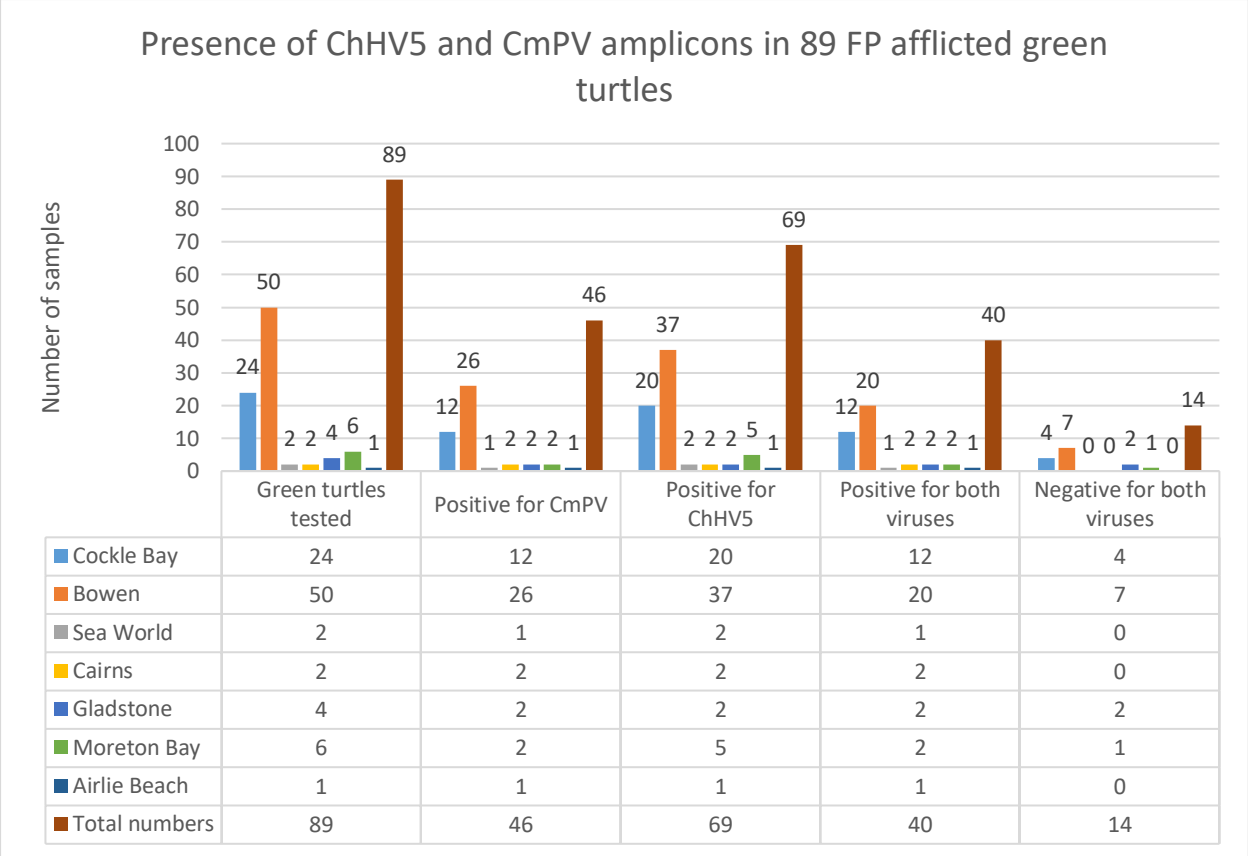
ChHV5 and CmPV sequences were detected by PCR in samples from turtles foraging all seven regions in this study. From 131 tumour tissues, 63 (48.09%) reacted for CmPV and 113 (86.25%) reacted for ChHV5. Both viruses were detected in 43.51% (57) of the samples and 9.16% of samples (12) did not react for both viruses in our assays. See figure 6-4 for region by region comparison.



	Tumour tissues	Positive for CmPV	Positive for ChHV5	Positive for both	Negative for both viruses
Cockle Bay	52	26	51	26	1
Bowen	53	26	39	20	8
Sea World	4	1	4	1	0
Cairns	3	3	3	3	0
Gladstone	4	2	2	2	2
Moreton Bay	11	3	10	3	1
Airlie Beach	4	2	4	2	0
Total numbers	131	63	113	57	12

Figure 6-4 The Tumour tissues collected from green turtles with FP foraging in a range of geographical regions tested positive for presence of ChHV5 and CmPV. In total 131 tumour tissues were tested, 63 samples reacted in PCR for CmPV, 113 for ChHV5, 57 for both viruses and 12 samples did not reacted for either of these viruses.

Tumour tissues were collected from 89 green turtles, but in some cases up to four samples were collected from individuals with multiple tumours making the total tally 131 samples. The detection of CmPV and /or ChHV5 amplicons varied from 0 to 100% of samples from individual animals. If one sample from one animal reacted in the PCR, the animal was considered positive for the presence of tested virus. Out of 89 green turtle samples, 69 (77.52%) tested positive for ChHV5, 46 (51.68%) for CmPV, 41 (46.06%) for both viruses and samples from 14 (15.73%) turtles tested negative for both (figure 6-5).



	Green turtles tested	Positive for CmPV	Positive for ChHV5	Positive for both viruses	Negative for both viruses
Cockle Bay	24	12	20	12	4
Bowen	50	26	37	20	7
Sea World	2	1	2	1	0
Cairns	2	2	2	2	0
Gladstone	4	2	2	2	2
Moreton Bay	6	2	5	2	1
Airlie Beach	1	1	1	1	0
Total numbers	89	46	69	40	14

Figure 6-5 The Tumour tissues collected from 89 green turtles with FP foraging in a range of geographical regions tested positive for presence of ChHV5 and CmPV. In total 89 green turtles were tested, 46 samples reacted in PCR for CmPV, 69 for ChHV5, 41 for both viruses and 14 samples did not react for either of these viruses.

To help visualising the presence of viruses in FP-afflicted turtles, the presence of CmPV and ChHV5 in samples from 89 green turtles are shown in Venn Diagrams (figure 6-6A). The same plots were made for Cockle Bay and Bowen, where the highest number of samples originated from (figure 6-6B and C). The overall results indicates that if the animal is positive for CmPV there is 70-100% chance for the animal to be ChHV5 positive too.

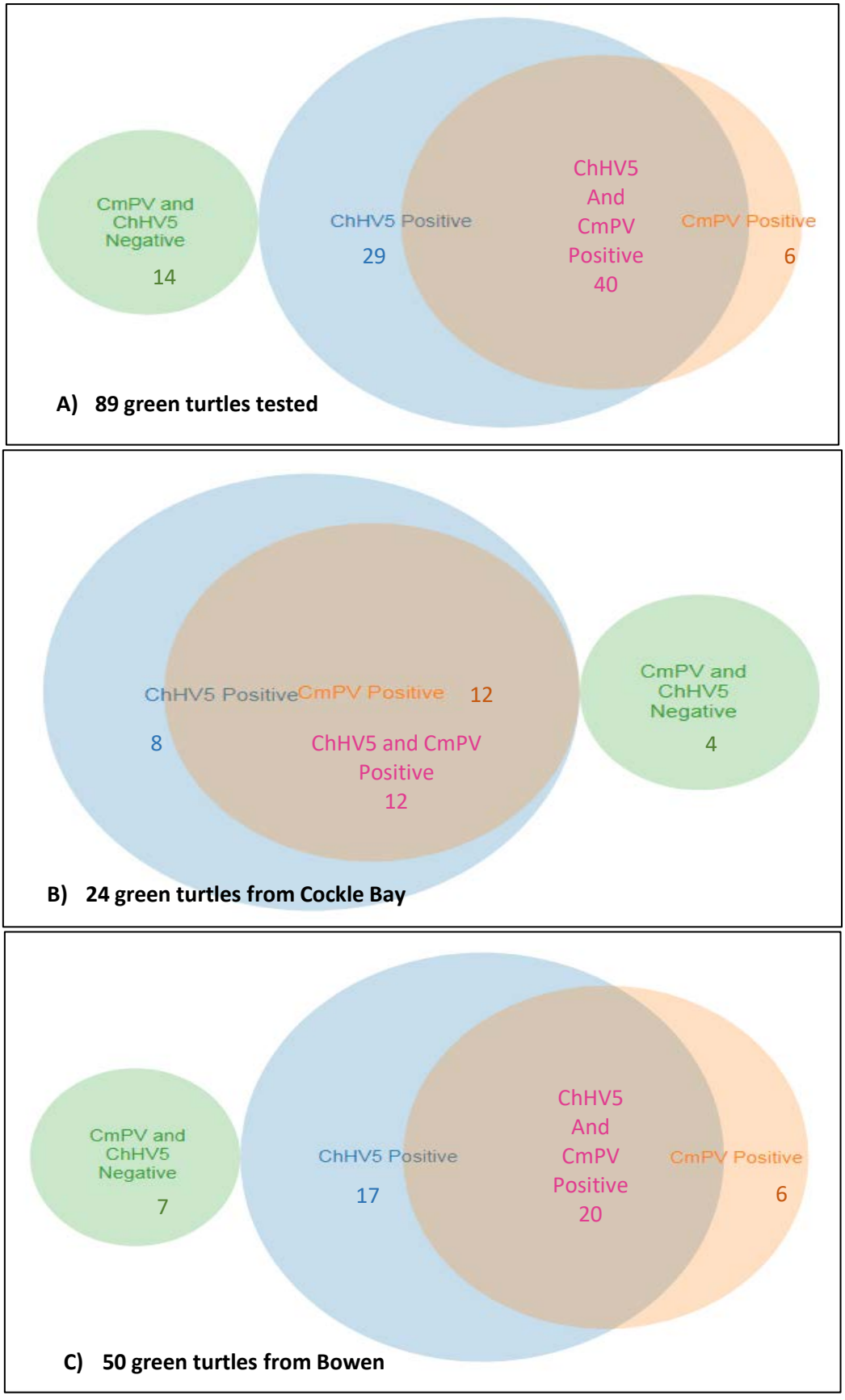
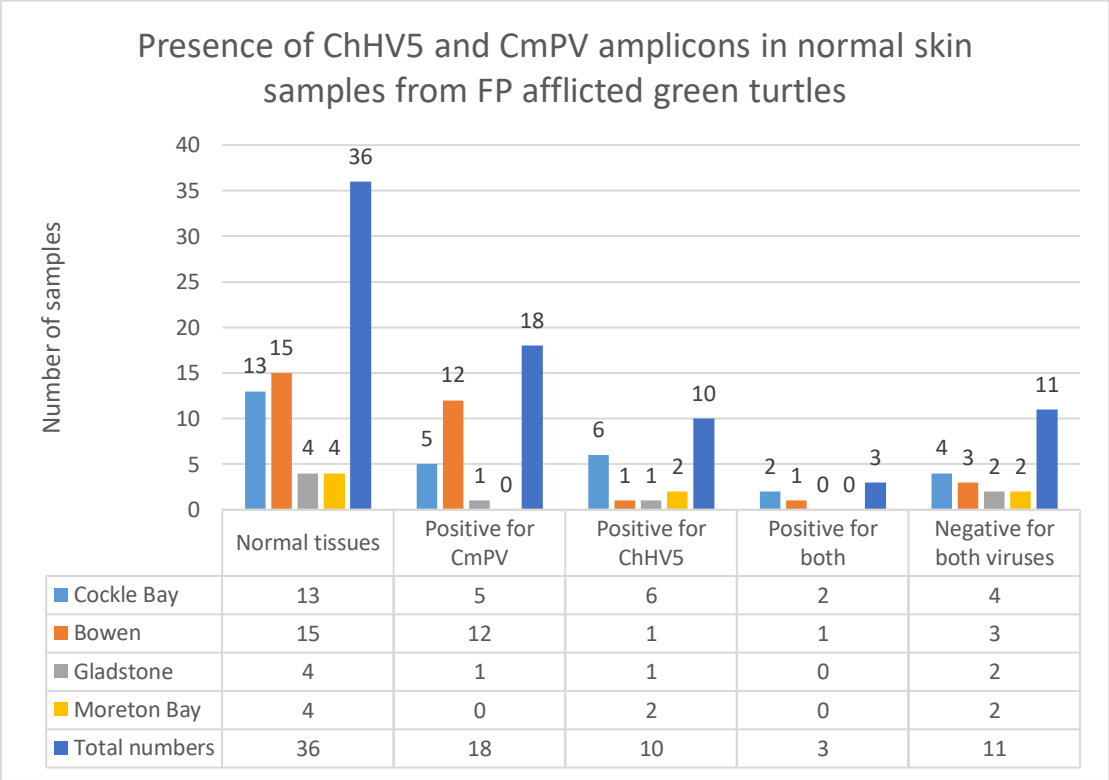


Figure 6-6 The presence of CmPV and ChHV5 in green turtle with FP tumours. A) 89 green turtles from east coast of Queensland; B) 24 green turtles from Cockle Bay, Townsville; C) 50 green turtles from Bowen

Both viruses were also detected in normal (tumour free) skin samples from green turtles with FP lesions. From 36 normal tissues tested for presence of ChHV5 and CmPV, 18 (50%) samples reacted in PCR for ChHV5, 10 (27.77%) for CmPV, 3 (8.33%) for both viruses and 11 (30.55%) samples did not react for either of these viruses (Figure 6-7).



	Normal tissues	Positive for CmPV	Positive for ChHV5	Positive for both	Negative for both viruses
Cockle Bay	13	5	6	2	4
Bowen	15	12	1	1	3
Gladstone	4	1	1	0	2
Moreton Bay	4	0	2	0	2
Total numbers	36	18	10	3	11

Figure 6-7 Normal skin samples from green turtles with FP foraging in Cockle Bay, Bowen, Gladstone and Moreton Bay tested positive for presence of ChHV5 and CmPV. In total 36 normal tissue samples were tested, 18 samples reacted in PCR for CmPV, 10 for ChHV5, 3 for both viruses and 11 samples did not react for either of these viruses.

A total of 47 samples from asymptomatic green turtles (clinically healthy) which were captured in Cockle Bay, Bowen and Gladstone and were investigated for the presence of ChHV5 and CmPV. Of those samples, 27 reacted in PCR for ChHV5 (57.44%) and 34 (72.34%) for CmPV. Samples which reacted for both viruses consisted of 46.8% of the samples (n=22) and 7 samples did not react for either of these viruses (14.89%) (Figure 6-8).

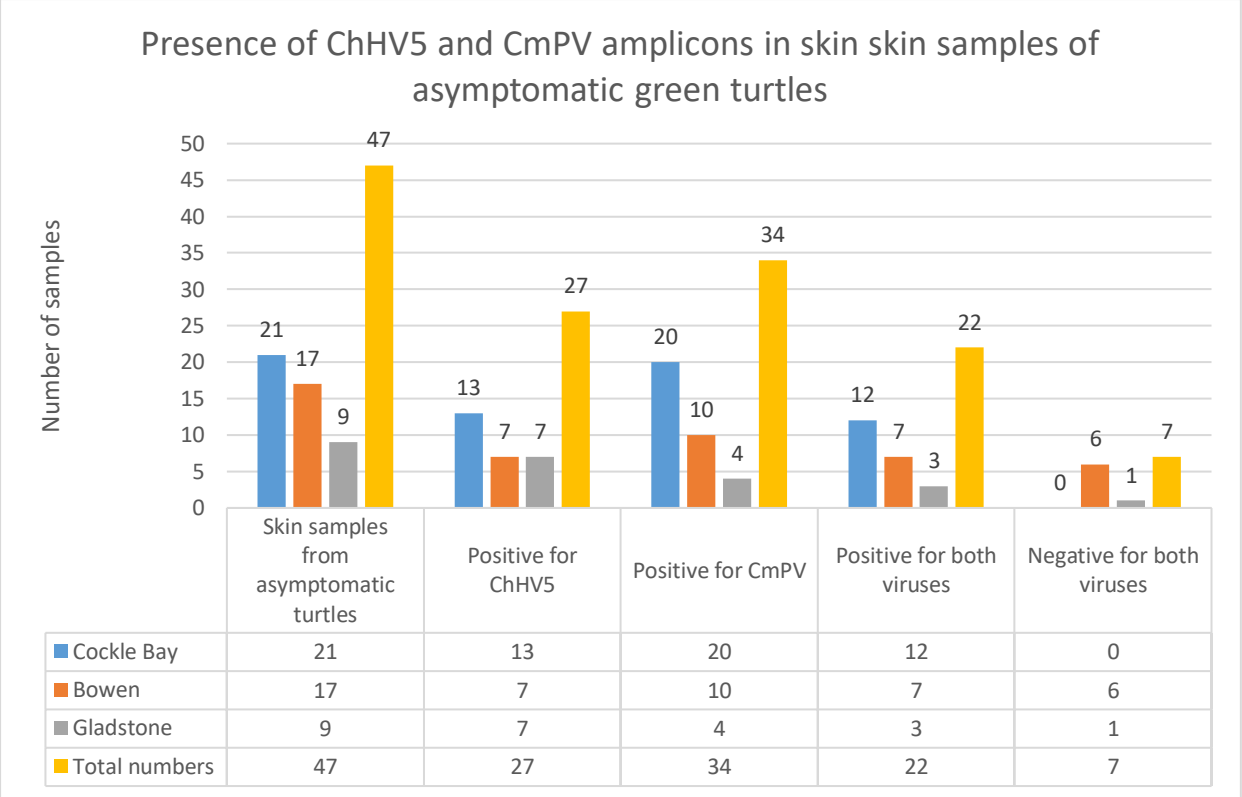


Figure 6-8 Normal skin samples from asymptomatic green turtles foraging in Cockle Bay, Bowen and Gladstone tested positive for presence of ChHV5 and CmPV. In total 47 normal tissue samples were tested, 27 samples reacted in PCR for ChHV5, 34 for CmPV, 22 for both viruses and 7 samples did not react for either of these viruses.

6.3.5. The copy number per cells of CmPV and ChHV5

The herpesviral load in 131 tumour tissues ranged from 0 to 226.1 (mean: 13.486; SD: 28.504) copies in each cell and the papilloma-viral load was from 0 to 2.54 (mean: 0.039 (≈1 copy in 25 cells); SD: 0.249). The raw data is provided in appendices 9, 10 and 11. The paired t test results show that by conventional criteria, the higher copy number per cell of ChHV5 compared with CmPV is considered to be statistically significant with a P value of less than 0.0001. The mean of ChHV5 minus CmPV equals 13.477; with a 95% confidence interval of this difference: from 8.544 to 18.351. Intermediate values used in calculations: t=5.425; df=130; standard error of difference = 2.479.

For each virus, a comparison was made between the viral loads in tumour tissues and normal skin samples of green turtles with FP lesions (table 6-5). The viral load per cell of ChHV5 in tumour tissues is higher than normal tissues of green turtles with FP lesions and is statistically significant (P value<0.0001). The viral load of CmPV is higher in normal skin samples but the difference is not statistically significant (P value=0.274). The t-test statistical values are presented in table 6-5.

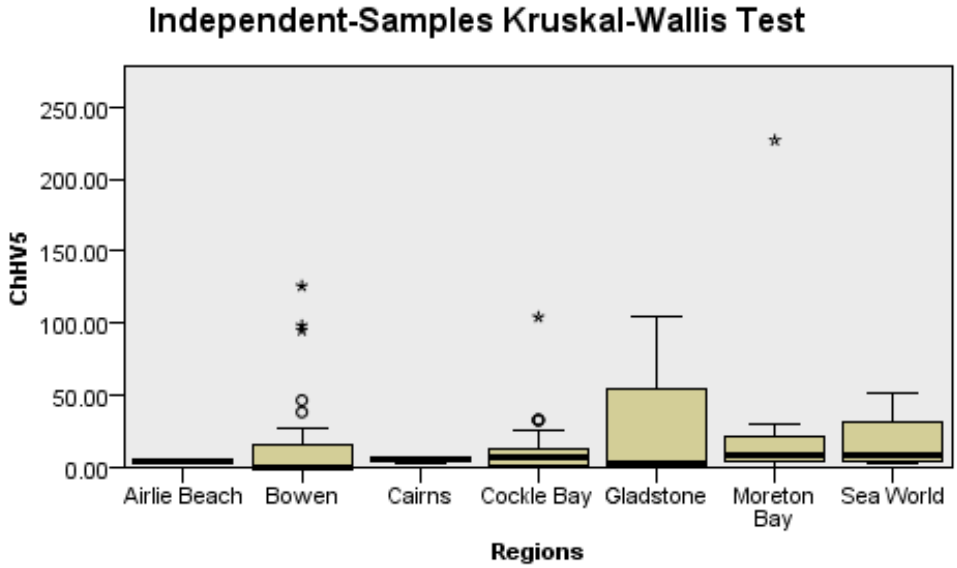
Table 6-5 A) Calculated copy numbers per cell and the paired t-test statistical values for the comparison between ChHV5 in tumour tissues and ChHV5 in normal tissues;

Groups	ChHV5 in tumour tissues	ChHV5 in normal tissues
Mean	13.486	0.004
Std. Deviation	28.504	0.018
Std. error of mean	2.490	0.003
Variance	812.494	0.000
Minimum	0.00	0.00
Maximum	226.91	0.08
N	131	36
The two-tailed P value is less than 0.0001. The mean of ChHV5 in tumour tissues minus ChHV5 in normal tissues equals 7.7; 95% confidence interval of this difference: From 4.783 to 10.616. Intermediate values used in calculations: t=5.360; df=35; standard error of difference = 1.437.		

B) Calculated copy numbers per cell and the paired t-test statistical values for the comparison between CmPV in tumour tissues and CmPV in normal tissues;

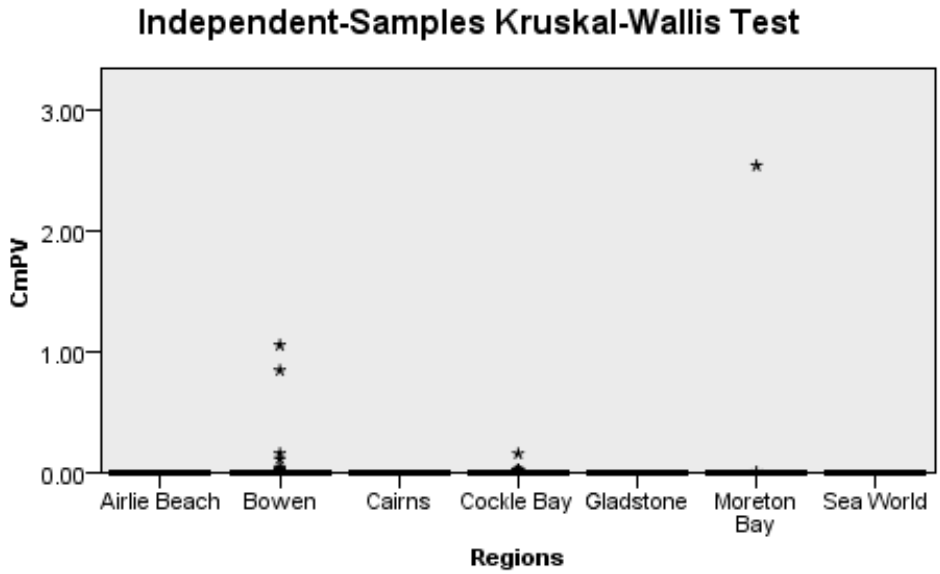
Group	CmPV in tumour tissues	CmPV in normal skin
Mean	0.0392	0.239
Std. Deviation	0.249	1.253
SEM	0.021	0.208
Variance	0.062	1.572
Minimum	0.00	0.00
Maximum	2.54	7.54
N	131	36
The two-tailed P value equals 0.274. The mean of CmPV in tumour tissues minus CmPV in normal skin equals -0.232; 95% confidence interval of this difference: From -0.656 to 0.192. Intermediate values used in calculations: t=1.109; df = 35; standard error of difference = 0.209.		

The distribution of ChHV5 and CmPV load is the same across the seven regions tested, as Kruskal–Wallis test by ranks resulted in a non significant difference between the ChHV5 and CmPV viral load in samples from these seven regions (ChHV5 P value=0.096; CmPV P value=0.946). The Stem and Leaf Plots (figures 6-9 and 6-10) were drawn to help visualising the viral load distributions in FP tumours from seven regions of Queensland.



Total N	131
Test Statistic	10.761
Degrees of Freedom	6
Asymptotic Sig. (2-sided test)	.096

Figure 6-9 The independent-samples Kruskal-Wallis test for the distribution of ChHV5 viral loads in seven regions of Queensland. The distribution of ChHV5viral load is the same in these regions (P=0.096).



Total N	131
Test Statistic	1.690
Degrees of Freedom	6
Asymptotic Sig. (2-sided test)	.946

Figure 6-10 The independent-samples Kruskal-Wallis test for the distribution of CmPV viral loads in seven regions of Queensland. The distribution of CmPVviral load is the same in these regions (P=0.946).

6.3.6. The eight CmPV positive green turtle samples from Chapter four

All of the eight animals were positive for both viruses in previous studies. The mean copy number/cell of the CmPV was 0.00701 and the normal skin samples were free of virus. The mean copy number /cell of the ChHV5 in these tumour tissues was 14.771.

6.3.7. Demographical analysis

The range of curved carapace length (CCL) was 25.7 to 101.1cm which was measured for 74 animals out of 89. There is a non-significant negative correlation between the viral loads and the CCL measurements which are shown in figure 6-11 and 6-12. The value of R^2, the coefficient of

determination, is 0.0025 for CmPV and the P value is 0.672. The R^2 and P values of the correlation between ChHV5 and CCL measurement are 0.0005 and 0.85, respectively.

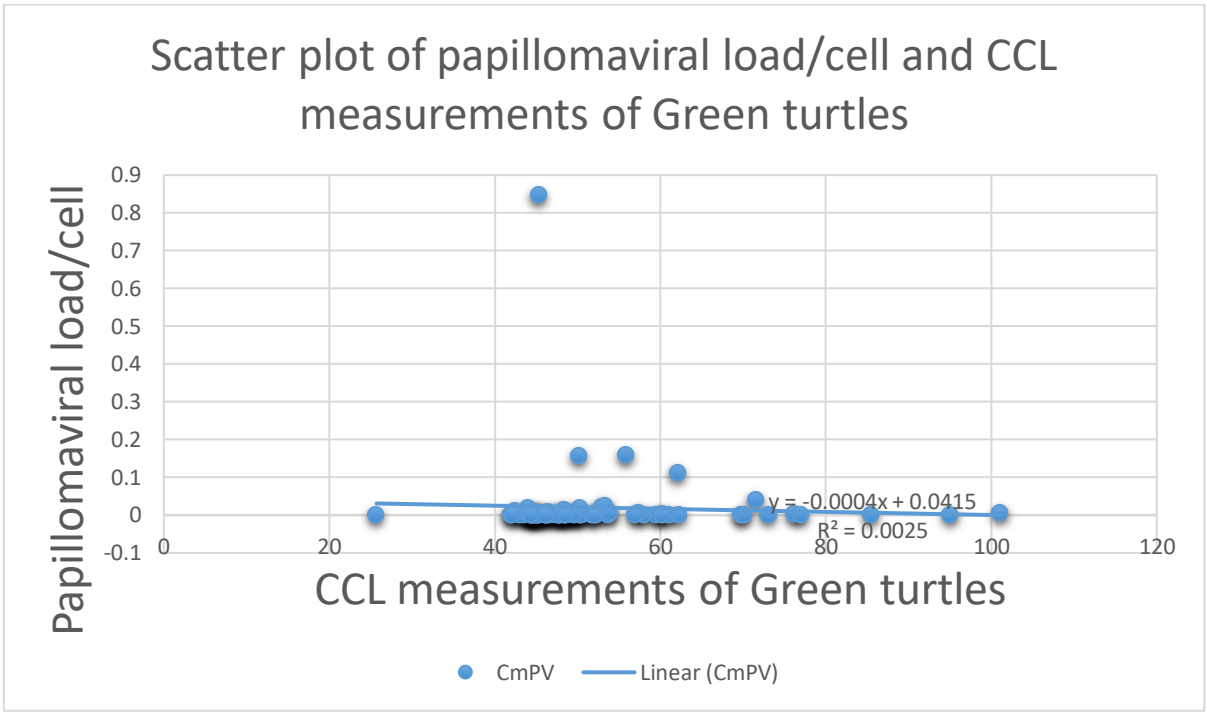


Figure 6-11 The non-significant negative correlation between the papilloma viral load and the CCL measurement: y = -0.0004x + 0.0415; Correlation Coefficient (r): -0.05; P=0.672.

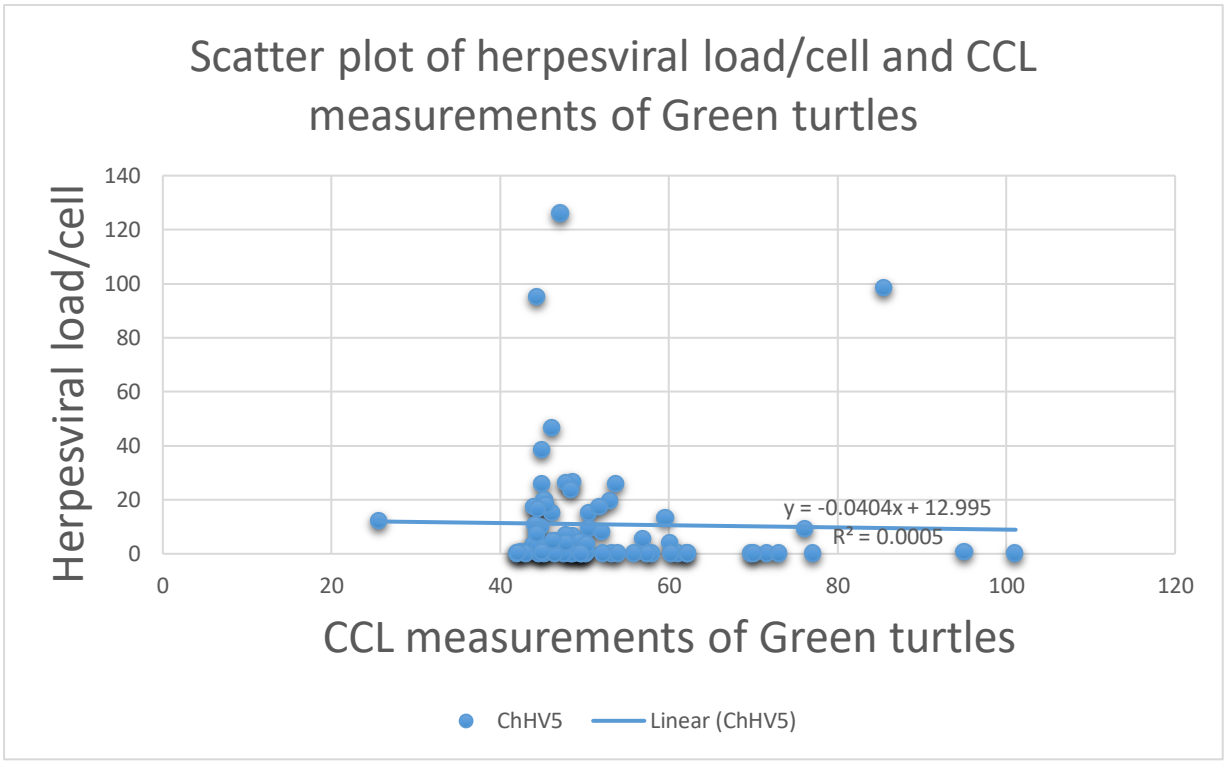


Figure 6-12 The non-significant correlation between the herpes viral load and the CCL measurement: y = -0.0404x + 12.995; (Correlation Coefficient (r): 0.022; p=0.85.

6.3.8. The host genomic DNA

The copy number per reaction of GAPDH is consistent in samples from green turtles with FP lesions, in other words, there is no significant difference between the quantities of tissues used for the qPCR reactions and subsequently viral load comparison (Figure 6-13) (P value: 0.121).

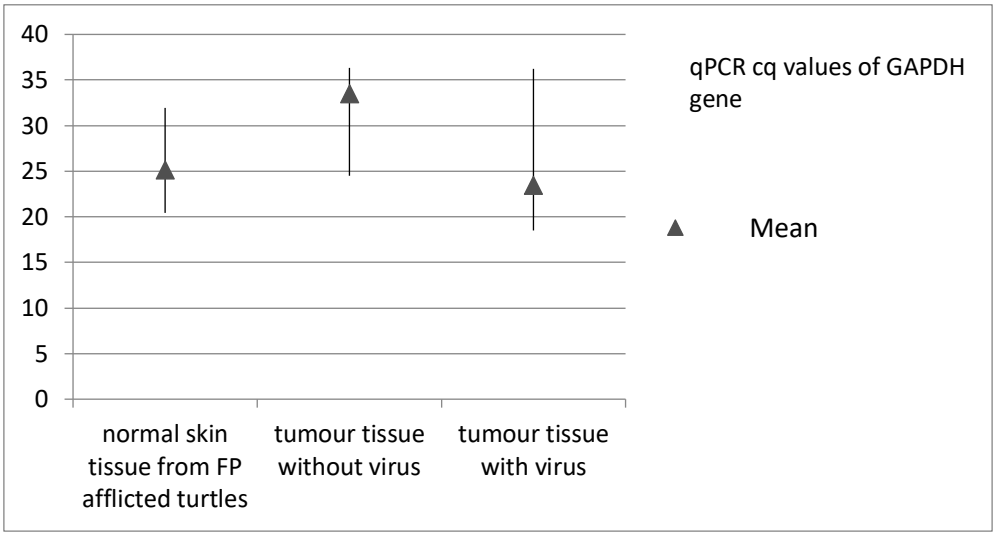


Figure 6-13 The cq value of host genomic DNA (GAPDH) in three different groups of samples from FP afflicted turtles.

As the starting tissue mass appeared to be similar in samples, after calculating the DNA extraction elute volume (50µL of extracted DNA in DNase free water to 20µL of PCR reaction) the estimated mean of ChHV5 viral loads appears to be approximately 3.707×10^5 copies/µL of DNA extract from tumour tissues and 36.36 copies/µL from normal skins of green turtles with FP lesions. For CmPV, the viral load is 4164.29 copies/µL of DNA extract from tumour tissues and 2201.75 copies/µL from normal skins of green turtles with FP lesions. This unit calculation enabled us to compare our results of ChHV5 viral loads with previous studies that used different protocols and calculated copies/cell, copies/µL of samples in PCR reaction or copies/µgr of DNA (table 6-6).

Table 6-6 The average copy number of ChHV5 in various units calculated in different studies in comparison to the current study

		Quackenbush *et al.,* 2001	Greenblatt, *et al.,* 2005	Page-Karjian *et al.,* 2015	Alfaro-Núñez *et al.,* 2016	Current study
Tumour tissues	Copies/cell	15.21	13.9[$]	-	15.61, s ± 12.5	13.486, SD 28.504
	copies/µL of samples in PCR reaction	-	-	-	4.9×10^4	3.707×10^5
	copies/µgr of DNA	3.3×10^4 to 4.9×10^6 [#]	-	3.24×10^8	-	4.630×10^5*
	Copies/cell		0.03[$]	-	0.02, s ± 0.00	0.004, SD 0.018

Normal skin	copies/µL of samples in PCR reaction	-	-	-	1.8×10^{1}	3.636×10^{1}
	copies/µgr of DNA	122 ± 82.67[#]	-	22722	-	45.45

[$] 13900 and 30 copies per 1000cells

[#] 3.3×10^{3} to 4.9×10^{5} per 100ng and 12.2 ± 8.267 per 100ng

* the mean was 1.145×10^{5} copies/µL of DNA, each µL of DNA contained approximately 80ngr of DNA and therefore, 1.144×10^{7} copies/µg of DNA was resulted

6.3.9. Presence of CcPV and CmPV in loggerhead turtles

Using the specific primer sets for loggerhead papillomavirus (Cc-Pap-99 PCR primers), CcPV was detected in tumour samples of loggerhead turtles while the negative controls did not produce any bands (figure 6-14).

The loggerhead and green samples were tested using a general PCR primer set to detect turtle papillomaviruses (Turt-Pap-218 primers), all green and loggerhead samples reacted in the PCR however did not produce the expected 218bp products (figure 6-15), the loggerhead tumour tissue samples amplified larger products in size compared to the green turtles. The tumour tissues of loggerhead samples did not react in CmPV-specific PCR assays and the green samples did not react in CcPV-specific PCRs. Both loggerhead and green turtle samples reacted for ChHV5 (data not shown here).

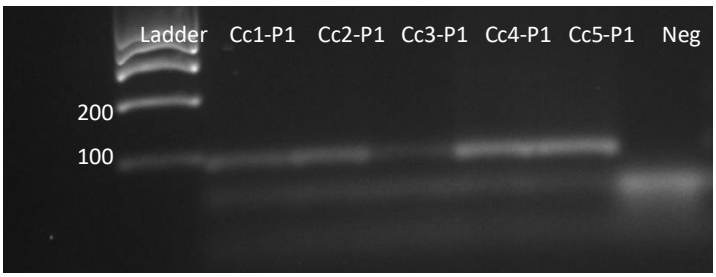


Figure 6-14 The agarose gel of tumour tissues from loggerhead turtles (Cc) tested by Cc-Pap-99 primer (P1). Lane one contains the ladder Cc1 to Cc5 represent the loggerhead samples which reacted in PCR and produced an approximate 99bp size band. The negative control is negative.

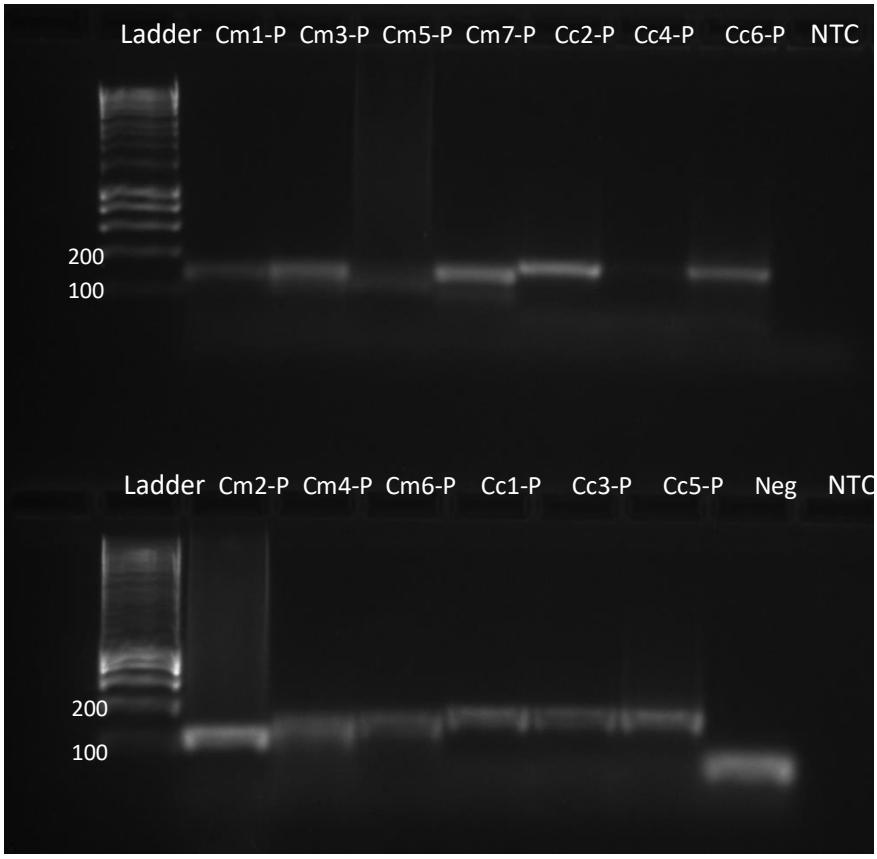


Figure 6-15 The agarose gel of tumour tissues from loggerhead (Cc) turtles and putative CmPV-positive samples of green (Cm) tested by Turt-Pap-218 PCR primers (P). Lane one contains the ladder; Cm and Cc samples are distributed unevenly to be able to compare any differences in band size. The negative control is negative along with Cc4-P. The bands are smaller than expected size (218bp).

6.4. Discussion

Although ChHV5 is turtles is closely associated with FP, other factors such as pollution and water temperature considered to trigger or contribute to the development of clinical disease (de Deus Santos *et al.,* 2015) which makes epidemiological studies challenging. The phylogeny and frequency of ChHV5 has been studied in different regions (i.e. Northern and Southern Pacific, Western Africa, Mediterranean Sea) and for various sea turtle hosts (i.e. green, loggerhead and hawksbill turtles) (Quanckenbush *et al.,* 2001; Greenblatt *et al.,* 2005; Page-Karjian *et al.,* 2015; Alfaro-Núñez *et al.,* 2016). The virus ChHV5 is present in FP tumours, in normal tissue of green turtles with FP lesions and in asymptomatic turtles. The latter groups in some cases harbour higher viral loads despite their health status and this is potentially due to a different stage of the disease process or the presence of a latent viral infection (Quanckenbush *et al.,* 2001; Greenblatt *et al.,* 2005; Page-Karjian *et al.,* 2015; Alfaro-Núñez *et al.,* 2016). Alfaro-Núñez *et al.,* (2016) suggested a near ubiquitous characteristic for ChHV5 and proposed the need for a co-factor to trigger the gross signs of the disease. In line with this theory,

Lawrancea *et al.* (2018) indicated that the presence of ChHV5 infection and the genetic divergence between viral lineages are not enough to explain disease development. Additionally, the Australian ChHV5 is examined mainly in terms of the genetic variations within the sea turtle populations (Ariel *et al.,* 2017) and the viral quantity of ChHV5 has only been evaluated in two Australian samples by Quanckenbush *et al.* (2001). The FP tumour samples were compared with FP samples from Hawaii, Florida, Barbados and Costa Rica, where the viral loads of Australian and Costa Rican samples were found to be lower than Barbadian, Hawaiian and Floridian samples (Quanckenbush *et al.,* 2001).

Two neoplastic disease-causing viruses were examined and proposed to have a potential role in the disease: retroviruses (Casey *et al.,* 1997) and papillomaviruses (Lu *et al.,* 2000). However, these intermittent studies were discontinued and the major focus remained on ChHV5. In early 2018, we published findings of the presence of CmPVs in eight green turtles with FP skin tumours explained in Chapter four (Mashkour *et al.,* 2018). There was a need for surveying a higher number of green turtles with FP tumours to assess the presence, prevalence and viral load of CmPV in these samples - preferably whilst evaluating the status of ChHV5.

This study is the first report of a herpes-papillomavirus concurrent infection in FP tumour tissues of green turtles. However, co-infections with herpes and papillomaviruses in immunodeficient humans or individuals with cervical diseases have been previously reported (Akbari and Elmi, 2017; Zhao *et al.,* 2012). Concurrent infections with these two viruses has also been reported in animals such as bottle nose dolphins (Cruz *et al.,* 2014). In this survey, ChHV5 and CmPV were both present in 43.51% of skin tumour tissues collected from 89 green turtles and in 8.33% of normal skin tissue from 36 green turtles with FP lesions. Both viruses were also present in 46.8% of 47 asymptomatic green turtles. The presence of herpes and papillomaviruses in normal skin tissue of animals with clinical disease and asymptomatic animals are consistent with previous studies on these viruses (Kullander *et al.,* 2013; Page-Karjian *et al.,* 2015; Alfaro-Núñez *et al.,* 2016) and are in line with the ubiquity and commensal nature of both viruses (Antosson *et al.,* 2000; Alfaro-Núñez *et al.,* 2016).

The viral loads of the two viruses in tumour tissues versus normal skin tissues were investigated. However, the comparison between the viral loads of these two viruses may not be meaningful and there might be a need to interpret the results for each virus independently. The reason being, when investigating herpesviruses, using herpes simplex virus (HSV) as an example, transmission is likely happening in the presence of high viral loads (more than 10^4 copies of DNA per cell) (Schiffer *et al.,* 2014) and the efficiency of viral reactivation is also affected by viral copy number (Sawtell *et al.,* 1998). Whereas in HPV-associated cervical carcinomas, the viral load of the genital HPVs is at least 1 viral genome per cell (Kullander *et al.,* 2008). Greater than 1000 copies per cell is considered a high viral

load for cervical cancer development and a risk factor for cervical intraepithelial neoplasia (Schlecht *et al.,* 2003). The copy number per cell is even lower in cutaneous HPVs; typically significantly lower amounts (1 copy/100 cells or less), are found in both cutaneous tumours and normal skin tissue of infected individuals (Kullander *et al.,* 2008). In addition, Forslund *et al.,* (2004) reported that the viral copy number of HPVs is higher in superficial layers of lesions versus tumour biopsies (Forslund *et al.,* 2004), thus wiping the skin would reduce the viral load. Wiping the tissue area before taking the sample was the protocol for this study (4.2.1. Animals and sampling protocols and 6.2.1. Animals and sample collection) and may have contributed to low copy numbers found in some samples.

The herpes viral loads in 131 tumour tissues was in a range from 0 to 226.91 viral genomes per cell (mean: 13.486; SD: 28.50). The copy number of ChHV5 per cell in Australian green turtles is compatible with previous studies on viral burdens in FP tumours from other regions (Greenblatt *et al.,* 2005; Page-Karjian *et al.,* 2015; Alfaro-Núñez *et al.,* 2016). The mean of the ChHV5 viral load in this study (13.486 copies/cell) is less than the load found in East Pacific samples (table 6-5) which is consistent with the study of Quackenbush in 2001 (Quackenbush *et al.,* 2001; Greenblatt *et al.,* 2005; Page-Karjian *et al.,* 2015; Alfaro-Núñez *et al.,* 2016). The ChHV5 viral loads in tumour tissues were significantly higher than in normal skin from diseased green turtles ($p=0.0001$), which is in line with previous studies (Greenblatt *et al.,* 2005; Page-Karjian *et al.,* 2015).

The papillomaviral load was from 0 to 2.54 (mean: 0.0392 (≈1 virus in 25 cells); SD: 0.249), approximately 4164 copies/µL of DNA extract from tumour tissues, which seems quite normal as described for cutaneous papillomaviruses where less than 1 viral DNA per 1000 host cells has been reported in cutaneous lesions in immunosuppressed humans and in non-melanoma skin cancers (Forslund *et al.,* 2003; Kullander *et al.,* 2008). There is no significant difference between CmPV viral loads in tumour tissues and normal skin samples of green turtles with FP lesions ($P=0.274$), however, the viral loads of the normal skins from the same animals were higher than tumour tissues. Such complexities were also reported for ChHV5. Clinically healthy marine turtles from distinct sites showed higher loads of ChHV5 when compared to FP-afflicted turtles (Alfaro-Núñez *et al.,* 2016). The heterogeneity of papilloma viral loads in abnormal skin growths has challenged other researchers as well (Forslund *et al.,* 2003; Hazard *et al.,* 2006; Kullander *et al.,* 2013). Analysing microdissected tissues to localise the viral DNA was suggested to acquire a more precise quantification (Forslund *et al.,* 2003), and also to consider the heterogeneity in studying the biology of the virus (Hazard *et al.,* 2006; Kullander *et al.,* 2013).

Papillomavirus may have a role as a cofactor in triggering fibropapillomatosis or tumour formation. Drawing a robust conclusion about the association between CmPV presence and FP needs more analysis in different regions and on different species of sea turtles.

Where possible, multiple tumours were analysed from one turtle. The results were inconsistent between individual turtles. However, the results showed that if tumour tissue from a turtle is not reacting in a screening test for the presence of CmPV and ChHV5, it is not proof that the rest of the turtle's body is free of the virus investigated. There is no significant correlation between the viral loads and the CCL measurement (as an indicator of the size and possibly the age of the turtle).

The loggerhead samples were positive for CcPV and ChHV5 and negative for CmPV. Green turtle samples which were positive for CmPV, were negative for CcPV (data not shown here). CmPV and CcPV appear to be species specific, however, further analyses on these samples and possibly more loggerhead samples are required, but were not within the scope of this project.

The two ubiquitous viruses, ChHV5 and CmPV, were both present in approximately half of the tumour tissues. The disease stage and severity of FP tumours in these green turtles were not recorded during sampling and it is therefore not possible to relate presence and viral load to disease severity or stage. Both viruses were found in all regions sampled; considering the potential risk for infection and transmission, further analysis on more tumour tissues, species and regions is required. In future studies involving screening for papillomaviruses of sea turtles, care must be taken to avoid wiping the skin and to also sample superficial layers of skin for improved detection.

How I achieved the aim of the chapter:

1. I amplified regions of *C. mydas* papillomavirus (CmPV), chelonid α-herpes virus 5 (ChHV5) and green turtle Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using PCR assays
2. I successfully cloned the three amplicons of CmPV, ChHV5 and GAPDH in pGEM®-T Easy Vectors
3. I linearising the plasmids using restriction enzymes as follows:
   - Bioinformatical analysis and selection of SacI and PstI
   - Treatment with enzymes
4. I developed probe-based PCR assays to specifically detect the target regions in standards, positive controls and unknown samples
5. I built calibration curves and standards for the three surveys via
   - Quantifying the dsDNA concentrations
   - Carrying out the calculation to find the mass of DNA
   - Preparing plasmid dilutions
   - Performing probe based PCR assays
6. I performing statistical analysis to be able to report the viral loads per cells of each virus and meaningfully compare them
7. I developed a PCR assay to screen the loggerhead samples for the presence of *C. mydas* papillomavirus and *Caretta caretta* papillomavirus

7. GENERAL DISCUSSION

Researchers have identified various risks and threats to marine turtles survival including: fishery bycatch, unsustainable take, habitat degradation, pollution and pathogens, and the controversial subject of climate change (Donlan *et al.,* 2010; Wallace *et al.,* 2011). All in all, the ultimate consequence of such threats is a decline in sea turtle populations and depletion of the genetic diversity (Wallace *et al.,* 2011). The Great Barrier Reef (GBR), the world's largest coral reef, is home to six of the seven species of sea turtles: Green, Loggerhead, Hawksbill, Flatback, Olive ridley, and Leatherback turtle (GBRMPA, 2018). The International Union for Conservation of Nature (IUCN) has listed five of those on the IUCN Red List of Threatened Species while the flatback turtle (*Natator depressus*), is reported as "Data Deficient" (IUCN, 2015).

Governmental and non-governmental groups are working on marine turtle conservation around the world, trying to create frameworks to prioritise conservation plans (Wallace *et al.,* 2011). It is generally accepted that infectious diseases can adversely affect local populations and reduce genetic diversities (Smith *et al.,* 2009; Wiethoelter *et al.,* 2015), and for an already endangered species such as sea turtles, emerging infectious diseases are a concern. To address this issue the disease drivers and contribution to other threats should be defined (Smith *et al.,* 2009; Smith *et al.,* 2006). Diseases are likely contributing to conservation challenges for marine turtles (Daszak *et al.,* 2000), although disease risk assessments are affected by low commercial importance, funding policies and the complexities of studying a migratory animal (Daszak *et al.,* 2000; Jensen, 2010). To address this, a comprehensive disease risk analysis was done to review sea turtle population "*health and disease determinants*" in one practical approach and to create an informed risk assessment for sea turtles. The DRA was carried out based on the published DRAs for other wildlife (Jackob-Hof *et al.,* 2014) and in collaboration with

veterinarians, researchers and care takers from various disciplines around the globe. As a result, the health hazards for sea turtles were defined and collated into infectious and non-infectious hazards. The risks of these hazards were assessed and a possible management plan was suggested for the highest ranked hazards, or in other words, the hazards estimated to have the highest conservation impact.

The disease risk assessment was done via a literature based review and also benefitted from opinions of the experts in the field by organising two international workshops and interviewing experts individually.

The major outcomes of the DRA were:

1. Immunosuppressed turtles are more prone to different diseases (Dobbs, 2001; Work *et al*., 2004; Alfaro *et al.,* 2008). There is a possible link between environmental pollution and immunosuppression and this in turn is linked to anthropogenic effects, however there is a need for more research into the impacts and to provide supportive information about causes and treatments. This requires multi-skilled tasks force to address anthropogenic impacts on environmental and turtle health.

2. The assessment step emphasises the data deficiencies in several aspects of sea turtle health investigation and conservation:

- The general health components are not easy to measure and there is a lack of specific standards to tell a sick and healthy turtle apart. More research is needed in this aspect to build on the published findings. Health surveillances during a long lifespan and including a large number of the sea turtles would help achieving this aim, an example can be the work by Flint *et al*. (2010)
- The link between presence of pathogens and disease aetiology is not clear in most cases (Manire *et al.,* 2017). The studies on infectious diseases of sea turtles can benefit from international collaborations, funding and advances in methodologies such as immunohistochemistry and qPCR.
- There are a limited number of social science studies on sea turtle conservation and general interaction with sea turtles, although sea turtles are an iconic part of marine ecotourism, and ecotourism has shown positive effects on human health (MacKenzie, 2004) and in spite of the importance of these species for indigenous communities (GBRMPA, 2018). Furthermore, social sciences are important to understand drivers and inhibitors in conservation and management initiatives.

3. Viruses were ranked as the least studied health hazards of sea turtles. In other words, higher numbers of parasites, bacteria and fungi have been detected, isolated and reported in sea turtles, while, to date, only five families of viruses are known to infect sea turtles. Bearing in mind that the debilitating disease fibropapillomatosis is believed to be caused by viruses (Jones *et al*., 2016), there is a need to further analyse sea turtles for presence and impacts of viruses.

In this project, a review of the literature showed that viruses of sea turtles have only been partially studied due to lack of suitable methods for viral diagnosis in these animals. Five families of viruses are described in sea turtles: herpesviridae, retroviridae, unclassified tornovirus, papillomaviridae and nodaviridae, however the studies are sporadic and the link to pathogenesis and disease manifestation for most of these viruses is not fully understood.

To address this particular gap we embarked on developing specific tools for viral diseases in sea turtles. Nine green turtle primary cell lines were successfully established and characterised for viral isolation. Seven days after inoculating the primary cultures with skin tumour homogenates, we observed cytopathic effects which were confirmed by molecular analysis to be caused by papillomavirus. However viral characterisation beyond this point was not applicable in cell culture. We then designed specific diagnostic qPCR primers and probes and identified papillomavirus directly in skin tumour tissues for the first time, and as explained before these tumours were only linked to ChHV5 before this.

The *C. mydas* papillomavirus was subsequently characterised through more PCR assays and sequencing. Further characterisation of the papillomavirus isolates through molecular analysis and sequencing revealed that these Australian papillomaviruses are closely related to CmPV-1 and CcPV-1, the green and loggerhead turtles papillomaviruses described in 2008 and 2009 (Manire *et al.,* 2008; Herbst *et al.,* 2009). The L1, partial E2 and long control region (LCR) of Australian CmPV isolates are 100% similar to CmPV-1. The Nuclear Localisation System (NLS) of the eight CmPV isolates are 100% similar to CmPV-1 and the (DNA binding domains (DBDs) are 99% similar. The only differences are two SNPs in one codon of DBD that is kept in this well-conserved gene, as both CTC and AUG codes translate for Leucine, substitution is synonymous.

Partial characterisation of the Australian CmPV isolates suggested that these isolates are likely to be from the same papilloma-viral type as CmPV-1 and from the same genus as CcPV-1 based on E1 and L1 sequences. Papillomavirus strains are considered to be from the same type if there is less than 10% divergence between them (Lange *et al.,* 2011; Chen *et al.,* 2015; Doorbar *et al.,* 2015) and from the same species if there is 60 to 70% similarity between the two (Chen *et al.,* 2015). *C. mydas* papillomavirus appears not to be affected by geographic distribution of the host. However from two

distinct regions the papillomavirus typing gene (L1) is identical between all the sequenced isolates. Herbst *et al*., suggested a low rate of evolution for non-mammalian PVs due to lower rates of metabolism (Herbst *et al*., 2009). Such interactions with the hosts may suggest a vertical viral transfer for CmPV. The similarities between green and loggerhead turtles papillomaviruses are enough to classify them in one species of PVs, however these viruses are still highly host-specific.

Rolling circle amplification (RCA) was not successful in amplifying the viral genome of these 8 tumour derived papillomaviruses. Integration or the very low copy number of episomal circular DNA in sample vials may be the reason behind the failure to amplify CmPV isolates via RCA. Such a problem was reported previously in amplifying human papillomavirus 33 (HPV-33) (de Oliveira *et al.,* 2017) which is known to have a low copy number of episomal viral load when found in tumours (Khouadri *et al.,* 2007). Future plans for sequencing the full genome of the Australian CmPV isolates, requires acquiring suitable samples harbouring higher copies of circular-episomal virus for RCA and following up with next generation sequencing. The cell culture supernatant was not used for this trial as papillomaviruses are normally grown in three-dimensional culture and the qPCR results showed that the titer did not increase significantly. The assumption was that the cell culture CPE and the vigorous growths of the cells were happening due to presence of E6 and E7 protein in the culture which was reported previously in the literature (Halbert *et al.,* 1991; DeFilippis *et al.,* 2003). In the RCA trial (section 5.2.4), 1 µL of each sample was used, which contained approximately 2000 copies of CmPV on average (Chapter six). The initial copy number of HPV used in successful RCAs is relatively similar: 100 copies of HPV-16 genome for example (Rector *et al.,* 2004), thus low copy number of the initial template was not an issue in RCA. But assuming the low copy number of some samples had interfered with the experiment, new samples from the viral load survey which harboured higher copy numbers (ie 6.58×10^4 and 8.86×10^3) and were collected from normal skin that might contain episomal viruses, will be used in the future upon approval of the sample providers.

A molecular survey of 131 skin tumour tissues and 36 normal skin tissues of 89 green turtle along with 47 asymptomatic green turtles was done to detect the presence of CmPV and ChHV5. These quantification assays led to several findings, one of which was a concurrent infection of CmPV and ChHV5 in approximately 50% of tumour tissues and skin from turtles without lesion. When possible, multiple tumours were analysed from one turtle. The inconsistent results implied that if a tumour tissue from a turtle is not reacting in a screening test for presence of CmPV and ChHV5, it is not an indication for that turtle to be free of the investigated viruses and multiple tumours should be tested per turtle.

This study was the first investigation of ChHV5-viral loads of FP-afflicted and healthy Australian green turtles done in Australia. It was also the first survey on CmPV-viral loads in these tumour tissues and normal skin samples. The viral loads of ChHV5 were consistent with previous surveys done in other countries indicating the presence of virus in tumour tissues along with tumour free tissues and even asymptomatic animals. (Quanckenbush *et al.,* 2001; Greenblatt *et al.,* 2005; Page-Karjian *et al.,* 2015; Alfaro-Núñez *et al.,* 2016). This characteristic suggests a near ubiquitous nature for the virus which is also seen for CmPVs. The viral load of ChHV5 in Australian samples is less than East Pacific turtle tumour samples (Quanckenbush *et al.,* 2001; Page-Karjian *et al.,* 2015; Alfaro-Núñez *et al.,* 2016).

The herpes-viral copynumber per cell was higher than the papilloma-viral load. The CmPV-viral loads were in line with cutaneous lesions in immunosuppressed human and in non-melanoma skin cancers (Forslund *et al.,* 2003; Kullander *et al.,* 2008). Previous studies on papillomaviruses found more virus in superficial layers of biopsied skins and wiping the skin reduced the viral loads. Unfortunately, wiping of the skin was a part of the sampling protocol in this study. The CmPV-viral load was lower in tumour tissues when compared to healthy skin from the same animal, though the difference was not significant. Such findings were also reported for ChHV5-viral loads in other regions (Alfaro-Núñez *et al.,* 2016). The severities of FP tumours in sampled green turtles were not recorded, so the disease stage is not known and there are no previous reports of CmPV viral loads to compare these results to, therefore drawing a conclusion about a possible correlation between presence of CmPV and FP stage appears premature. The eight CmPV positive turtles that were characterised in Chapter four and five of this thesis were also included in the viral load study (Chapter six). All of the eight animals were positive for both viruses while the normal skin samples were negative as reported in Chapter four.

Loggerhead samples were positive for CcPV and ChHV5 and negative for CmPV. The papillomaviruses of these two sea turtles appear to be species specific. In this study, no CcPV was found in any green turtle tested and also the two loggerhead turtles were negative for CmPV. While ChHV5 is showing an association to geographic distribution of the host, CmPV as mentioned before, is host specific and is not showing any differences in different locations. Further analysis on these samples will be done in future projects to characterise the viruses.

In this project, methods were established and successfully used to discover papillomaviruses in green and loggerhead turtles and the detected CmPV were partially characterised through molecular analysis. The virus is present in several regions and in diseased and healthy green turtles as expected from commensal papillomaviruses. Future studies need to include samples from more animals and regions to clarify any association between presence of the virus and diseases of green or other sea turtles.

Sea turtles are ancient and long lived inhabitants of oceans worldwide. They serve as health indicators for the environment, but their health is not well assessed. Conservation plans have accepted infectious disease as a threat to endangered species, but the uncertainty surrounding disease aetiology still remains. Therefore, regional management units have been encouraged to rectify data deficiencies around turtle infectious diseases. Sea turtle virology is developing and is forcasted to benefit from future advances in methodology. Multidisciplinary research is encouraged to promote the collective knowledge on turtle health assessment and disease origin. And hopefully, we will be able to minimise the threats to these ancient species.

7.1. THE PROJECT ACHIEVEMENTS ACCORDING TO GENERAL AIMS AND FUTURE DIRECTIONS

1. To do a structured Sea Turtle Disease Risk Analysis (DRA) in collaboration with the experts in the field (Chapter three)

The DRA steps were carried out according to published guidelines. Two international expert workshops were facilitated and veterinarians, sea turtle health researchers and care takers were consulted. Current knowledge and details pertaining to the health and diseases of sea turtles were collated and analysed and the DRA for sea turtles will be published in an open access journal.

2. To develop and assess virological methods to study the presence and impacts of viruses in green turtles from the northern Great Barrier Reef (Chapter four)

Cell culture establishment and molecular methods development were successful and instrumental in finding a new virus in the investigated region: *Chelonia mydas* papillomavirus. The results were published in the journal of virological methods. The established methods will be used in the future to further analyse the samples for the presence of other viruses such as iridovirus and adenovirus.

3. To characterise the Australian *Chelonia mydas* papillomavirus isolates (Chapter five)

The Australian *Chelonia mydas* papillomavirus isolates from chapter four were partially characterised using the designed primers. The Australian isolates were therefore compared to CmPV-1 and CcPV-1. Suitable samples (with higher viral loads based on chapter six) were determined for rolling circle amplification (RCA) and next generation sequencing (NGS) and full genome sequencing. Primer walking and designing overlapping primers is an alternative option if RCA is not successful, because the virus is small and non-segmented.

4. To determine the level of co-infection of ChHV5 and CmPV in fibropapillomas and normal skin biopsies of green turtles from the northern Great Barrier Reef (Chapter six)

The first molecular survey on presence and viral loads of Australain *Chelonia mydas* papillomaviruses was done in this project along with the first survey on the viral loads of Australian chelonid herpesvirus 5. The results indicated that CmPV may be a cofactor in the development of clinical fibropapillomatosis in green turtles, however future studies on more geographical regions and species are required to provide a robust conclusion.

8. References

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9. APPENDICES

Appendix 1. The bacterial pathogens of sea turtles

1.1. Gram negative bacteria

Disease hazard	Region reported	presence in sea turtle		Outcome of infection (lesion, clinical sign and/or disease) sign in individuals; ease of spread, rate of spread; is a test, quarantine, treatment available to detect the disease?	zoonotic/transmissible to companion animals	Correlation with climatic/anthropogenic events	Key reference
		Captive populations	Wild populations				
Achromobacter spp	Canary Island		Loggerhead (*Caretta caretta*)	opportunistic pathogens Heterophilic scleritis	Can be found in aquatic species		(Orós *et al.*, 2005)
Acinetobacter anittratus	Florida	*	cloacal and nasopharyngeal swabs of green turtle (*Chelonia mydas*) with and without FP	opportunistic and able to infect tissues damaged by trauma ulcerative dermatitis and rhinitis and stomatitis, shell disease, bronchopneumonia in captivity		anthropogenic effects can cause trauma and trigger infection	(Aguirre, 1992; Aguirre *et al.*, 1994)
Acinetobacter lwoffi			cloacal swabs of Green turtle (*Chelonia mydas*) with and without FP	A low frequency isolation in the survey			(Aguirre *et al.*, 1994)
Acinetobacter calcoaceticus	Northern Australia	Loggerhead (*Caretta caretta*)	Hatchling, juvenile and adults Green turtle (*Chelonia mydas*),	Oral lesions and conjunctivitis ulcerative stomatitis, obstructive rhinitis-pneumonia	Evidences of conjunctivitis in		(Campbell and Glazebrook, 1990;

			Loggerhead (*Caretta caretta*), Hawksbill turtle (*Eretmochelys imbricate*)	Detection: clinical signs and microbial culture of caseous lesions	human, potentially zoonotic		Glazebrook *et al.,*1993; Glazebrook and Campbell, 1990)
Aeromonas hydrophilia	Spain, Hawaii, Australia	Green turtle (*Chelonia mydas*) Loggerhead (*Caretta caretta*)	Hatchling, juvenile and adults Green turtle (*Chelonia mydas*), Loggerhead (*Caretta caretta*), Hawksbill turtle (*Eretmochelys imbricate*), Leatherback turtle (*Dermochelys coriacea*); Hawksbill turtle (*Eretmochelys imbricate*)	Traumatic ulcerative skin lesions (align with *Vibrio alginolyticus* and *Staphylococcus* spp) ulcerative stomatitis, obstructive rhinitis-pneumonia complex and fibropapillomatosis 9 cases of salt gland infection and mortality in Loggerhead (*Caretta caretta*) (Along with *Staphylococcus* spp., and *Vibrio alginolyticus* (Orós *et al.,* 2011a)) Detection: clinical signs and microbial culture of caseous lesions	Potentially zoonotic	Boat strike, by catching fishing line	(Buller, 2014; Campbell and Glazebrook, 1990; Glazebrook *et al.,* 1993; Glazebrook and Campbell, 1990; Orós *et al.,* 2011a; Work *et al.,* 2003)
Aeromonas sorbia	Hawaii		Green turtle (*Chelonia mydas*)	Found in blood samples, no pathogenicity determined Low frequency isolation in the survey			(Work *et al.,* 2003)
Aeromonas popoffii	Hawaii		Green turtle (*Chelonia mydas*)	Found in blood samples, no pathogenicity determined Low frequency isolation in the survey			(Work *et al.,* 2003)
Aeromonas caviae	Hawaii		Green turtle (*Chelonia mydas*)	Found in blood samples, no pathogenicity determined Low frequency isolation in the survey			(Work *et al.,* 2003)
Alcaligenes faecalis	Jekyll Island, Georgia, USA		Loggerhead (*Caretta caretta*)	High frequent in failed hatching			(Awong-Taylor *et al.,* 2008)
Aureobacterium spp.	Thailand	Green turtle (*Chelonia mydas*) *Eretmochelys imbricata*	Green turtle (*Chelonia mydas*)	Isolated from oral cavity and liver in captivity Can be from normal skin flora without pathogenicity			(Chuen-Im *et al.,* 2010)

Burkholderia spp. And B. cepacia	Hawaii; Canary Island		Loggerhead (*Caretta caretta*)	heterophilic blepharitis, heterophilic stomatitis and obstructive rhinitis Low frequency isolation in the study (Work *et al.,* 2003)			(Orós *et al.,* 2005)
Citrobacter spp.	Oman sea and Persian Gulf, Florida	*	Black Sea Turtles (*Chelonia mydas agassizi*)	opportunistic and able to infect tissues damaged by trauma ulcerative dermatitis and rhinitis and stomatitis, shell disease, bronchopneumonia in captivity Egg contaminant		anthropogenic effects can cause trauma and trigger infection	(Aguirre, 1992; Chinnadurai and Devoe, 2009)
Citrobacter diversus	Hawaii		swabs of healthy Green turtle (*Chelonia mydas*)	Isolated in 1 sample out of 22 along with *E. coli*			(Aguirre *et al.,* 1994)
Citrobacter freundii	East Caribbean sea, central Mediterranean Sea, Florida	*	Green turtle (*Chelonia mydas*) with spirorchid and with and without FP; Leatherback turtle (*Dermochelys coriacea*); Loggerhead (*Caretta caretta*) nesting beaches of olive ridley turtle (*Lepidochelys olivacea*) and green turtle (*Chelonia mydas*)	Opportunistic pathogen. may correlate to hatching failure Cutaneous ulceration which can be septicemic, sloughing skin, Resistant to ampicillin and Shows 17% to 100% resistance to antibiotic tested by Foti *et al*.	Can infect many species can be a risk to egg consumers		(Aguirre, 1992; Buller, 2014; Foti *et al.,* 2008; Foti *et al.,* 2009; Keene *et al.,* 2014; Raidal *et al.,* 1998)
Citrobacter youngae	Green turtle (*Chelonia mydas*) cloaca and the mouth of aquatic chelonians (*Podocnemis expansa and P. unifilis*)		egg surface of Leatherback turtle (*Dermochelys coriacea*)	may correlate with hatching failure Resistant to ampicillin and amoxicillin with clavulanic acid			(Fichi *et al.,* 2016; Zieger, Trelease, Winkler, Mathew, & Sharma, 2009)

Citrobacter brakii	central Mediterranean Sea		Loggerhead (*Caretta caretta*)	A low frequent isolation with *Citrobacter freundii*			(Foti; *et al.,* 2008)
Enterobacteriacae							
Edwardsiella spp.	Oman sea Persian Gulf; Thailand Northwestern Mexico	Green turtle (*Chelonia mydas*) *Eretmochelys imbricata*	green turtle (*Chelonia mydas*) in nesting beaches Black sea turtle (*Chelonia mydas agassizii*)	Potentially pathogenic and opportunistic in sick sea turtles Can be from normal skin flora without pathogenicity collected in oviductal samples in nesting beaches *Edwardsiella tarta* is resistant to Streptomycin			(Al-Bahry *et al.,* 2012; Raidal *et al.,* 1998)
Enterobacter aerogenes		Leatherback turtle (*Dermochelys coriacea*)	Cloacal swabs of Green turtle (*Chelonia mydas*) with and without FP Leatherback turtle (*Dermochelys coriacea*) (dead in net)	Found in samples, no pathogenicity determined			(Aguirre *et al.,* 1994)
Enterobacter agglomerans			from cloacal and nasopharyngeal swabs of healthy Green turtle (*Chelonia mydas*)	Found in nasal and cloacal samples, no pathogenicity determined			(Aguirre *et al.,* 1994)
Enterobacter cloacae	East Caribbean Sea	Leatherback turtle (*Dermochelys coriacea*)	from cloacal and nasopharyngeal swabs of Green turtle (*Chelonia mydas*) with and without FP Loggerhead (*Caretta caretta*) Leatherback turtle (*Dermochelys coriacea*) (dead in net)	Opportunistic pathogen. may correlate to hatching failure resistant to ampicillin and Amoxicillin with clavulanic acid	can be a risk to egg consumers		(Fichi *et al.,* 2016)
Escherichia coli	Central Mediterranean Sea, Oman	Green turtle (*Chelonia mydas*)	Loggerhead (*Caretta caretta*)	Coliforms resistant to Tetracycline	Can infect many species	high	(Foti; *et al.,* 2008; George,

	sea and Persian Gulf	(Campbell & Glazebrook, 1990)	swabs of healthy Green turtle (*Chelonia mydas*) Leatherback turtle (*Dermochelys coriacea*)		zoonotic	temperature may increase the number of E. coli	1997; Orós *et al.,* 2005)
Hafnia alvei	Florida, Hawaii		coacal swabs of FP Green turtle (*Chelonia mydas*)	has been isolated from clinical specimens of animals but the information about pathogenicity is not enough	commensals of terrestrial reptiles also can infect fish can be a risk to human		(Aguirre, 1992; Aguirre *et al.,* 1994)
Klebsiella oxytoca	has been reported in nesting beaches, Florida		from cloacal swabs of FP Green turtle (*Chelonia mydas*)	Pathogenicity not determined. Can be from normal flora without pathogenicity			(Aguirre, 1992; Buller, 2014)
Klebsiella pneumoniae		hatchling and juvenile green turtle (*Chelonia mydas*) and Loggerhead (*Caretta caretta*)	hatchling and juvenile green turtle (*Chelonia mydas*) and Loggerhead (*Caretta caretta*)	correlated with lower hatching success in natural and relocated nests of Loggerhead (*Caretta caretta*)s along with other *Enterobacteriaceae* associated with ulcerative stomatitis in captive hatchling and juvenile green turtle (*Chelonia mydas*) and Loggerhead (*Caretta caretta*)	Opportunistic and can infect Many species		(Santoro *et al.,* 2006b; Wyneken *et al.,* 1988)
Morganella morganii	East Caribbean sea, Oman sea and Persian Gulf	Leatherback turtle (*Dermochelys coriacea*)	cloacal swabs of healthy Green turtle (*Chelonia mydas*)	Opportunistic pathogens. may correlate to hatching failure resistant to ampicillin and Amoxicillin with clavulanic acid	can be a risk to egg consumers		(Zieger *et al.,* 2009)
Plesiomonas	Northwestern Mexico		Black sea turtle (*Chelonia mydas agassizii*)	Potentially pathogenic and opportunistic in sick sea turtles			(Zavala-Norzagaray *et al.,* 2015)
Providencia (Proteus) spp.	nesting beaches of Oman sea and Persian Gulf, Florida, Canary Island		Green turtle (*Chelonia mydas*) Black sea turtle (*Chelonia mydas agassizii*)	Can be from normal skin flora without pathogenicity: Costa Rica most frequently isolated from lesions in Canary Island and indicated as one	Many reptile species	May be due to pollution in the habitat	(Aguirre, 1992; Al-Bahry *et al.,* 2012; Orós *et al.,* 2005)

				of the causes of the diseases and mortality can be egg contaminant maybe resistant to ampicillin, streptomycin and tetracycline			
Proteus mirabilis	Costa Rica Ionian Sea; Sicilian Channel, South Tyrrhenian Sea		Loggerhead (*Caretta caretta*); Green turtle (*Chelonia mydas*)	Can be from normal skin flora without pathogenicity: Costa Rica			(Foti *et al.,* 2009; Santoro *et al.,* 2006b)
Proteus penneri	Georgia, USA		Loggerhead (*Caretta caretta*)	Hatching failure in Jekyll Island Georgia, USA			(Awong-Taylor *et al.,* 2008)
Proteus vulgaris	nesting beaches of Oman sea and Persian Gulf; Costa Rica; Canary Islands; Ionian Sea; Sicilian Channel, South Tyrrhenian Sea		Clinically healthy Green turtle (*Chelonia mydas*) Loggerhead (*Caretta caretta*)	Indicated as non-pathogenic constituent of microflora in costa Rica Reported to relate with Loggerhead (*Caretta caretta*) hatching failure in Jekyll Island, Georgia, USA Shows 17% to 100% resistance to antibiotic tested by Foti *et al*. (Foti *et al.,* 2009)		May be due to pollution in the habitat	(Al-Bahry *et al.,* 2012; Awong-Taylor *et al.,* 2008; Foti *et al.,* 2009; Santoro *et al.,* 2006b)
Providencia rettgeri	Ionian Sea; Sicilian Channel, South Tyrrhenian Sea		buccal cavity and cloacae of Loggerhead (*Caretta caretta*)	Shows 62.5% to 94.1% resistance to antibiotic tested by Foti *et al*. : carbenicillin, cephalothin, oxytetracycline and amoxicillin			(Foti *et al.,* 2009)
Shigella	Oman sea and Persian Gulf		green turtle (*Chelonia mydas*) in nesting beaches	collected in oviductal samples no pathogenicity was resistant to ampicillin and maybe tetracycline			(Al-Bahry *et al.,* 2012)

Flavobacterium spp		Green turtle (*Chelonia mydas*), Loggerhead (*Caretta caretta*), E. imbricate	Hatchlings, juvenile and adults Green turtle (*Chelonia mydas*), Loggerhead (*Caretta caretta*), Hawksbill turtle (*Eretmochelys imbricate*)	ulcerative stomatitis, obstructive rhinitis-pneumonia complex and fibropapillomatosis The frequent bacteria in keratoconjunctivitis, ulcerative blepharitis, Salt- secreting gland infection, Peritonitis along with *Pseudomonas* spp. Detection: clinical signs and microbial culture of caseous lesions	Potentially zoonotic		(Campbell and Glazebrook, 1990; Glazebrook *et al.,* 1993; Glazebrook and Campbell, 1990)
Leptospira interrogans	Baja California		Green turtle (*Chelonia mydas*)	Leptospirosis Detection: culture	zoonosis and can infect many species; sea turtles may act as reservoirs of some serotypes		(Aguirre *et al.,* 2006)
Moraxella spp	Western Australia		Green turtle (*Chelonia mydas*)	Isolated from liver, lung and kidney along with mycotic infections	opportunistic environmental pathogens		(Raidal *et al.,* 1998)
Pasteurella	Canary Island		Loggerhead (*Caretta caretta*), Green turtle (*Chelonia mydas*) and Leatherback turtle (*Dermochelys coriacea*)	Pneumonia, gastrointestinal opportunistic In mixed infections causing catarrhal, fibrinous, necropurulent and necrotising enteritis Egg contaminant	Can infect many species Potentially zoonotic		(Orós *et al.,* 2004a)
*Photobacterium damselae (*subspecies *damselae)*	Tasmania, Australia; Toscany, Italy		Leatherback turtle (*Dermochelys coriacea*) Loggerhead (*Caretta caretta*) Green turtle (*Chelonia mydas*) with and without FP	ulcers and haemorrhagic septicaemia, congested lung showing nodules in parenchyma, blood in body cavity Pathogenicity not determined. Can be from normal flora without pathogenicity	sharks, dolphins and shrimps, wild and cultivated fish		(Fichi *et al.,* 2016; Obendorf *et al.,* 1987)
Pseudomonas spp.	Hawaii, Australia, East	*	Green turtle (*Chelonia mydas*) with FP	Opportunistic pathogen. may correlate to hatching failure	can be a risk to egg consumers potentially zoonotic	infections in the salt glands due to the	(Aguirre *et al.,* 1994; Campbell and Glazebrook,

	Caribbean sea, central Mediterranean Sea		Leatherback turtle (*Dermochelys coriacea*) Loggerhead (*Caretta caretta*), Hawksbill turtle (*Eretmochelys imbricate*), Hatchlings, juvenile and adults	Ulcerative stomitis and dermatitis along with vibrio alginolyticus The frequent bacteria in keratoconjunctivitis, ulcerative blepharitis, Salt- secreting gland infection, Peritonitis along with *Flavobacterium* spp. resistant to ampicillin, Amoxicillin with clavulanic acid and Sulfamethoxazole-Trimethoprim		removal of foreign material	1990; Glazebrook and Campbell, 1990; Zieger *et al.,* 2009)
Pseudomonas putrefaciens			Green turtle (*Chelonia mydas*) with FP	Pathogenicity not determined. Can be from normal flora without pathogenicity Detection: clinical signs and microbial culture of caseous lesions	second most frequent bacteria in Aguirre 1994		(Aguirre *et al.,* 1994)
Pseudomonas putida	Hawaii	Green turtle (*Chelonia mydas*)	cloacal and nasopharyngeal swabs of Green turtle (*Chelonia mydas*) with and without FP	A frequent isolation in the survey (21/32)			(Aguirre *et al.,* 1994)
Pseudomonas fluorescens	Green turtle (*Chelonia mydas*), Loggerhead (*Caretta caretta*), E. imbricate	14.4% along with Ps. Aeruginosa (non-oxidative pseudomonads) in C.mydas	cloacal and nasopharyngeal swabs of Green turtle (*Chelonia mydas*) with and without FP	ulcerative stomatitis, obstructive rhinitis-pneumonia complex and fibropapillomatosis	the most abundant in FP turtles in the survey		(Aguirre *et al.,* 1994)
Pseudomonas aeruginosa	central Mediterranean Sea, Florida	*	nasopharyngeal swabs of Green turtle (*Chelonia mydas*) Loggerhead (*Caretta caretta*)	Can be from normal flora without pathogenicity opportunistic and able to infect tissues damaged by trauma ulcerative dermatitis and rhinitis and stomatitis, shell disease, bronchopneumonia in captivity		anthropogenic effects can cause trauma and trigger infection	(Aguirre, 1992; Buller, 2014)

				Shows 94.1% resistance to antibiotic tested by Foti *et al*. (Foti *et al.,* 2009)			
Pseudomonas stutzeri	Florida		nasopharyngeal swabs of Green turtle (*Chelonia mydas*) with FP	Pathogenicity not determined. Can be from normal flora without pathogenicity			(Aguirre, 1992; Aguirre *et al.,* 1994)
Salmonella spp	Tortuguero National Park, Costa Rica/ Western Australia (group O type B)/ Oman sea and Persian Gulf	Green turtle (*Chelonia mydas*)		Death and illness (enteritis and septicaemia as possible manifestations of reptilian) Can infect eggs resistant to ampicillin	Can infect any species (salmonellosis in humans) Sea turtles can be carriers	Human can be the source of infection in captivity	(Alfaro *et al.,* 2006; Buller, 2014; Raidal *et al.,* 1998)
Salmonella chester	Australian Northern Territory		Salmonella was isolated from partially cooked Green turtle (*Chelonia mydas*)		Outbreak of gastroenteritis due to Green turtle (*Chelonia mydas*) meat consumption		(O'Grady and Krause, 1999)
Salmonella enteritidis /enterica	Yorke Island/ Eastern Caribbean	Green turtle (*Chelonia mydas*)/ hawksbill	Leatherback turtle (*Dermochelys coriacea*) in nesting beaches	diffuse lymphocytic and Foci (<1mm) in liver a of a captive turtle resistant to ampicillin			(Campbell and Glazebrook, 1990; Dutton *et al.,* 2013)
Salmonella regent			Hawksbill turtle (*Eretmochelys imbricate*)	catarrhal colitis			(Alfaro *et al.,* 2006)
Serratia marcescens	Canary Island		Loggerhead (*Caretta caretta*)	Opportunistic pathogen Can associate with Fibrinous and necrotizing enteritis in a mixed infection			(Orós *et al.,* 2005)
Serratia odorifera			cloacal fluid and nest chamber of Olive ridley turtle (*Lepidochelys olivacea*) and Green turtle (*Chelonia mydas*)	Opportunistic pathogen. may correlate to hatching failure	can be a risk to egg consumers		(Keene *et al.,* 2014)

Vibrio alginolyticus	Pacific Ocean, Gulf of California, Canary Island	juvenile Green turtle (*Chelonia mydas*) E.imbricata	hatchling and juvenile Green turtle (*Chelonia mydas*) agassizi and Olive ridley turtle (*Lepidochelys olivacea*) Loggerhead (*Caretta caretta*) 50% Green turtle (*Chelonia mydas*) with FP	important causes of Mortality in sea turtles ulcerative dermatitis frequently seen; Second frequent bacteria in ulcerative stomatitis lesions exudative bronchopneumonia and/or granulomatous pneumonia, traumatic skin lesions, granulomatous nephritis, renal abscesses, and necrotizing and/or granulomatous hepatitis cam infect eggs global spread reported recently particularly clone O3:K6 no specific treatment, common antibiotics, normally resistant to antibiotics (primarily to ampicillin)	causing gastroenteritis related to consumption of meat and egg can infect fish, mollusks, crustaceans, cnidarians	ulcerative dermatitis in boat strike and bycatch Human can be the source of infection in captivity	(Orós *et al.,* 2004b; Orós *et al.,* 2005; Zavala-Norzagaray *et al.,* 2015; Orós *et al.,* 2011a)
Vibrio harveyi	Hawaii		Green turtle (*Chelonia mydas*)	Common in blood samples, no pathogenicity determined No more info	Can be a risk to human can infect Molluscs, crustaceans, fish		(Work *et al.,* 2003)
Vibrio tubiashii	Hawaii		Green turtle (*Chelonia mydas*)	Found in blood samples, no pathogenicity determined No more info	can infect molluscs, crustaceans, fish		(Work *et al.,* 2003)
Vibrio campbelli	Hawaii		Green turtle (*Chelonia mydas*)	Found in blood samples, no pathogenicity determined No more info	Can be a risk to human Can infect Molluscs, crustaceans, fish		(Work *et al.,* 2003)
Vibrio parahaemolyticus		Green turtle (*Chelonia mydas*) agassizi and Olive ridley turtle (*Lepidochelys olivacea*)	Loggerhead (*Caretta caretta*)	fungal and mixed bacterial infection of the skin global warming favors spread treatment: common antibiotics; normally resistant to antibiotics (primarily to ampicillin)	Can be a risk to human	Temperature elevation favors global increase of pandemic V. parahaemolyticus	(Fichi *et al.,* 2016; IUCN *et al.,* 2015; Zavala-Norzagaray *et al.,* 2015)

Vibrio cholera			Black sea turtle (*Chelonia mydas agassizii*) and Olive ridley turtle (*Lepidochelys olivacea*)	treatment: common antibiotics; normally resistant to antibiotics (primarily to ampicillin) susceptible to ciprofloxacin, SXT, tetracycline, and chloramphenicol	V. cholerae virulence genes in sea turtles caught in China	sea surface temperature can correlates with outbreaks	(IUCN *et al.,* 2015; Lu *et al.,* 2006)
Vibrio mimicus			Olive ridley turtle (*Lepidochelys olivacea*)	Reduced viability of eggs	*turtle egg caused diarrhea in 33 people (1991-1994 Costa Rica) *otitis in people who swim in estuarine water in Costa Rica (Acuña *et al.,* 1999)		(Acuña *et al.,* 1999; Buller, 2014)
Vibrio damsela	Eastern coast of Tasmania		Leatherback turtle (*Dermochelys coriacea*) also collected from Green turtle (*Chelonia mydas*) with and without FP (cloacal and nasopharyngeal swabs)	cause of death in a Leatherback turtle (*Dermochelys coriacea*); endocardia thrombus valvular endocarditis and septicaemia route of infection intestinal lesions	can infect fish, shark	anthropogenic effects caused intestinal lesions	(Aguirre *et al.,* 1994; Obendorf *et al.,* 1987)
Vibrio fluvialis			Green turtle (*Chelonia mydas*) with FP (cloacal and nasopharyngeal swabs)	Found in samples, no pathogenicity determined			(Aguirre *et al.,* 1994)
Vibrio aestuarianus	Hawaii		Green turtle (*Chelonia mydas*) with FP	Found in blood samples, no pathogenicity determined			(Work *et al.,* 2003)

1.2. Gram positive bacteria

Disease hazard	Region reported	presence in sea turtle		Outcome of infection (lesion, clinical sign and/or disease) sign in individuals; ease of spread, rate of spread; is a test, quarantine, treatment available to detect the disease?	zoonotic/transmissible to companion animals	Correlation with climatic/anthropogenic events	Key reference
		Captive populations	Wild populations				
Bacillus spp	Florida, Canary Island		FP afflicted Green turtle (*Chelonia mydas*)	Can be from normal skin flora without pathogenicity Fibrinous and necrotizing enteritis ulcerative stomatitis, obstructive rhinitis-pneumonia complex and fibropapillomatosis May correlate to hatching failure	can be a risk to egg consumers		(Aguirre, 1992; Orós, Torrent *et al.,* 2005)
Corynebacterium spp.	Thailand	Green turtle (*Chelonia mydas*) (Hawksbill turtle (*Eretmochelys imbricate*)		Can be from normal skin flora without pathogenicity. Opportunistic pathogen			(Chuen-Im *et al.,* 2010; Guthrie *et al.,* 2010)
Corynebacterium (diphteroids)			nasopharyngeal swabs of Green turtle (*Chelonia mydas*) with and without FP	non-pathogenic, commensals of skin and upper respiratory tract frequent Gram positive isolation in the survey	Can be found in aquatic species		(Aguirre, Balazs *et al.,* 1994)
Enterococcus spp	East Caribbean sea, central Mediterranean Sea, Oman sea and Persian Gulf		egg, hatchling and juvenile Green turtle (*Chelonia mydas*), olive ridley turtle (*Lepidochelys olivacea*), Leatherback turtle	Opportunistic pathogen. may correlate to hatching failure pneumonia, anorexia or poor appetite and lethargy, joint inflammation, radiographic evidence of osteomyelitis and excessive GI tract gas, persistent	can be a risk to human	Urban sewages can cause antibiotic resistant if found a way to ocean	(Innis *et al.,* 2014; Zieger *et al.,* 2009)

			(*Dermochelys coriacea*)	hypoglycemia, cutaneous wounds and subcutaneous masses commercially available test kit, bacteriologic culture of blood, electrophoresis Once specific *Enterococcus* infection treatment starts, recovery may happen. But no pharmacokinetic studies are available. May be resistant to ampicillin.			
Enterococcus faecalis and faecium	Fichi, New England Aquarium, Bostn;	Leatherback turtle (*Dermochelys coriacea*)	recognised during rehabilitation of cold-stunned Kemp's ridley turtle (*Lepidochelys kempii*) deep tissue infection in a Loggerhead (*Caretta caretta*)	bladder, brain, intestine, kidney, liver, lung and muscle can be infeted septicemia and osteomyelitis in sea turtles at the New England Aquarium treatment: may be resistant to ampicillin and amoxicillin amoxicillin–clavulanic acid,		This bacterium may be introduced through wounds from anthropogenic causes or predation	(Fichi *et al.,* 2016; Innis *et al.,* 2014)
Lactobacillus spp	Hawaii		cloacal and nasopharyngeal swabs of Green turtle (*Chelonia mydas*) with and without FP	A part of microbiota			(Aguirre *et al.,* 1994)
Lactococcus garviae	Tuscany, Italy		Loggerhead (*Caretta caretta*) and Green turtle (*Chelonia mydas*)	Detected using PCR. No pathogenic studies carried out	Fish, Molluscs and Crustaceans, identified in a bacterial epidemic in aquatic invertebrates, such as the giant freshwater prawn	Climate change may influence the threat levels associated with such exotic pathogens	(Fichi *et al.,* 2016)
(Lactobacillales) Aerococcus viridans	Canary Islands, Spain		Loggerhead (*Caretta caretta*)	first report of esophageal diverticulum in sea turtles, maybe due to ingestion of infected lobster	crustaceans		(Torrent *et al.,* 2002)
Micrococcus spp.		*	nasopharyngeal and cloacal swabs of	Can be from normal skin flora without pathogenicity	Normal flora in marine environments		(Raidal *et al.,* 1998)

			Green turtle (*Chelonia mydas*) with and without FP				
Staphylococcus spp. (beta-haemolytic)	Canary Island, Costa rica, Australia, Georgi, USA	Juvenile Green turtle (*Chelonia mydas*) and Hawksbill turtle (*Eretmochelys imbricata*)	swabs of nesting Green turtle (*Chelonia mydas*), Loggerhead (*Caretta caretta*), and olive ridley turtle (*Lepidochelys olivacea*), may contain *S. aureus, S. cromogenes, S. epidermis*, and *S. intermediu*	Normal flora in marine environments but are opportunistic Fibrinous exudative pericarditis; ulcerative oesophagitis and stomatitis; gastritis and hepatitis	the most abundant skin-colonising bacteria on human body and the cause of nosocomial infections	Humans may come in contact with turtle eggs and transfer these bacteria or cause resistant to antibiotics	(Alfaro *et al.,* 2006; Chuen-Im *et al.,* 2010; Keene, 2012; Santoro *et al.,* 2006; Orós *et al.,* 2011a)
Staphylococcus aureus	Easern Pacific		nasopharyngeal swabs of healthy Green turtle (*Chelonia mydas*) olive ridley turtle (*Lepidochelys olivacea*)	common skin flora, but some strains may produce cytotoxins and cause necrotic tissue and pneumonia may be resistant to Erythromycin		Human may come in contact with turtle eggs and transfer these bacteria or cause resistant to antibiotics	(Keene, 2012)
Staphylococcus epidermidis	Florida	*	nasopharyngeal swabs of Green turtle (*Chelonia mydas*) with and without olive ridley turtle (*Lepidochelys olivacea*) eggs	opportunistic and able to infect tissues damaged by trauma ulcerative dermatitis and rhinitis and stomatitis, shell disease, bronchopneumonia in captivity	Opportunistic and can be a risk to human	anthropogenic effects can cause trauma and trigger infection	(Aguirre, 1992)
Staphylococcus xylosus	Canary Island		Loggerhead (*Caretta caretta*)	pneumonia, Fibrinous exudative pericarditis, nephritis	Cutaneous lesions in chicken, sheep and mice		(Orós *et al.,* 2005)
Staphylococcus lentus	Tuscany, Italy		Loggerhead (*Caretta caretta*)	Detected using PCR. No pathogenic studies carried out	Side Necked Turtle (Phrynops geoffranus)		(Fichi *et al.,* 2016)
Staphylococcus sciuri	Hawaii		Green turtle (*Chelonia mydas*)	Found in blood samples, no pathogenicity determined Low frequency isolation in the survey			(Work *et al.,* 2003)

Alpha-hemolytic Streptococcus	Canary Island; Padre Island	Green turtle (*Chelonia mydas*) (Campbell & Glazebrook, 1990)	nasopharyngeal swabs of healthy Green turtle (*Chelonia mydas*)	Opportunistic pathogen Bilateral Chronic Shoulder Infections (along with Corynebacterium spp. and Nocardia spp.) in a chelonian mydas Padre Island, TX, USA led to mortality.			(Guthrie *et al.,* 2010)
non-haemolytic streptococcus			Green turtle (*Chelonia mydas*) with and without FP (nasopharyngeal swabs)	High frequent isolation in the survey			(Aguirre *et al.,* 1994)
Streptococcus group C	Thailand	Green turtle (*Chelonia mydas*) Hawksbill turtle (*Eretmochelys imbricata*)		ulcerative stomatitis along with beta-haemolytic *Staphylococcus* spp			(Chuen-Im *et al.,* 2010; Raidal *et al.,* 1998)

1.3. Not defined by gram staining

Disease hazard	Region reported	presence in sea turtle		Outcome of infection (lesion, clinical sign and/or disease) sign in individuals; ease of spread, rate of spread; is a test, quarantine, treatment available to detect the disease?	zoonotic/transmissible to companion animals	Correlation with climatic/anthropogenic events	Key reference
		Captivity	Wild				
Chlamydia psittaci		Cayman turtle farm		Chlamydiosis systemic infection and evidence of epicarditis, myocarditis, hepatitis splenitis, pneumonia, and nephritis	Can infect many species/ known human pathogens	Humans can be the source of infection in captivity	(Arena *et al.,* 2014; Homer *et al.,* 1994)
Mycobacterium avium	From French Frigate Shoal rehabilitated in University of Hawaii	Green turtle (*Chelonia mydas*)		Tuberculosis, commonly cutaneaous lesions, anorexia, lethargy and wasting focal granulomas characterised by central necrosis seen in liver, lung,	*Mycobacterium* spp. can be potentially zoonotic	Humans in captivity or seabirds in nesting beaches can be cause of the infection	(Brock *et al.,* 1976; Campbell and Glazebrook, 1990)
Mycobacterium chelonae	Adriatic coast of Italy	Kemp's ridley turtle (*Lepidochelys kempii*) captive for rehabilitation	a stranded Loggerhead (*Caretta caretta*) (observation of nodules on internal organs)	osteolytic lesions, osteoarthritic diseases of synovial joints and systemic disease		Humans can be the source of infection in captivity	(Greer *et al.,* 2003; Nardini *et al.,* 2014)
Mycobacterium haemophilum	Atlantic coast of Florida	Leatherback turtle (*Dermochelys coriacea*)		disseminated mycobacteriosis mostly affecting the nervous system	Can infect various reptiles	Humans can be the source of infection in captivity	(Donnelly *et al.,* 2016)
mycobacterial pneumonia		Loggerhead (*Caretta caretta*)		tilted-swimming respiratory infections are most often fatal and may be contagious The bacterial agent wasn't isolated resistant to streptomycin treatment: NMFS STF with 5 mg/kg injectable enrofloxacin		Zoonotic	(Leong *et al.,* 1989; Lutz *et al.,* 2002)

Dermatophilus chelonae			Green turtle (*Chelonia mydas*); Loggerhead (*Caretta caretta*)	Dermatophilosis, Present in a mixed infection leading to cutaneous ulceration along with *V. alginolyticus*			(Buller, 2014)
Shewanella putrefaciens	Hawaii		commonly (26%) isolated from the cloaca Loggerhead (*Caretta caretta*) Green turtle (*Chelonia mydas*)	Low frequency bacteria isolated from Green turtle (*Chelonia mydas*)			(Work *et al.,* 2003)

1.4. Mixed bacterial infections

Bacteria involved	region reported	Presence in sea turtles		Disease	Anthropogenic Effect and Climatic event	Key references
		Captivity	Wild			
Alginolyticus, A. hydrophila, Pseudomonas spp., and Flavobacterium spp	Australia	* Largely seen in hatchlings and juvenile	Green turtle (*Chelonia mydas*)	Integumentary Digestive Respiratory traumatic ulcerative dermatitis, ulcerative stomatitis, obstructive rhinitis and bronchopneumonia	Fishing hooks, Boat strike	(Campbell and Glazebrook, 1990; Glazebrook and Campbell, 1990)
Aeromonas hydrophila, Citrobacter spp., Escherichia coli, Proteus spp., Vibrio alginolyticus, *and Staphylococcus spp*			Loggerhead (*Caretta caretta*)	Granulomatous nephritis and renal abscesses		(Orós *et al.,* 2005)
Klebsiella pneumoniae, Enterobacter agglomerans, E. cloacae, Escherichia coli, Klebsiella oxytoca, K pneumoniae, and Serrana (serratia) marcescens	nesting beach of Costa Rica		Green turtle (*Chelonia mydas*)	Egg failure		(Santoro *et al.,* 2006b)
Pseudomonas aeruginosa and Flavobacterium sp	Australia		Green turtle (*Chelonia mydas*)	Eyelids: ulcerative lesions		(Campbell and Glazebrook, 1990)

Bacillus sp Escherichia coli, Pasteurella spp., Proteus spp., Serratia marcescens, Staphylococcus spp., Streptococcus spp., and Vibrio alginolyticus	Canary Island; East Caribbean sea		Loggerhead (*Caretta caretta*); Green turtle (*Chelonia mydas*) with and without FP; Leatherback turtle (*Dermochelys coriacea*)	digestive lesions exudative bronchopneumonia and/or granulomatous pneumonia	Fishing hooks, Boat strike	(Orós *et al.,* 2004)
Aeromonas hydrophila, Citrobacter spp., Escherichia coli, Proteus spp., Staphylococcus spp., and Vibrio alginolyticus infections	Canary Island		Loggerhead (*Caretta caretta*); Green turtle (*Chelonia mydas*); Leatherback turtle (*Dermochelys coriacea*)	Necrotizing and/or granulomatous hepatitis		(Orós *et al.,* 2004a)
Burkholderia cepacia , Pseudomonas spp., Staphylococcus spp., and Achromobacter spp	Canary Island		Loggerhead (*Caretta caretta*)	Heterophilic scleritis		(Orós *et al.,* 2005)
Serratia mareescens (and Aeromonas spp., Bacillus spp., Enterobacter spp., Escherichia coli, Klebsiella spp., Pasteurella spp., Proteus spp., Pseudomonas spp., Serratia marcescens, Staphylococcus spp., and Vibrio spp.)		*	Nesting Green turtle (*Chelonia mydas*)	bronchopneumonia, integumental lesions, obstructive rhinitis, traumatic ulcerative dermatitis, ulcerative shell disease, and ulcerative stomatitis abscesses of the salt-secreting gland and peritoneal wall	Fishing hooks, Boat strike	(Santoro *et al.,* 2006b)

Appendix 2. The fungal pathogens of sea turtles

Disease hazard	Region reported	presence in sea turtle		Outcome of infection (lesion, clinical sign and/or disease) sign in individuals; ease of spread, rate of spread; is a test, quarantine, treatment available to detect the disease?	zoonotic/transmissible to companion animals	Correlation with climatic/anthropogenic events	Key reference
		Captive populations	Wild populations				
Acremonium spp.	Shoalwater, Heron Reef, Peak Island, Australia;		Green turtle (*Chelonia mydas*); Loggerhead turtle *(Caretta caretta)*; Flatback (*Natator depressus*)	Isolated from nesting females' cloaca	Can rarely infect humans	Were isolated in ocean sediments and at 30°C, these isolates were halotolerant and even halophilic	(Phillott *et al.,* 2002; Rédou *et al.,* 2015)
Allescheria spp.	Nancite, Costa Rica		Olive ridley (*Lepidochelys olivacea*)	Isolated from failed eggs shell and chamber	Can infect immunosuppressed hosts		(Mo *et al.,* 1990)
Alternata arborescens	Rio Grande do Sul, Southern Brazil		Loggerhead turtle *(Caretta caretta)*;	Phaeohyphomycosis, Kidney Nodules, peritonitis and nephritis (along with *C. cladosporioides*)	emerging opportunistic mycotic infections agents of domestic animals		(Domiciano *et al.,* 2014)
(Pseud)allescheria boydii	Heron Island, Wreck Island, Peak Island, Mon Repos, Milman Island, Australia;		Green turtle (*Chelonia mydas*); Loggerhead turtle *(Caretta caretta)*; Flatback (*Natator depressus*); Hawksbill (*Eretmochelys imbricata*);	Found in failed eggs			(Phillott and Elmorse, 2004)
Aspergillus spp.	Turkey, pacific coast of Costa Rica		Green turtle (*Chelonia mydas*); Loggerhead turtle *(Caretta caretta)* hatchlings	skin diseases in captive marine turtles Airborne infection and can be present in successful nests. Has been found in many failed nests of different sea turtles	Immunosuppressed sea turtles	Can produce Immunosuppressive mycotoxins, such as gliotoxin at higher temperatures	(George, 1997; Güçlü *et al.,* 2010; Keene, 2012; Phillott *et al.,* 2004)

			Kemp ridely (Lepidochelys kempii) juvenilles Olive ridley (*Lepidochelys olivacea*) cloacal fluid	Focal, dry, black areas on flippers of hatchlings Necrotic lesions on head, neck and shell of kemp ridley Potential treatment Fluconazole topical iodine		water pollution can trigger infection	
Aspergillus niger	Woongarra Coast, AUS		Loggerhead turtle *(Caretta caretta)*	Isolated from egg chamber			(Keene, 2012)
Absidia	Fethiye, Turkey, Nancite, Costa Rica		Loggerhead turtle *(Caretta caretta)* nest; Olive ridley (*Lepidochelys olivacea*)	Isolated from nest. May correlate with hatching failure	an emerging cause of mucormycosis	Absidia can correlate with nitrification increase (greenhouse gas production)	(Güçlü *et al.,* 2010; Smith, 2010; Woo *et al.,* 2012)
Candida albicans	Canary Island		Loggerhead turtle *(Caretta caretta)*;	systemic mycotic infection and Intestinal candidiasis Potential treatment Fluconazole	Immunosuppressed human and anima		(Orós *et al.,* 2004b; Orós, *et al.,* 2005)
Cephalosporium sp		immunocompromised Kemp's ridley (*Lepidochelys kempii*)		necrotic lesions on the head, neck, and shell	Plant pathogen		(Leong *et al.,* 1989)
Cephalosporium curtipes var. uredinicola	Woongarra Coast, AUS;			Isolated from failed eggs			(Keene, 2012)
Cladosporium sp	Cayman Turtle Farm, USA; Yanıklar, Fethiye, Turkey; Woongarra Coast, AUS; Heron Reef and North West Island in Australia; Playa	Green turtle (*Chelonia mydas*)	Green turtle (*Chelonia mydas*); *Caretta caretta* Olive ridley (*Lepidochelys olivacea*) egg chamber	Has been isolated from egg chamber, egg shell and cloacal fluid. May correlate with hatching failure pneumonic lesions in captivity	Immunosuppressive sea turtles	prefer lower temperatures The infection is more probable in popular beaches (anthropogenic effects)	(Jacobson *et al.,* 1979; Keene, 2012; Phillott *et al.,* 2002)

	Grande, Costa Rica						
Cladosporium cladosporioides	Rio Grande do Sul, Southern Brazil		Loggerhead turtle *(Caretta caretta)*;	Phaeohyphomycosis, Kidney Nodules, peritonitis and nephritis (along with *Alternata arborescens*)	emerging opportunistic mycotic infections agents of domestic animals		(Domiciano *et al.,* 2014)
Colletotrichum acutatum	Florida	immunocompromised Kemp's ridely (*Lepidochelys kempii*)	Juvenile	mycotic nephritis, pneumonia, granulomatous hepatitis and granulocytic hyperplasia of the bone marrow of the carapace	very rarely known to cause disease in human		(Manire *et al.,* 2002)
Chrysosporium	Yanıklar, Fethiye, Turkey;		Loggerhead turtle *(Caretta caretta)*;	Frequently isolated from sand and egg shell of failed eggs	Emerging infection in snakes (Allender *et al.,* 2011)	Isolated from nails. Handling the eggs can cause infection	(Güçlü *et al.,* 2010)
Cunninghamella	Nancite, Costa Rica; Eastern Australian Nests		Olive ridley (*Lepidochelys olivacea*)	Isolated from failed eggs shell and chamber			(Mo *et al.,* 1990; Phillott *et al.,* 2002)
Cylindrocarpon	Yanıklar, Fethiye, Turkey;		Loggerhead turtle *(Caretta caretta)*;	Isolated from nest chamber of failed eggs			(Güçlü *et al.,* 2010)
Drechslera spp.			Hawksbill (*Eretmochelys imbricata*);	necrotic lesions on the head, neck, and shell	Isolated from sea birds' cloaca but haven't been isolated from turtle eggs (Phillott and Elsmore, 2004)		(Sison *et al.,* 1990)
Emericella	Yanıklar, Fethiye, Turkey;		Loggerhead turtle *(Caretta caretta)*;	Isolated from nest chamber of failed eggs			(Güçlü *et al.,* 2010)
Eretmochelys imbricata			*Lepidochelys kempii* during rehabilitation	Lung, liver and kidney infection along with *Colletotrichum acutatum*			(Manire *et al.,* 2002)
Geotrichum spp.	Philippines; Playa Grande, Costa Rica	Hawksbill (*Eretmochelys imbricata*);	Olive ridley (*Lepidochelys olivacea*) cloacal fluid Loggerhead turtle *(Caretta caretta)* hatchlings	necrotic lesions on the head, neck, and shell isolated from cloacal fluid and may correlate with hatching failure	immunocompromised	Cooler temperatures, between 25-37°C increase the growth rate	(Keene, 2012; Sison *et al.,* 1990)
Gliocladiopsis	Nancite, Costa Rica		Olive ridley (*Lepidochelys olivacea*)	Isolated from failed eggs			(Mo *et al.,* 1990)

Fusarium spp.	Playa Grande, Costa Rica; St Croix, U.S. Virgin Islands; Raine Island, Australia; Isla de la Plata at Machalilla National Park, Ecuador	Hawksbill (*Eretmochelys imbricata*); Lepidochelys kempi	Olive ridley (*Lepidochelys olivacea*) cloacal fluid; leatherback sea turtle (*Dermochelys coriacea*); Caretta caretta	Dermatomycosis (normally shell is infected) necrotic skin lesions more often in captivity Pneumonic lesion Known fungus of nest chamber; Probably infects a non-viable egg before spreading to other viable eggs	Failed eggs of all sea turtle species	nonhalophiles at 25 and 30°C	(Alfaro *et al.,* 2006; Keene, 2012; Orós *et al.,* 2004c; Rédou *et al.,* 2015; Sarmiento-Ramírez *et al.,* 2014)
Fusarium oxysporum	Columbia; Heron Island, Wreck Island, Peak Island, Mon Repos, Milman Island, Australia;		leatherback sea turtle (*Dermochelys coriacea*); Flatback (*Natator depressus*); Hawksbil (*Eretmochelys imbricata*);	excrete mycotoxins in sea turtle eggs			(Phillott *et al.,* 2004)
Fusarium scirpi		Green turtle (*Chelonia mydas*)		pneumonic lesions in captivity			(Campbell and Glazebrook, 1990)
Fusarium solani	(Bahamas, Spain); Columbia; Woongarra Coast, Heron Island, Wreck Island, Peak Island, Mon Repos, Milman Island, Australia; Boavista, Cape Verde	Eggs Adult and baby *Caretta caretta* in captivity	Green turtle (*Chelonia mydas*) *C. caretta Lepidochelys kempi*; leatherback sea turtle (*Dermochelys coriacea*); Flatback (*Natator depressus*); Hawksbill (*Eretmochelys imbricata*);	skin diseases in captive marine turtles cutaneous abscess in *Lepidochelys kempii* mass mortalities in natural and relocated nests of the sea turtle species (excrete mycotoxins in sea turtle eggs) 100% mortality of *Caretta caretta* nests in Boavista, Cape Verde May have an effect on hatchlings fitness	immunosuppressed sea turtles, Potentially zoonotic		(Cabanes *et al.,* 1997; Orós *et al.,* 2004c; Sarmiento-Ramírez *et al.,* 2014; Sarmiento-Ramírez *et al.,* 2010)

Fusarium falciforme, Fusarium keratoplasticum	Raine Island, Australia		Green turtle (*Chelonia mydas*) Eggs	normally seen is nesting beaches, reduce hatching success (able to kill up to 90% of the embryos)	Able to infect stressed sea turtles		(Sarmiento-Ramírez *et al.,* 2014)
Homodendrum	Nancite, Costa Rica		Olive ridley (*Lepidochelys olivacea*)	Isolated from failed egg shells and chamber			(Mo *et al.,* 1990)
Mucor spp.	Göksu Delta, Turkey; Yanıklar, Fethiye, Turkey; Nancite, Costa Rica; Heron Island, Australia		Olive ridley (*Lepidochelys olivacea*) egg shells; Loggerhead turtle *(Caretta caretta)*; *Chelonia mydas*	May correlate with hatching failure	an emerging cause of mucormycosis	temperatures lower than 37°C favors the growth	(Güçlü *et al.,* 2010; Keene, 2012; Woo *et al.,* 2012)
Paecilomyces spp.	Australia	immunocompromised Kemp's ridely (*Lepidochelys kempii*) *C. mydas*	Green turtle (*Chelonia mydas*) Juvenille, adult, eggs Olive ridley (*Lepidochelys olivacea*)	Granulomatous pneumonia necrotic lesions on the head, neck, and shell detection: histopathology and culture	Can infect human		(Leong *et al.,* 1989)
Paecilomyces lilacinus (Purpureocillium lilacinum)	Woongarra Coast, AUS; Cayman Turtle Farm, USA;	Green turtle (*Chelonia mydas*); *Caretta caretta*	Loggerhead turtle *(Caretta caretta)*; Hawksbill (*Eretmochelys imbricata*);	Purpureocilliosis disseminated granulomas buoyancy abnormality and pneumonia in captivity leading to death (Detected using histopathology after necropsy) Isolated from failed eggs	Immunosuppressed humans or animals. Has been reported in several cases of reptiles and also chelonians		(Jacobson *et al.,* 1979; Posthaus *et al.,* 1997; Schumacher *et al.,* 2014)
Penicillium sp	Yanıklar, Fethiye, Turkey; Mon Repos, Australia; Peak Island, Australia; Milman Island, Australia; Nancite, Costa Rica		hatchlings Juvenile Loggerhead turtle *(Caretta caretta)*; Flatback (*Natator depressus*); Hawksbill (*Eretmochelys imbricata*);Olive ridley	skin diseases in captive marine turtles Detection: culture (pneumonic lesions) lung is affected, dissemination of other organs is possible Can correlate to hatching failure	Turtles and tortoises	grow best at temperatures below 37°C; halotolerant at 25°C	(Alfaro *et al.,* 2006; Keene, 2012; Phillott *et al.,* 2002; Rédou *et al.,* 2015)

			(*Lepidochelys olivacea*) cloacal fluid, eggs and nesting area				
Phialophora spp.	Peak Island, Australia		Flatback (*Natator depressus*);	Isolated from cloacal samples of interesting turtles			(Phillott *et al.,* 2002)
Rhodotorula spp.			adult, eggs Green turtle (*Chelonia mydas*)	Bronchopneumonia in captivity	sea turtle various species, can infect human		(Campbell and Glazebrook, 1990)
Saksenaea vasiformis	Nancite, Costa Rica		Olive ridley (*Lepidochelys olivacea*)	Isolated from failed egg shells and chamber			(Mo *et al.,* 1990)
Scedosporium aurantiacum	Woongarra Coast, AUS		Loggerhead turtle *(Caretta caretta)*;	Isolated from failed eggs			(Keene, 2012)
Scolecobasidium constrictum		immunocompromised *Lepidochelys kempii*	Hawksbill (*Eretmochelys imbricata*);	skin diseases in captive marine turtles Pulmonary mycoses infections necrotic lesions on the head, neck, and shell			(Leong *et al.,* 1989)
Sporotrichium spp.	Cayman Turtle Farm, USA;	Green turtle (*Chelonia mydas*)		pneumonic lesions and buoyancy abnormality along with *Cladosporium spp*., and *Paecilomyces spp*			(Jacobson *et al.,* 1979)
Thielavia	Yanıklar, Fethiye, Turkey		Loggerhead turtle *(Caretta caretta)*;	Isolated from egg shells, can correlate with hatching failure			(Güçlü *et al.,* 2010)
Trichophyton spp			Olive ridley (*Lepidochelys olivacea*)	ulcerative lesions on flipper, pneumonia	cause forms of dermatophytosis in human		(Orós *et al.,* 2011a)
Unknown Fungi		Hawksbill (*Eretmochelys imbricata*);		Crusty yellow lesions on neck and flippers; Congestion of intestinal blood vessels			(Bailey, 2008)

Appendix 3. The parasites of sea turtles

Disease hazard	Region reported	presence in sea turtle		Outcome of infection (lesion, clinical sign and/or disease) sign in individuals; ease of spread, rate of spread; is a test, quarantine, treatment available to detect the disease?	zoonotic/transmissible to companion animals	Correlation with climatic/anthropogenic events	Key reference
		Captive populations	Wild populations				
Protozoa							
Caryospora cheloniae and a genotype most closely related to species of *Schellackia*	Grand Cayman Island; Queensland, Australia; Canary Islands, Spain; northwestern Africa	green turtle (*Chelonia myda*)	green turtle (*Chelonia myda*) adult and sub-adults Loggerhead turtle *(Caretta caretta* Leatherback turtle *(Dermochelys coriacea)*	Coccidiosis Granulomatous encephalitis, enteritis and, thyroiditis and nephritis; gastritis mass mortalities in south-east of Queensland, Australia Detection: histopathology, faecal floatation, blood smear and buffy coat examination	Not zoonotic		(Chapman *et al.,* 2016; Gordon, 2005; Orós *et al.,* 2005)
Cryptosporidium parvum	Oahu and the western shores of Maui, Hawaii		green turtle (*Chelonia myda*) Adult and egg	No correlation was found to stranding Detection from faecal and intestinal samples (culture and microscopically)	Possibly zoonotic; turtles can carry the oocytes and infect human; emerging food borne pathogen	Raw sewage disposal may cause the pollution	(Aguirre *et al.,* 2006; Graczyk *et al.,* 1997)
Eimeria carettae	Martin County, Florida; Atlantic sea		stranded Loggerhead turtle *(Caretta caretta)*	Oocysts were found in the faeces but no correlation was found with stranding			(Upton *et al.,* 1990)
Entamoeba invadens	?	green turtle (*Chelonia myda*) hatchlings; Loggerhead turtle *(Caretta caretta)*	Leatherback turtle *(Dermochelys coriacea)*	mortality in hatchlings in captivity;	Turtles can be sub-clinical carriers of amoebiasis; Possibly zoonotic		(Aguirre *et al.,* 2006)
Metazoa							

Nematodes								
Family/Genus	Species							
Acanthocephala								
Anisakis spp.	--	Canary Island; Western Australia	*	Loggerhead turtle (*Caretta caretta*)	Ulcer in stomach and intestine, dominant between helminths; Granulomatous hepatic serositis Haemorrhagic and ulcerative disease resulting from larval migration Samples are collected during necropsy and gross examination	A wide range of marine hosts; Sea turtles can act as accidental or paratenic hosts zoonotic	Captive turtles were fed by fresh sardines (the reason of infection)	(Orós *et al.,* 2005; Santoro *et al.,* 2010b)
	Anisakis pegreffii	Central Mediterranean of southern Italy		Loggerhead turtle (*Caretta caretta*)	Samples are collected during necropsy and gross examination Detection: PCR and histochemistary.			(Santoro *et al.,* 2010b)
	Sulcascaris sulcata	Brazil; Mediteranean Sea and Western Pacific; Australia; Uruguay; Atlantic Ocean, Florida		Loggerhead turtle (*Caretta caretta*); green turtle (*Chelonia myda*)	Esophagus and small intestine can be infected	Being a carnivore Loggerhead turtle (*Caretta caretta*) is the main host		(Santoro *et al.,* 2010b; M. R. Werneck *et al.,* 2008)
Cucullanidae	*Cucullanus cauettae*	Western Australia; Mediteranean Sea		Loggerhead turtle (*Caretta caretta*)	Were found in intestine			(Santoro *et al.,* 2010a)
Echinocephalus spp.spp.		Western Mediterranean		Loggerhead turtle (*Caretta caretta*)	Immature worms	Main host: Elasmobranchs		(Aznar, *et al.,* 1998)
	Hysterothylacium	Adriatic Sea		Loggerhead turtle (*Caretta caretta*)	generalist helminth found with a low frequency			(Gracan *et al.,* 2012)
Gnathostomatidae	*larval gnasthostome Echinocephalus sp*							

Kathlanidae	*Kathlania leptura*	Sri Lanka, Mauritania, and Brazil; Egypt, Western Australia, the Mediterranean and Ossabaw Island, Georgia, U.S.A.		Loggerhead turtle (*Caretta caretta*); green turtle (*Chelonia myda*); Olive ridely (*Lepidochelys olivacea*)	Large intestine can be infected and it can be an intense infection.	bivalves, cephalopods, crustaceans, and fishes		(Lester *et al.,* 1980; M. R. Werneck *et al.,* 2008)
	Tonaudia tonaudia	Costa Rica		Loggerhead turtle (*Caretta caretta*); green turtle (*Chelonia myda*); Olive ridely (*Lepidochelys olivacea*)	Were found gastrointestinal tract			(Santoro *et al.,* 2009)
Oxyuridae spp.spp.		Western Mediterranean		Loggerhead turtle (*Caretta caretta*)	Immature worms			(Aznar *et al.,* 1998)
Taxon: Platyhelminthes								
Pronopsis psenopsis		Western Mediterranean		Olive ridely (*Lepidochelys olivacea*)	Adult worms			(Aznar *et al.,* 1998)
Sub taxon: Trematodes								
Angiodictyidae	*Deuterobaris intestinalis*	Costa Rica		green turtle (*Chelonia myda*)	Were found in intestine; specialist parasites			(Santoro *et al.,* 2006a)
	Microscaphidium reticulare	Costa Rica		green turtle (*Chelonia myda*)	The second most prevalent and the most abundant trematode in the study; only immature parasites were found in; were found in intestine; specialist parasites	Were found in birds and fish	Stress can trigger the infection	(Santoro *et al.,* 2006a)
	Microscaphidium warui	Costa Rica		green turtle (*Chelonia myda*)	only immature parasites were found in; were found in urinary bladder; specialist parasites	Were found in birds and fish	Stress can trigger the infection	(Santoro *et al.,* 2006a)
	Octagium hyphalum (hiphalum)	Queensland, Australia; Costa Rica		green turtle (*Chelonia myda*)	Only immature parasites were found in Costa Rica; Were found in large intestine	Were found in birds and fish	Stress can trigger the infection	(Santoro *et al.,* 2006a)
	Polyangium linguatula	Costa Rica		green turtle (*Chelonia myda*)	Were found in intestine			(Santoro *et al.,* 2006a)

Aspidogastridae	*Lophotaspis vallei*	Costa Rica		Loggerhead turtle (*Caretta caretta*)	The only non-digenean trematode reported. Were found oesophagus and stomach			(Santoro *et al.,* 2009)
Brachycoeliidae	*Cymatocarpus solearis*	Brazil		Hawksbill turtle (*Eretmochelys imbricata*)	Were found in stomach			(Werneck *et al.,* 2015)
Calycodidae Family	*Calycodes anthos*	Brazil; Adriatic Sea; East, Central and Western Mediterranean		Loggerhead turtle (*Caretta caretta*)	Were found in large intestine; Host specific; low frequency and only in small juveniles			(Gracan *et al.,* 2012; Werneck *et al.,* 2008)
Clinosomatidae	*Clinostomum complanatum*	Caribbean Sea, Costa Rica		green turtle (*Chelonia myda*)	Only immature parasite found in esophagus; specialist parasites	A generalist in birds		(Santoro *et al.,* 2006a)
	Enoditrema megachondrus	Central and Western Mediterranean; North-eastern Atlantic		Loggerhead turtle (*Caretta caretta*)	Host specific; The dominant species in Western Mediterranean and North-eastern Atlantic			(Gracan *et al.,* 2012; Santoro *et al.,* 2010a)
Hemiuroidea sp		West Mediterranean		Loggerhead turtle (*Caretta caretta*)	Found in stomach and intestine. But turtle GI is probably having a defense against them (only sexually immature parasite found)	Fish parasites probably recruited through shared preys		(Santoro *et al.,* 2010a)
Pachypsolidae	*Pachypsolus irroratus*	Adriatic Sea; East, Central and Western Mediterranean		Loggerhead turtle (*Caretta caretta*)	Were found in stomach and intestine; were frequently found in heavy infections and throughout all classes of turtles. Mostly infecting juveniles Host specific			(Gracan *et al.,* 2012; Santoro *et al.,* 2010a)
Paramphistomidae	*Schizamphistomoides erratum*	Costa Rica		green turtle (*Chelonia myda*)	Were found in large intestine			(Santoro *et al.,* 2006a)
	Schizamphistomoides scleroporum	Costa Rica		green turtle (*Chelonia myda*)	Were found in intestine and stomach			(Santoro *et al.,* 2006a)
Pronocephalidae	*Adenogaster serialis*	East Mediterranean		green turtle (*Chelonia myda*)	Were found in intestine			(Santoro *et al.,* 2010a)
	Charaxicephaloides spp.spp.	Costa Rica		green turtle (*Chelonia myda*)	Were found in stomach			(Santoro *et al.,* 2006a)

	Charaxicephalus robustus	Costa Rica		green turtle (*Chelonia myda*)	Were found in intestine and stomach			(Santoro *et al.,* 2006a)
	Cricocephalus albus	Brazil; coast of Espírito Santo		Hawksbill turtle (*Eretmochelys imbricata*) green turtle (*Chelonia myda*)	Were found in esophagus, stomach, small and large intestine	Main host: marine turtle		(Werneck *et al.,* 2015; Gomes *et al.,* 2017)
	Cricocephalus resectus	Costa Rica		green turtle (*Chelonia myda*)	Were found in esophagus and stomach	Occasionally been isolated from the marine French angelfish		(Santoro *et al.,* 2006a)
	Cricocephalus megastomus	Costa Rica		green turtle (*Chelonia myda*)	Were found in esophagus and stomach			(Santoro *et al.,* 2006a)
	Desmogonius desmogonius	Costa Rica		green turtle (*Chelonia myda*)	Were found in esophagus and stomach; specialist parasites			(Santoro *et al.,* 2006a)
	Diaschistorchis pandus	East Mediterranean; Brazil		Loggerhead turtle (*Caretta caretta*); *Eretmochelys imbricata*	Were found in small intestines			(Santoro *et al.,* 2010a; Werneck *et al.,* 2015)
	Himasomum lobatus	Costa Rica		green turtle (*Chelonia myda*)	Were found in intestine; generalist			(Santoro *et al.,* 2006a)
	Metacetabulum invaginatum	Brazil		Hawksbill turtle (*Eretmochelys imbricata*)	Were found in small intestine			(Werneck *et al.,* 2015)
	Pleurogonius longiusculus; P. linearis; P. sindhii; P. solidus	Costa Rica		green turtle (*Chelonia myda*)	Were found in intestine;			(Santoro *et al.,* 2006a)
	Pleurogonius trigonocephalus	East, Central and Western Mediterranean; Egypt		Loggerhead turtle (*Caretta caretta*)	Were found in intestine			(Gracan *et al.,* 2012; Santoro *et al.,* 2010a)
	Pronocephalus obliquus	Brazil		Hawksbill turtle (*Eretmochelys imbricata*)	Were found in stomach and high intestine; specific to the host			(Werneck *et al.,* 2015)
	Pyelosomum cochlear	Costa Rica		green turtle (*Chelonia myda*)	Infected more than 50% of the studied turtles; were found in urinary bladder			(Santoro *et al.,* 2006a)

	Pyelosomum renicapite	Brazil		Loggerhead turtle (*Caretta caretta*)	Were found in large intestine			(Santoro *et al.,* 2010a; Werneck *et al.,* 2008)
	Rameshwarotrema uterocrescens	Costa Rica		green turtle (*Chelonia myda*)	Were found in esophagus; generalist			(Santoro *et al.,* 2006a)
Rhytidodidae	*Rhytidodes gelatinosus*	Adriatic Sea; Central and Western Mediterranean; Egypt		Loggerhead turtle (*Caretta caretta*)	Were found in stomach and intestine; mostly infecting juveniles			(Gracan *et al.,* 2012; Santoro *et al.,* 2010a)
	Rhytidodoides intestinalis	Costa Rica			Were found in gall bladder; specialist parasites			(Santoro *et al.,* 2006a)
	Rhytidodoides similis	Costa Rica			Were found in gall bladder and liver; specialist parasites			(Santoro *et al.,* 2006a)
Spirochidae family		USA; Australia; India, Pakistan		various species of sea turtles between 41 and 98% in Australia in last 25 years Adults, juvenile and possibly eggs	important cause of stranding and mortality in sea turtles worldwide (up to 40% mortality in Australia) Obstruction of blood vessels; normally cardiovascular and gastrointestinal system are affected. In heavy infestations bronchopneumonia and septicemia–toxemia is also reported Tissue damages are open doors for secondary bacterial infections (*Salmonella, Escherichia coli, Citrobacter, and Moraxella spp.*) Detection: Histopathological studies of affected tissues	No zoonotic		(Flint *et al.,* 2015; Lutz *et al.,* 2002; Wolke *et al.,* 1982)
Spirocozohiae family	*Amphiorchis caborojoensis*	Brazil		Hawksbill turtle (*Eretmochelys imbricata*)	Found in body wash			(Werneck *et al.,* 2015)

	Amphiorchis solus Simha and Chattopadhyaya	Costa Rica		green turtle (*Chelonia myda*)	Found in intestine			(Santoro *et al.,* 2006a)
	Carettacola, Hapalotrema	Atlantic seaboard (Florida to Massachusetts)		Loggerhead turtle (*Caretta caretta*); green turtle (*Chelonia myda*)	acute inflammatory response in intestine (blood fluke eggs) along with Neospirorchis			(Wolke *et al.,* 1982)
	Carettacola hawaiiensis	Hawaii; Australia		green turtle (*Chelonia myda*); Loggerhead turtle (*Caretta caretta*)	Blood flukes in hepatic vessels. Gross lesions included variously sized, lobulated tumours, serous atrophy of fat, and oedema in the subcutaneous tissues and in the pectoral and coracoid muscles.			(Graczyk *et al.,* 1995)
	Carettacola stunkardi	Brazil; USA; Panama;		Hawksbill turtle (*Eretmochelys imbricata*); green turtle (*Chelonia myda*)	Found in liver and body wash			(Werneck *et al.,* 2015)
	Haplotrema spp.spp.	Australia; Taiwan; Florida, USA		Green turtle (*Chelonia myda*) and Hawksbill turtle (*Eretmochelys imbricata*) Loggerhead turtle (*Caretta caretta*)	Blood flukes from heart and major vessels Granulomata along with Learedius spp.spp. severe enteric lesions in Loggerhead turtle (*Caretta caretta*)			(Chen *et al.,* 2012; Cribb *et al.,* 2010)
	Hapalotrema dorsopora	Hawaii; Australia; Atlantic seaboard (Florida to Massachusetts)		moribund green turtle (*Chelonia myda*); Loggerhead turtle (*Caretta caretta*)	Blood flukes from heart and major vessels Eggs were recovered from intestine scraping			(Graczyk *et al.,* 1995; Wolke *et al.,* 1982)
	Hapalotrema postorchis	Costa Rica		green turtle (*Chelonia myda*)	Can infect great vessels and heart; specialist parasite			(Santoro *et al.,* 2006a)
	Haemoxenicon spp.	Atlantic seaboard		Loggerhead turtle (*Caretta caretta*)	The eggs were found in turtles			(Wolke *et al.,* 1982)

	Learedius learedi	Bermudan waters, Baja, California; Hawaii; Australia; Atlantic seaboard (Florida to Massachusetts) (the most prevalent 97.5% from Costa Rica in a study by Santoro *et al.,* 2006a)		green turtle (*Chelonia myda*); Black sea turtle, (*Chelonia mydas agassizi*); Loggerhead turtle (*Caretta caretta*)	adult nematodes Blood flukes in heart chamber and major blood vessels (Cardiovascular spirorchidiasis along with Hapalotrema dorsopora and Carettacola hawaiiensis) Were found in great vessel; heart; esophagus; gall bladder; liver and intestine Eggs were recovered from intestine scraping Generalist parasite			(Graczyk *et al.,* 1995; Santoro *et al.,* 2006a; Wolke *et al.,* 1982)
	Metacetabulum invaginatum	coast of Espírito Santo, Brazil		green turtle (*Chelonia myda*)				(Gomes *et al.,* 2017)
	Monticellius indicum	Costa Rica; Cosast of Rio de Janeiro, Brazil		green turtle (*Chelonia myda*) Loggerhead turtle (*Caretta caretta*)	Were found in heart; specialist parasite GI tract, endocine system and heart of loggerhead turtle			(Santoro *et al.,* 2006a; Werneck *et al.,* 2017)
	Neoctangium travassosi	Queensland, Australia; Puerto Rico; coast of Espírito Santo, Brazil		green turtle (*Chelonia myda*) Hawksbill turtle (*Eretmochelys imbricata*)	Found in GI tract			(Blair, 1987) (Gomes *et al.,* 2017)
	Octangium sagitta	Queensland, Australia		green turtle (*Chelonia myda*)	Found in GI tract			(Blair, 1987)
	Neospirorchis spp.spp. And schistosomatoides	Costa Rica		green turtle (*Chelonia myda*) from Bermudan waters	adult nematodes			(Santoro *et al.,* 2009)
	Monticellius	Atlantic seaboard (Florida to Massachusetts)		Loggerhead turtle (*Caretta caretta*);	Eggs were recovered from intestine scraping			(Wolke *et al.,* 1982)

Styphlotrematidae	*Styphlotrema solitaria*	West Mediterranean; Brazil; Florida, Costa Rica;		Loggerhead turtle (*Caretta caretta*); Hawksbill turtle (*Eretmochelys imbricata*)	Were found in stomach, small and large intestine	a generalist species found only in sea turtles		(Santoro *et al.,* 2010a; Werneck *et al.,* 2015; Werneck and Silva, 2012)
Telorchiidae	*Orchidasma amphiorchis*	Brazil, Mexico, Italy, Florida, Australia, Adriatic Sea; Oaxaca, Japan; Mediterranean Sea		Loggerhead turtle (*Caretta caretta*), green turtle (*Chelonia myda*); Hawksbill turtle (*Eretmochelys imbricata*)	The most frequent species (897 parasites in 18 turtles) in Small intestine infections. It can be an intense infection	Main host: marine turtles; Teleost		(Gracan *et al.,* 2012; Santoro *et al.,* 2010a; Werneck *et al.,* 2015)
Cestodes								
Ancistrocephalus imbricatus		Western Mediterranean		Loggerhead turtle (*Caretta caretta*)	Found in stomach and intestine	Main host: Teleosts		(Aznar *et al.,* 1998)
Nybelinia spp.spp.		Western Mediterranean		Loggerhead turtle (*Caretta caretta*)				(Santoro *et al.,* 2010a)
Tentacularia coryphaenae		Western Mediterranean		Loggerhead turtle (*Caretta caretta*); green turtle (*Chelonia myda*)?	Found in stomach and intestine; Larvae	Main host: Elasmobranchs		(Aznar *et al.,* 1998)
Trypanorhynchidae	*Lacistorhynchus or Eutetrarhynchus?*	Egyptian Coast		Loggerhead turtle (*Caretta caretta*)	Larvae	Main host: Elasmobranchs		(Santoro and Mattiucci, 2009)
Acanthocephalan								
Bolbosoma spp.spp.		Western Mediterranean		Loggerhead turtle (*Caretta caretta*)				(Santoro *et al.,* 2010a)
Rhadinorhynchus pristis		Western Mediterranean		Loggerhead turtle (*Caretta caretta*)	Were found in intestine (only found in 1 location)			(Santoro *et al.,* 2010a)
Anelides								
Diplotesticulata, Oligochaeta		Turkey			Found in sand and empty egg shells			(Aymak *et al.,* 2005)

Hydroides spp.spp. and Loimia spp.spp.		Brazil		Hawksbill turtle (*Eretmochelys imbricata*)				(Alfaro *et al.,* 2006)
Ozobranchus branchiatus		Caribbean coast; Atlantic Ocean; Costa Rica; Australia; pacific coast of Mexico; Hawaii; Florida and North Carolina		Loggerhead turtle (*Caretta caretta*), green turtle (*Chelonia myda*); Olive ridely (*Lepidochelys olivacea*)	found on the skin around the throat, dorsal surface of the neck, and axillary region of the flippers; may cause severe skin lesions, deep cutaneous erosion, eye injuries and even host death Generalist parasite Possible vector for FP herpes viruses	Can complete the reproductive cycle on sea turtles		(George, 1997; Rodenbusc *et al.,* 2012; Santoro and Mattiucci, 2009)
Ozobranchus margoi		Hawaii; Rio Grande do Sul, Brazil; Tobago, West indies		Adult Loggerhead turtle (*Caretta caretta*); green turtle (*Chelonia myda*)	Possible vector for FP herpes viruses; may cause severe skin lesions, deep cutaneous erosion, eye injuries and even host death Detection: visual expection of external surfaces	Can complete the reproductive cycle on sea turtles Not zoonotic		(George, 1997; Rodenbusch *et al.,* 2012)
Arthropodes (da Silva *et al.,* 2016)								
Arachnida (Archnoidea) mites (Acaridae)	*Rizoglyphus spp.*	Mexico		Leather back turtle (*Dermochelys coriacea*)	Mites embedded in skin The correlation with hatching failure is not clear Detection: uncertain	Unknown if its able to cause diseases in human		(Glazebrook and Campbell, 1990; Vivaldo *et al.,* 2006)
Chelonibiidae and Platylepadidae	*Chelonibia spp, Platylepas spp*	Japan, Mexico and Atlantic populations; Mediterranean, Florida		Loggerhead turtle (*Caretta caretta*)	External barnacles attached to carapace and plastron, species are different over larger expanse of space.	Not able to cause diseases in human		(Santoro and Mattiucci, 2009)
Coleoptera	*Cardiophorine spp. and Agriotine spp.*	northern Cyprus		Loggerhead turtle (*Caretta caretta*)				(Aymak *et al.,* 2005; McGowan *et al.,* 2001)
	Elater spp.	Turkey		green turtle (*Chelonia myda*); Loggerhead turtle *(Caretta caretta)*	Found in sand and damaged eggs			(Aymak *et al.,* 2005)

	Lanelater sallei	Bill Baggs Cape Florida State Park		Loggerhead turtle (*Caretta caretta*)	Beetle larvae eggs caused a prominent turtle egg damage in the study (3 times worse than the damages occurred by raccoons)			(Donlan *et al.,* 2004)
	Omorgus suberosus	Floreana Island; Quinta Playa; Galá-pagos Islands ; Oaxaca, Mexico		green turtle (*Chelonia myda*); Olive ridely (*Lepidochelys olivacea*)	Predation on turtle egg and causing mortality		climate change could also increase the impact; Rainfall increase increases the number of beetles	(Zárate *et al.,* 2013)
	Pimelia sp	Alata beach, Dalaman beach Turkey; Dalyan İztuzu		green turtle (*Chelonia myda*)*;* Loggerhead turtle *(Caretta caretta)*	The most prominent invertebrate in the nest; may correlate with hatching failure and egg damage found in both sand and egg Were found mostly on top of the nest Occurs in nest closer to vegetation			(Aymak *et al.,* 2005; Katılmış *et al.,* 2006)
Corallanidae	*Excorallana spp.*	North-East Tobago, West Indies		Hawksbill turtle (*Eretmochelys imbricata*)	Isolated from eyelid			
Diptera	*Mus cidae*	Dalaman, Turkey		green turtle (*Chelonia myda*)*;* Loggerhead turtle *(Caretta caretta)*	Less damage compared to Pimelia spp. And/or occurs on the egg shells opened by Pimelia larvae			(Katılmış *et al.,* 2006)
	Platystomatidae (Duomyia foliata McAlpine and Plagiostenopterina enderleini Hendel)	central Queensland, Australia		green turtle (*Chelonia myda*)*;* Loggerhead turtle *(Caretta caretta)*	preferentially infest dead embryos and necrotic materials			(Hall and Parmenter, 2006)
	Sarcophagid (Eumacronychia sternali)	pacific coast of Mexico; Michoacán, Mexico			reducing hatching success			(McGowan *et al.,* 2001)

	Sarcophagidae (Phrosinella spp. and Eusenotainia spp.)	Mexico, Turkey		Leather back turtle (*Dermochelys coriacea*); olive ridley (*Lepidochelys olivacea*)	The correlation with hatching failure is not clear			(McGowan *et al.,* 2001)
	Sarcotachina aegyptiaca	Cyprus in the eastern Mediterranean; Pacific coast of Mexico		green turtle (*Chelonia myda*) and Loggerhead turtle (*Caretta caretta*); Hawksbill turtle (*Eretmochelys imbricata*);	Preferentially infest dead embryos; may out-compete other species of insects. In some studies evidences found to correlate with hatching failure		variation in nest temperatures, asynchronous incubation and chemical signals associated with early hatching may attract adult flies; higher temp reduces development time	(McGowan *et al.,* 2001)
	Sarcophaga (Parasarcophaga) crassipalpis, Sarcotachina subcylindric	Mediterranean sea		Loggerhead turtle (*Caretta caretta*), green turtle (*Chelonia myda*)	may be primary factors in the reduction of the success of a nest or only affect moribund eggs or embryos			(Broderick and Hancock, 1997)
	Sarcophagidae (Wohlfahrtia spp.)	Mediterranean sea		green turtle (*Chelonia myda*)	may be primary factors in the reduction of the success of a nest or only affect moribund eggs or embryos			(Broderick and Hancock, 1997)
	Eumacronychia sternalis	east Pacific; Costa Rica; Mexico;		green turtle (*Chelonia myda*); Hawksbill turtle (*Eretmochelys imbricata*);	30% reduction in hatching success in *C. mydas* nests on the east coast of Mexico			(Lopes, 1982)
Hymenoptera	*Formicidae (Ants)*	Rio Grande, Brazil		Hawksbill turtle (*Eretmochelys imbricata*);	Attacking the hatchlings after being hatched, cases of blindness, head and flipper			(da Silva *et al.,* 2016)

					attack; They can attack the embryo and cause still birth; Nest closer to vegetation are more vulnerable			
	Brachymeriapodagarica	Mediterranean Sea		green turtle (*Chelonia myda*)	may be primary factors in the reduction of the success of a nest or only affect moribund eggs or embryos may exert some Natural biological control over the sarcophagid fly infestations.			(Broderick and Hancock, 1997)
Orthoptera	*Gryllotalpidae (Scapteriscus didactylus)*	French Guiana		Leatherback turtle (*Dermochelys coriacea*)	prey on the eggs, up to 40% damage in this study			(Maros *et al.,* 2003)
Phoriodae	*Megaselia scalaris*	Tortuguero, Costa Rica, Mediterranean sea		Hawksbill turtle (*Eretmochelys imbricata*); green turtle (*Chelonia myda*)	Feeding on weak hatchling or dead embryos			(Broderick and Hancock, 1997; Hall and Parmenter, 2006)

Appendix 4. The viruses of sea turtles

Disease hazard		Species/region reported	presence in sea turtle		Outcome of infection (lesion, clinical sign and/or disease) sign in individuals; ease of spread, rate of spread; is a test, quarantine, treatment available to detect the disease?	zoonotic/transmissible to companion animals	Correlation with climatic/anthropogenic events	Key references
			Captive populations	Wild populations				
Herpesvirus	Chelonid fibropapilloma-associated herpesvirus (CFPHV) (*Chelonid alphaherpesvirus 5*)	Reported in tropical and sub-tropical oceans worldwide Found recently in new regions such as Malaysia, Mexico	*Chelonia mydas; Caretta caretta*	(more than 50% of) *Chelonia mydas; and Caretta caretta; Dermochelys coriacea; Eretmochelys imbricate; Lepidochelys kempii; Lepidochelys olivacea; Natator depressa*	External and internal tumors mostly in immunocompromised turtles The excessive growth of tumors can be life threatening and makes the turtle prone to susceptible to secondary infections and opportunistic pathogens; a likely debilitating synergy with spirorchidiasis Increases the risk of entanglement in monofilament line or other debris no significant difference in prevalence between males and females Transmission from a rare disease to a global threat in a short period Detection is based on Molecular analysis and histopathology, non culturable to date	The disease has spread between all sea turtles No evidence has been found to prove transmission to other species rather than sea turtles Not zoonotic	Climate changes, stress and pollution seem to have correlation with disease manifestation	(Herbst *et al.,* 1999; Jones *et al.,* 2016; Lackovich *et al.,* 1999; Work *et al.,* 2004) And many more
	Gray-patch disease (GPD) (Chelonid	Cayman turtle farm	Up to 1 year old *Chelonia mydas*		Circular popular lesion on skin that could spread and be lethal or spontaneously resolved	No data	Overcrowding turtle tanks and higher	(Haines, 1978; Rebell *et al.,* 1975)

	herpesvirus 1				May lead to secondary bacterial infection Control: Strict hygiene and quarantine procedures for a minimum of 3 months		temperature may correlate with the disease	
	lung, eye, trachea disease (LETD) (Chelonid herpesvirus 6)	Florida	*Chelonia mydas*	*Chelonia. mydas; Caretta caretta*	Lesions in eye lung and trachea Mortality can reach 70% Could transmit by direct contact Detection by molecular tool, histopathology and ELISA (antibody detection) The only marine turtle herpes virus successfully isolated in cell culture	No data	The virus may inactivate above 30°C	(Coberley *et al.,* 2001; Curry *et al.,* 2000)
	loggerhead genital-respiratory herpesvirus (LGVR)	Florida		*Caretta caretta*	respiratory and genital lesions diagnosis: histopathology, Molecular analysis on sample from ulcerative tissue	Possible vector: Marine leeches		(Stacy *et al.,* 2008)
	loggerhead orocutaneous herpesvirus (LOCV)	Florida		*Caretta caretta*	oral and cutaneous lesions diagnosis: histopathology, Molecular analysis on sample from ulcerative tissue	Possible vector: Marine leeches		
Papillomavirus	*Chelonia mydas* papillomavirus 1 (CmPV-1)	East Central Coast of Florida		*Chelonia. mydas*	Skin lesions resolved after several months and left scars/pitted skin Histopathology and PCR	Generally species specific		(Herbst *et al.,* 2009; Manire *et al.,* 2008)
	Chelonia mydas papillomavirus and *Caretta caretta* Papillomavirus isolates	North East Australia		*Chelonia. Mydas; Caretta caretta*	Green and loggerhead turtle skin tumours along with ChHV5 The possible correlation with FP is under investigation			(Mashkour *et al.,* 2018)
	Caretta caretta Papillomavirus 1 (CcPV-1)	North East Florida		*Caretta caretta*	Skin lesion, resolved after several months Histopathology and PCR	Generally species specific		(Herbst *et al.,* 2009; Manire *et al.,* 2008)

Retrovirus		Hawaiian islands		*Chelonia mydas* with FP	Incidental finding, no prove for correlation with FP disease, no correlation with any clinical disease Was found with laboratory test: conventional reverse transcriptase assay			(Casey *et al.,* 1997)
Tornovirus	sea turtle tornovirus 1 (STTV1)	Lake Worth Lagoon, Forida		*Chelonia mydas* with FP	Different variations of virus have been found in infected turtles No correlation with FP (it hasn't been found in most FP infected turtles) but may cause co-infections, may be commensal to turtle or an opportunistic infection			(Ng *et al.,* 2009)
Betanodavirus		Tuscany, Italy		*Caretta caretta*	Isolated from eye and lung No evidence of pathogenic roles in sea turtles yet Chlorine can inactivate the virus	Can infect teleost fish and cause nervous necrosis Can infect marine invertebrates Sea turtles can be a carrier for this virus	Higher temperature can inactivate the virus	(Fichi *et al.,* 2016)

Appendix 5. Non-infectious diseases of sea turtles

	Health Problem	Region Reported	Species Affected		Explanation: Is the aetiology clear? The effect on individuals, population? Is there any treatments? Is mortality, morbidity reported?	Key References
			Captive Population	Wild Population		
Physical problems	Injuries	All regions	*	*	-Due to predator bites, by-catch or accidents. Can happen quite often and lead to infection, minor scars and/or deep wounds. Mortality may occur if the injury is traumatic. -Appropriate modifications to vessel operation and configuration can reduce the threats. -Aggressive males may bite females during mating Captive turtles are prone to injuries in overcrowded facilities. -Existence of rehabilitation centres in the area to surrender injured or caught turtles for healing period followed by releasing may help the population	(Crane, 2013; Gilman *et al.,* 2006; Work *et al.,* 2010)
	Missing organs	All regions		*	-Loss of an entire flipper or two is seen in sea turtles -Predation and accident can cause amputation -Mono-filament fishing lines can tie around the flipper an lead to injuries or amputation -Female turtles missing one or two flippers can successfully come ashore for nesting -If the injury is fresh the creatine kinase (CK) value is high due to muscle damage	(Deem *et al.,* 2006)
	Carapace/Plastron disorders				-Fracture and traumatic erosion is common and happens due to predation or accident -Traumatic injuries in carapace can cause lesions in lungs or kidneys -Wound management is required according to established protocols (see reference (Mettee, 2014b))	(Orós *et al.,* 2005)

					-Deformation and lack of ossification is also observed	
	Buoyancy problems	All regions		*	-The disability to submerge make the turtle prone to predation, accidents and being washed ashore -The common cause is trapped gas in intr-acoelomic cavity In GI: due to ingestion of foreign bodies, obstructions, intusseseption or constipation/obstipation In lung: due to pneumonia (generally pulmonary diseases), trauma leading to lung tear or internal tumors -Spinal cord or brain trauma can also cause buoyancy disorder -The metabolic cost of breathing is much higher for positive buoyant turtles -If the buoyancy disorder lasts for more than a month the turtle is not allowed to be released and to avoid lung malformation external weights should be applied to normalise flotation	(Mettee, 2014a; Orós *et al.,* 2005; Schmitt *et al.,* 2005)
Nutritional problems	Malnutrition	All regions	*	*	-Physical damages, high parasitic load or suffering from chronic diseases can lead to loss of appetite, nutritional deficiencies and cachexia -Anthropological or natural habitat changes can negatively alter the nutrient intake -Brevetoxin intoxicated turtles show low foraging activities that can lead to malnutrition -Morphologically the animal has concave plastron, sunken eyes and muscular atrophy -Blood test can exhibit anemia, hypoproteinemia and hypoglycemia -In captivity, improper diet can cause GI obstruction and malnutrition	(Fauquier *et al.,* 2013; Lutz and Musick, 1996)
	Metabolic bone disease		*		-low levels of calcium and high phosphorus in the diet can induce demineralization of the bones and even lead to fracture -Gelatin based diets, ultraviolet radiation and vitamin D3 supplements can help reducing the sign	(Lutz and Musick, 1996)

	Iron deficiency		*	Kemp ridely (Lepidochelys kempii), Loggerhead turtle (Caretta caretta)	-Anemic turtles can be found listless, floating and hyperventilating -In blood tests, packed cell volumes (PCVs) are lower than normal -In captivity, diets only based on fish and squid reported to develop anemias	(Lutz and Musick, 1996)
Environmental factors	Climate change consequences	*		*	In case of alteration in marine biodiversity, sea turtles' nutrition will be affected	(Hawkes *et al.,* 2009)
	Hypothermic stunning	Gulf of mexico, new England, Western Europe, Persian Gulf, Indian Lagoon, Florida		green (*Chelonia mydas*), Kemp's ridley (*Lepidochelys kempii*), loggerhead (*Caretta caretta*), and hawksbill (*Eretmochelys imbricata*)	-Large numbers of stranding is reported due to cold stunning -When a turtle is exposed to low temperature water and this situation lasts for a while, it might affect the turtle in different way (especially smaller turtles): water temperature=10°C floating and not able to function, decreased heart rate, decreased circulation Water temperature=5to 6°C shock, pneumonia, death may occur. -Salt glands functionality may decrease due to cold stunning -Shallow waters can show more temperature fluctuations -Rehabilitation is helpful: depending on the condition fluid therapy, antimicrobial treatment, medications, and prolonged care maybe required	(Davenport, 1997; Shaver *et al.,* 2017)
Anthropogenic problems	Entanglements	*		*	-Entanglement in man-made objects such as fishing gears can cause minor and major injuries -If the entanglement keeps the turtle submerged for a while anoxia can occur -Due to interactional behavior of sea turtles, flipper entanglement is more probable -In pelagic stages, entanglement in buoyant objects is happening frequently	(Adimey *et al.,* 2014; Carr, 1987)
	Accidents and injuries				-Collision with maritime traffic: fishing vessels, (oil and cargo) tankers, beach leisure facilities happens in different regions -It can cause mortality or injuries The injuries may be minor or major (See Physical problems>Injuries for more detailes)	(Mendonca and Abi-Aoun, 2009)

	By-catch injuries	All regions	Not applicable	All species	-Trawl and grill net and fishing lines can keep the turtle submerged for a long time and put the turtle in the risk of drowning or an anoxic state -Loggerheads interact with more fisheries than any other sea turtle species. A loggerhead turtle was reported injured by an eagle ray spine in a trawl net in Florida. -These methods may help reducing the risk: turtle exclusion devices (TEDs); larger round shape hooks; using fish bait; avoiding bycatch hotspots; setting the gears deeper, reducing the soaking time and retrieving during the day	(Adimey *et al.,* 2014; Bezjian *et al.,* 2014; Gilman *et al.,* 2006)
	Debris ingestion			* green turtles (herbivorous) and leatherback turtles (gelatinivorous), are more likely to ingest debris Kemp ridely is less likely to ingest debris	-Turtle ingest debris frequently and these foreign objects may affect the health condition -Young turtles in pelagic stages are at higher risk of eating or entangling in buoyant wastes -Plastic is one of the main debris, tar and crude oil are the other examples -Debris can block the GI and accumulate intestinal gas; cause local ulcerations; interfere with metabolism; be toxic for the body	(Brodie *et al.,* 2014; Camedda *et al.,* 2014; Carr, 1987)
	Problems with chemical and organic pollutant			Green and hawksbill are more prone to be impacted by oil splits	-Chemical pollutants are not biodegradable and normally persist in the release site. Such pollutants like oil can cause fouling, entanglement, external necrosis and skin sloughing, it also can interfere with salt gland functionality -Agricultural waste can elevate the nutrient level in the ocean and stimulate harmful algal and cyanobacterial blooms. This incidence can directly harm the turtles (stranding and mortality is also reported) or correlate with other diseases such as Fibropapillomatosis -High concentration of PCBs, OC insecticides, persistent organic pollutants, algal toxins, metals and trace elements can cause a variety of adverse health effects such as immunosuppression /immunostimulation	(Brodie *et al.,* 2014; Deem *et al.,* 2009; Fauquier *et al.,* 2013)

					-Immunosuppressive cofactors are likely to correlate with fibropapillomatosis	
Medical problem	Neurological disease			Brevetoxin in Florida Loggerhead, *carettaa caretta* green, *Chelonia mydas*, and Kemp's ridley, *Lepidochelys kempii*	-Diagnosis is not easy but neurological signs can be atypical behaviors such as circling or nonresponsiveness, head bobbing, muscle twitching, and jerky body movements -Neurologic spirorchiidiasis is reported in loggerheads. Normally diagnosis is postmortem -Brevetoxin produced by dinoflagellate can cause neurological signs. Stranding and mortality is also reported Dehydration, systemic antihistamine treatment and supportive care seems to be helpful in this situation -Pollutant metals can induce lesions in the central nervous system	(Deem *et al.,* 2009; Flint *et al.,* 2010; Jacobson *et al.,* 2006; Manire *et al.,* 2013)
	Pulmonary disease	Frequently reported	*	*	-Pneumonia is a common pulmonary disease, the aetiology can be infectious or non-infectious -Traumatic injuries in the carapace can also lead to pulmonary lesions -Pulmonary diseases can lead to buoyancy disorders	(Orós *et al.,* 2005)
	Debilitated Turtle Syndrome			* Loggerhead turtles *Chelonia mydas* and Lepidochelys olivacea	-The cause is unknown, cold stunning can be an initial cause -End stage disease with following signs: emaciated, lethargic, hypoglycemic, anemic, and heavily covered with epibiota Secondary infections may also occur, Turtle may be immunosuppressed -Rehabilitation can be helpful: fluid therapy, treating anemia, hyperglycemia, antimicrobial treatments, nutritional supports, medical treatments due to diagnosed internal problems	(Fernández *et al.,* 2015; Norton, 2014; Sloan, 2011)
	Immunomodulation					

*The information is not enough or the species/region is not reported.

Appendix 6. The handbook for Sea Turtle Disease Risk Analysis

The workshop objective is to prioritise the importance of infectious and non-infectious diseases for conservation, surveillance and research.

Step 1: Forming small groups for discussions (5 minutes)

You will work in small groups to carry out a sea turtle disease risk analysis (DRA) following the process outlined in this workbook.

You will have about 5 minutes to become acquainted with your group members and their contributions to sea turtle health.

Step 2: Hazard identification (15 minutes)

During this step of any health risk assessment you would normally use books, journals and many more resources to gather information about infectious and noninfectious diseases that threaten the animals' survival.

For this workshop this information is being provided for you.

Note: The list provided here is based on current shared knowledge and there are many more bacterial, viral, fungal and parasitic infections, present and influencing disease in sea turtles. The exact epidemiology and clinical manifestation of many of the pathogens that are known to affect sea turtles are not thoroughly understood. The same applies to noninfectious diseases where the aetiology and the influence on the population may not be completely studied.

To make the process easier, you will see:

- Non-infectious diseases are described in one table.
- The pathogens have been categorised in four different tables of bacteria, fungi, parasites and viruses.
- **Each group will only evaluate one category.**

Read the information carefully about sea turtle health hazards (Material 1: Pathogen tables and health problems table).

Step 3: Hazard ranking (60 minutes)

We will work on each part and will have a discussion after this step.

Considering the time and budget restraints to do a full health assessment on all of these hazards, if you must choose only three hazards that represent the highest potential risk to sea turtle health, which three would you select? Fill the table below and write why your group has chosen these three hazards.

Hazards	Reasoning
1.	
2.	

3.

4.

5.

Step 4: One Health (30 minutes)

A successful DRA should consider the study population in the context of the environment. Disease exposure not only affects a population but also the habitat, humans and companion animals. "*One Health*" considers the inter-dependent health of the environment, humans and animals.

Use *Material 1: Pathogen table* to evaluate the risk associated with physical contact with sea turtles. This can be from a human's perspective; for example, the risk of transferring infections through meat and egg consumption. The other aspect is the risk of pathogen transmission from humans to sea turtles; for example, handling the turtles in rehabilitation centres or nest relocation.

We will work on this in our groups and will have a discussion after this step.

If you are working on pathogen tables answer questions 1 to 4 and if you are working on non-infectious health problems answer question 5 and 6:

1. What is the main zoonotic pathogen of concern in your group?

2. Are there any pathogens being transferred from human to sea turtles?

3. What is the main problematic pathogen in captivity?

4. Are there any pathogens to be considered as a risk for aquaculture?

5. What is your insight about the cultural dimensions of interacting with sea turtles?

6. What is the socio-economic advantage of sea turtle conservation?

Step 5: General discussion about sea turtle health (10 minutes)

Now that we have finished reviewing sea turtle health hazards we can start a general conversation about putting conservation in practice. In other words, how can we use this information to help conservation?

Appendix 7. The handbook for management workshop

Introduction to group members

Introduction to DRA presented by Narges Mashkour.

Part one: Reviewing the risk assessment outcomes from previous workshops

Infectious health hazards	
Parasite	*Spirorchiidae*
	Annelids
	Arthropods
Virus	Herpesvirus in association with fibropapillomatosis
	Papillomavirus
Bacteria	*Enterobacteriaceae* and antibiotic resistant bacteria
	Strep iniae, salmonella typhimurium, Ecoli
	Pseudomonas spp. Klebsiella
Fungi	*Fusarium solani*
	Penicillium spp.
	Cladosporium spp.
Non-infectious health hazards	
	Anthropogenic (Plastic, by-catch, boat strike)
	Environmental
	Medical

Part two: Defining management options

In this section “management options” will be proposed and for each option the level of “effectiveness” and “feasibility” is ranked **from 1 being the least feasible/effective to 10 the most feasible/effective**. Desirably, based on these criteria a decision about that option is made.

Non-infectious Health hazards: Macro plastic

Management Option	Effectiveness	Feasibility	Decision

Infectious Health Hazard: *Enterobacteriaceae* and antibiotic resistant bacteria

Management Option	Effectiveness	Feasibility	Decision

Part three: Critical control points for a mock translocation scenario and diagram

Consider a scenario for translocating a clutch of egg from Raine Island to mainland. Discuss the high and medium priority critical control points.

Appendix 8. Mock Clutch Translocation

8.1. Problem description

A clear problem description that is not influenced by political and social issues would help the efficiency of DRA (Hartley and Sainsbury, 2017). The scope is determined through precise information such as scientific name, exact locations, and number of animals to be translocated and the frequency of such actions. The goal of the risk analysis should be stated **clearly** (Jakob-Hoff *et al.,* 2014). For example, for clutch translocation to identify and assess the likelihood of the hazard(s) being introduced and spreading or becoming established in (the area of translocation)'.

Information to assist in identifying hazards, assessing risks and exploring options to manage risk can be found in the current DRA.

8.2. Risk communication

A risk communication strategy should be developed for two purposes: 1) identifying interested parties (stakeholders and experts); 2) defining the timing and the means of communication with them (Jakob-Hoff *et al.,* 2014).

8.3. Hazard identification

The hazards likely to be associated with the species under consideration should be identified. (The hazard identification process is not as exhaustive as explained in appendices 1-5. Only putative disease-causing-hazards are considered that relate to the population, the region and the scenario under

consideration). An example for clutch translocation is shown in following table, this table can vary for different translocation scenarios, regions and species of marine turtles. Table 1 is derived from appendices 1-5, see these sections for references.

Table1. Potential disease hazards for sea turtle egg clutch translocation

Infectious Hazards			
Fungi	Parasites	Gram Negative Bacteria	Gram Positive Bacteria
Allescheria spp. *(Pseud)allescheria boydii* *Absidia* *Cephalosporium* *curtipes var·* *uredinicola* *Cladosporium sp* *Chrysosporium* *Cunninghamella* *Cylindrocarpon* *Emericella* *Fusarium solani* *Homodendrum* *Saksenaea vasiformis* *Scedosporium* *aurantiacum* *Thielavia*	*Diplotesticulata, Oligochaeta* ***Coleoptera*** *Elater spp., Lanelater sallei, Omorgus suberosus, Pimelia sp* ***Diptera*** *Sarcophaga (Parasarcophaga) crassipalpis, Sarcotachina subcylindric* ***Orthoptera*** *Gryllotalpidae (Scapteriscus didactylus)*	*Citrobacter freundii* *Citrobacter youngae* *Enterobacter cloacae* *Morganella* *morganii* *Proteus penneri* *Proteus vulgaris* *Pseudomonas spp.* *Serratia odorifera* *Vibrio mimicus*	*Bacillus spp* *Enterococcus spp*
Non-infectious hazards			
Terrestrial predators Poaching			

Marine predators

Considering the hazard table (table 1) the following questions should be answered to decide if a risk assessment is required. **There should be sufficient capability and confidence to rule out the presence of pathogens, or to claim that they are not hazardous** (Jakob-Hoff *et al.,* 2014).

"3.2 Is the live animal or germplasm under consideration a potential vehicle for the pathogenic agent?

3.3 Is the pathogenic agent present in the area from which the animals or germplasm are sourced?

3.4 Are there zones from which the animals or germplasm will be sourced that are free of the pathogenic agent?

3.5 Is the pathogenic agent already present in the area to which animals or germplasm are to be translocated and which will be affected by the planned activity?" (Page 47, Jakob-Hoff *et al.,* 2014).

8.4. Risk assessment

Risk assessment should be conducted for each hazard and the populations of interest. For example, for clutch translocation, potentially susceptible species may be terrestrial and aquatic predators as well as humans if the hazard has zoonotic potential. High risk pathogens should be selected. The criteria by which the hazard is selected depends on the translocation situation and can be done through expert workshops, paired rankings or scenario trees. Table 2 is an example of risk assessment using paired ranking. Table 2 is only an example and some pathogens such as Coleoptera (beetles) may not be an issue in some regions. The health risk ranking should be done through expert ranking and the risk can vary from negligible to minimal, moderate or high.

Table2. The health risk of pathogen for translocated eggs

Pathogen	Health risk	Evaluation
Fungi	High Or Moderate Or Minimal Or Negligible	
Fusarium solani		Widely distributed, associated with mass mortalities in relocated nests. zoonotic
Cladosporium sp		Hatching failure is reported. Not enough data
Parasites		
Coleoptera		Different species of beetles are found in nests
Diptera		Dead-decayed eggs were reported to be attacked by flies.
Bacteria		
Enterobacter		Widely spread component of gastrointestinal flora; zoonotic
Citrobacter freundii		A confirmed contaminant of eggs. Poses risk in translocation to the handler and local wildlife.

A scenario tree can also be drawn to identify the various biological (risk) pathways leading to the translocated animals and also susceptible animals or humans being exposed to translocated animals. Figure 1 shows an example of a scenario tree for clutch translocation. In this scenario tree, release assessment was also considered to be able to estimate the impact on the environment and ecosystem. When the assessment is done the critical control points should be defined and ranked with high or medium priority (red triangles in figure 1).

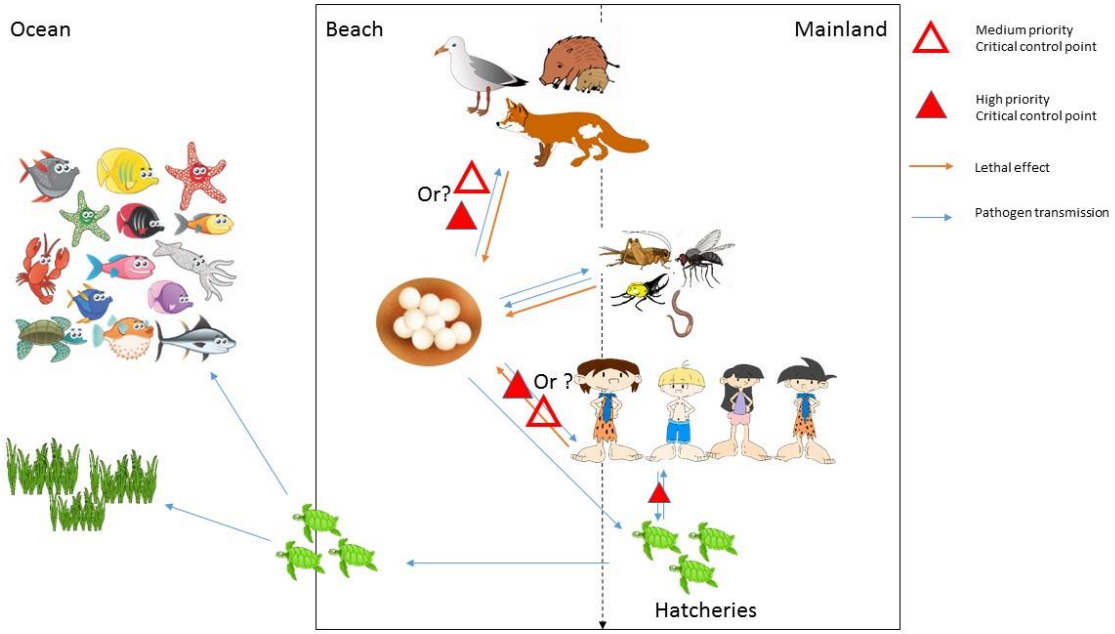

Figure1. Possible pathogen transmission and lethal effects on sea turtle eggs and hatchling and application of critical control points.

The results or conclusions from each "*risk assessment*" procedure should be summarised and provided for risk management (Risk estimation).

8.5. Risk management

Risk evaluation: If the risk estimate is greater than the acceptable level for stakeholders, options for mitigating the risk should be evaluated.

Option evaluation and selection: An objective will be formulated that clearly defines the risk mitigations and is technically, operationally and economically feasible and ideally is based on scientific principles and a risk analysis (Jakob-Hoff *et al.,* 2014).

8.6. Implementation

The final decision should be made to implement the risk mitigation measure(s). The overall procedure needs to be followed up by monitoring and review. In some cases if the risk is high and there is no applicable measures to mitigate the risk the translocation may be abandoned.

Appendix 9. The viral loads of the tumour tissues

Sample	Cq	Calculated Concentration: copy number pe reaction (2 microliter)	Sample	Cq	Calculated Concentration: copy number pe reaction (2 microliter)	Sample	Cq	Calculated Concentration: copy number pe reaction (2 microliter)	Copy number per cell	
pCmPV-E1			pChHV5-Dpol			pGAPDH			pCmPV-E1	pChHV5-Dpol
CB83FP			CB83FP	21.1	1400048.781	CB83FP	23.05	109745.482	0	25.51446775
CB93FP			CB93FP	22.12	878573.1218	CB93FP	22.75	132187.0975	0	13.29287258
CB94FP			CB94FP	22.49	736462.9071	CB94FP	24.12	56987.51198	0	25.84646641
CB95FP			CB95FP	41.2	102.1897954	CB95FP	36.19	34.12850754	0	5.988529985
CB96FP			CB96FP			CB96FP	26.75	11295.93774	0	0
CB97FP			CB97FP	24.41	296245.1397	CB97FP	24.71	39526.73865	0	14.98960703
CB98FP			CB98FP	44.6	20.40152056	CB98FP	25.56	23485.08794	0	0.001737402
CB99FP1			CB99FP1	23.14	540504.6625	CB99FP1	22.33	171407.7509	0	6.306653693
CB99FP2	37.19	54.6728344	CB99FP2	23.69	417018.5785	CB99FP2	23.07	108785.4873	0.001005149	7.666805357
CB100FP?			CB100FP?	44.49	21.44204264	CB100FP?	31.33	677.4223531	0	0.063304798
CB101FP1	31.75	384.6	CB101FP1	24.54	652100	CB101FP1	23.47	136000	0.005655882	9.589705882
CB101FP2	31.32	871.7	CB101FP2	22.08	3186000	CB101FP2	22.42	194900	0.0089451	32.69368907
CB101FP3	37.74	37.96307389	CB101FP3	22.02	921489.5968	CB101FP3	21.91	220937.9126	0.000343654	8.341615849
CB102FP1	33.36	702.1854418	CB102FP1	32.86	5360.51066	CB102FP1	23.85	67088.32587	0.020933163	0.159804574
CB102FP2	35.85	133.3025839	CB102FP2	32.44	6554.884259	CB102FP2	23.94	63568.34527	0.004193993	0.20623108
CB102FP3	30.16	5045	CB102FP3	27.16	1125	CB102FP3	25.07	64680	0.155998763	0.034786642
CB103FP1	34.25	88.65	CB103FP1	24.87	249500	CB103FP1	23.13	257500	0.000688544	1.937864078
CB103FP2	35.14	214.8987852	CB103FP2	23.6	434832.9644	CB103FP2	22.93	118051.5232	0.003640763	7.366833609
CB103FP3	35.55	163.0797526	CB103FP3	24.9	234421.1233	CB103FP3	24.52	44396.27703	0.007346551	10.56039555
CB104FP1			CB104FP1	22.29	808530.1955	CB104FP1	22.52	151929.5974	0	10.643485
CB104FP2			CB104FP2	26.08	134093.741	CB104FP2	26.84	10722.72351	0	25.0111347

CB104FP3	37.24	52.90798329	CB104FP3	24.8	245959.9869	CB104FP3	24.22	53662.31291	0.001971886	9.166954367
CB105FP1	36.73	74.38659681	CB105FP1	23.35	489490.7393	CB105FP1	22.57	147616.5777	0.001007835	6.631920981
CB105FP2	34.3	19.25	CB105FP2	24.25	469800	CB105FP2	21.6	295900	0.000130112	3.175397094
CB106FP1	36.6	80.92030092	CB106FP1	25.38	186848.6155	CB106FP1	24.85	36285.01739	0.00446026	10.29894039
CB106FP2	34.5	328.3374066	CB106FP2	24.39	298552.9124	CB106FP3	25.12	30872.87331	0.021270285	19.34079212
CB106FP3	37.84	35.47350732	CB106FP3	26.32	119518.3344	CB106FP2	24.17	55331.86893	0.001282209	4.320054127
CB107FP1			CB107FP1	23.44	470056.8638	CB107FP1	23.14	104041.1657	0	9.035978418
CB107FP2			CB107FP2	22.37	780593.9994	CB107FP2	19.24	1140585.018	0	1.368760745
CB107FP3	38.63	20.87030313	CB107FP3	24.74	252964.2795	CB107FP3	20.08	682013.6321	6.1202E-05	0.741815904
CB108FP1			CB108FP1	38.32	198.2660174	CB108FP1	27.33	9170.036412	0	0.043242144
CB108FP2	37.36	17.05146573	CB108FP2	38.16	214.5014009	CB108FP2	25.58	26865.93887	0.001269374	0.015968279
CB108FP3			CB108FP3	42.76	23.16679909	CB108FP3	30.41	1384.618455	0	0.03346308
CB109FP1	26.68	694.8	CB109FP1	24.21	195500	CB109FP1	19.36	1549000	0.000897095	0.252420917
CB109FP2	32.36	477.2608003	CB109FP2	25.55	95185.50273	CB109FP2	24.28	59923.14797	0.015929096	3.176919303
CB109FP3	32.76	367.4269812	CB109FP3	25.86	81824.71332	CB109FP3	24.71	46010.18118	0.015971551	3.556809003
CB110FP1			CB110FP1?	22.15	491515.1013	CB110FP1	22.52	176833.8079	0	5.55906257
CB110FP2	36.67	27.00222199	CB110FP2+CB111N!	25.12	116724.7147	CB110FP2	24.58	49669.29144	0.00108728	4.700075695
CB110FP3	33.63	205.3986292	CB110FP3	21.15	795979.1509	CB110FP3	23.96	72820.46818	0.005641233	21.86141261
CB111FP1			CB111FP1	22.02	522285.4042	CB111FP1	23.49	97321.2027	0	10.73322955
CB111FP2			CB111FP2	22.01	526739.0007	CB111FP2	23.49	97293.37677	0	10.82784909
CB111FP3			CB111FP3	21.65	626337.6126	CB111FP3	22.94	136207.9449	0	9.1967853
CB112FP1			CB112FP1	32.55	3219.140503	CB112FP1	35.47	61.73183365	0	104.2943426
CB112FP2			CB112FP2	28.43	23636.45108	CB112FP2	30.34	1442.774656	0	32.7652707
CB112FP3			CB112FP3	26.39	63443.50607	CB112FP3	28.75	3841.370131	0	33.03170687
CB113FP1			CB113FP1	37.93	239.0366687	CB113FP1	35.93	46.51104798	0	10.27870491
CB113FP2			CB113FP2	39.01	141.8268039	CB113FP2	34.94	85.73170137	0	3.308619838
CB113FP3			CB113FP3	33.56	1974.157853	CB113FP3	33.07	270.6072041	0	14.5905787
CB114FP1	35.47	59.85473766	CB114FP1	20.08	1338103.303	CB114FP1	22.76	152346.9864	0.000785769	17.56652146
CB114FP2	32.28	88.32	CB114FP2	20.59	2134000	CB114FP2	22.76	262900	0.00067189	16.23430962

CB114FP3	33.47	227.4780431	CB114FP3	22.21	478559.1843	CB114FP3	22.99	132242.7493	0.00344031	7.237586736
CB115FP	NA		CB115FP	35.34	836.8712639	CB115FP	25.56	27311.15381	0	0.061284212
BW99FP	33.62	206.0745297	BW99FP	24.72	141624.3677	BW99FP	24.2	62956.17473	0.006546603	4.499141454
BW01FP	30.4	523.4	BW01FP		8.714	BW01FP	24.72	78790	0.01328595	0.000221196
BW107FP	36.21	36.66759807	BW107FP	19.24	2008167.135	BW107FP	21.49	332241.647	0.000220728	12.08859367
BW108FP	38.07	10.60037167	BW108FP	32.34	3566.9259	BW108FP	26.27	17655.55486	0.001200797	0.404057072
BW116FP	36.53	29.57064361	BW116FP			BW116FP	30.26	1517.904677	0.038962451	0
BW117FP			BW117FP			BW117FP	32.78	323.4764779	0	0
BW118FP	30.34	535.6	BW118FP	33.22	393.3	BW118FP	24.14	1551000	0.000690651	0.000507157
BW119FP	32.07	222	BW119FP	37.77		BW119FP	26.07	18500	0.024	0
BW120FP			BW120FP	29.35	32681.30064	BW120FP	31.7	664.4832939	0	98.36605657
BW121FP			BW121FP			BW121FP	25.68	26796.37404	0	0
BW122FP	31.74	177.6	BW122FP		15.31	BW122FP	25.12	84850	0.004186211	0.000360872
BW123FP			BW123FP	24.1	381673.2175	BW123FP	25.08	38733.69955	0	19.70755295
BW124FP1	32.38	47.64	BW124FP1	23.83	55650	BW124FP1	21.25	300600	0.000316966	0.370259481
BW124FP2	35.1	124.4032283	BW124FP2	31.98	9514.501549	BW124FP2	25.08	38719.78658	0.006425822	0.491454235
BW125FP?	35.56	91.28410744	BW125FP?			BW125FP?	30.79	1160.758819	0.157283504	0
BW126FP1	31.23	139	BW126FP1	33.49	540	BW126FP1	24.46	63690	0.004364892	0.016957136
BW126FP2			BW126FP2	38.26	504.0661459	BW126FP2	25.35	32806.77569	0	0.030729393
BW127FP			BW127FP	40.94	144.0536022	BW127FP	32.28	465.1104798	0	0.619438213
BW128FP	33.87	282.9769781	BW128FP	31.29	13146.20703	BW128FP	24.46	56709.25264	0.009979923	0.463635348
BW129FP	36.23	58.69068693	BW129FP			BW129FP	30.94	1059.88981	0.110748658	0
BW130FP	37	34.98911202	BW130FP	21.17	1500457.138	BW130FP	21.12	441319.3066	0.000158566	6.799870821
BW131FP			BW131FP	22.03	1001654.333	BW131FP	23.06	134246.2166	0	14.92264525
BW132FP			BW132FP	21.53	1266036.015	BW132FP	24.21	66100.50522	0	38.30639449
BW134FP			BW134FP	36.48	1161.983806	BW134FP	32.43	423.0933201	0	5.492801474
BW135FP			BW135FP	30.49	19154.51354	BW135FP	31.35	823.3693747	0	46.52714598
BW136FP	37.16	31.38806475	BW136FP	22.09	977766.8613	BW136FP	23.32	114030.6758	0.00055052	17.14918998
BW137FP			BW137FP	22.77	711360.8179	BW137FP	24.55	53662.31291	0	26.51249189

BW138FP	35.55	92.18530801	BW138FP			BW138FP	33.51	218.294449	0.844595989	0
BW139FP	35.73	81.82150148	BW139FP	23.41	526334.1282	BW139FP	23.29	116521.0969	0.001404407	9.034143041
BW140FP	33.24	18.1	BW140FP	36.19		BW140FP	18.5	173400	0.000208766	0
BW141FP			BW141FP			BW141FP	31.53	735.9959432	0	0
BW142FP			BW142FP	22	1015824.868	BW142FP	23.34	112528.0754	0	18.05460306
BW143FP	36.51	48.73993067	BW143FP	31.77	10522.63384	BW143FP	25.89	23596.39168	0.004131134	0.891884995
BW144FP			BW144FP	20.8	1783867.822	BW144FP	25.13	37523.27144	0	95.08061286
BW145FP			BW145FP	36.88	959.547603	BW145FP	35.27	73.93350553	0	25.95704332
BW146FP?			BW146FP?	33.07	5716.798378	BW146FP?	23.28	116980.2248	0	0.097739569
BW147FP?			BW147FP?			BW147FP?	36.25	40.52847226	0	0
BW148FP?			BW148FP?			BW148FP?	34.5	118.6636938	0	0
BW149FP?			BW149FP?			BW149FP?	33.51	218.5727083	0	0
BW150FP?			BW150FP?	41.53	108.8701901	BW150FP?	33.19	265.181147	0	0.821100529
BW151FP			BW151FP			BW151FP	36.33	38.62239581	0	0
BW151FP?			BW151FP?			BW151FP?	32.27	466.9191655	0	0
9231			9231	16.62	7890963.2	9231	19.77	1897728.67	0	8.316218565
QA15979	31.09	691.5	QA15979	16.1	67500000	QA15979	20.46	1073000	0.00128891	125.8154706
QA15980	29.34	700.6	QA15980	20.78	5460000	QA15980	21.5	630000	0.002224127	17.33333333
QA4962			QA4962	19.31	3593647.479	QA4962	21.12	440901.9176	0	16.30134656
K52464	31.14	989.7	K52464	29.19	6731	K52464	21.72	457500	0.004326557	0.029425137
K92663	37.29	56.70053568	K92663	20.83	994366.63	K92663	21.4	649735.5491	0.000174534	3.060834924
K93038	34.85	289.6608823	K93038	30.04	10591.46215	K93038	22.15	396380.4239	0.00146153	0.053440894
K93074			K93074	20.71	1050643.894	K93074	19.12	2893897.093	0	0.726110059
K93640			K93640	18.29	3464088.309	K93640	19.89	1747468.629	0	3.964692987
K97483			K97483	17.9	4198526.854	K97483	22.31	357145.8575	0	23.51155286
K97625	22.71	273600	K97625	33.54	229.2	K97625	21.18	519700	1.052915143	0.000882047
MB01FP1			MB01FP1	18.36	3357201.993	MB01FP1	20.2	1426079.096	0	4.70829704
MB01FP2	33.07	138.2	MB01FP2	20.53	2960000	MB01FP2	22.72	262700	0.001052151	22.53521127
MB01FP3	33	76.03	MB01FP3	20.13	3597000	MB01FP3	23.05	239200	0.000635702	30.07525084

MB03FP1			MB03FP1	20.22	1341747.155	MB03FP1	22.22	380937.0307	0	7.044456413
MB03FP2			MB03FP2	20.65	1085462.921	MB03FP2	22.04	428380.2475	0	5.067754304
MB03FP3			MB03FP3	19.63	1793179.888	MB03FP3	23.46	168068.6388	0	21.3386614
MB04FP1			MB04FP1	20.03	1473330.687	MB04FP1	21.41	644726.8811	0	4.570402538
MB04FP2			MB04FP2	19.77	1671718.166	MB04FP2	22.27	366606.6749	0	9.119954873
MB05FP			MB05FP	22.11	526739.0007	MB05FP	23.85	130350.5859	0	8.081881599
MB06FP1			MB06FP1			MB06FP1	31.13	1102.324359	0	0
MB07FP1	38.2	30.86611942	MB07FP1	32.77	2760.824939	MB07FP1	36.94	24.33377892	2.536894868	226.9129631
GS52FP1	31.93	465.8	GS52FP1	31.44	873.4	GS52FP1	23.17	172900	0.005388086	0.01010295
GS72FP	38.23	30.16768898	GS72FP	25.03	124822.1629	GS72FP	25.01	60702.27411	0.000993956	4.112602525
GS73FP			GS73FP			GS73FP	24.93	63999.64724	0	0
GS74FP			GS74FP			GS74FP	24.47	87067.34618	0	0
CN01FP1	34.89	280.9117268	CN01FP1	15.26	15494466.99	CN01FP1	22.6	295511.4146	0.00190119	104.8654382
CN01FP2	37.42	52.11943279	CN01FP2	17.94	4125649.821	CN01FP2	20.15	1471991.886	7.08148E-05	5.605533371
CN02FP1	32.07	150.3	CN02FP1	24.97	198700	CN02FP1	25.63	53480	0.005620793	7.430815258
SW01FP1			SW01FP1	20.04	1464828.366	SW01FP1	20.18	1452513.733	0	2.016956305
SW01FP2			SW01FP2	20.44	1200041.812	SW01FP2	21.64	555127.375	0	4.323482741
SW02FP1			SW02FP1	22.56	421877.0474	SW02FP1	24.84	68090.05947	0	12.39173679
SW02FP2	36.99	69.20469355	SW02FP2	18.33	3409430.534	SW02FP2	23.81	133954.0443	0.00103326	50.90448075
AB01FP1			AB01FP1	21.8	615406.0576	AB01FP1	22.3	360484.9696	0	3.414322979
AB01FP2	35.7	163.9058531	AB01FP2	22.58	419042.9406	AB01FP2	22.37	345041.5764	0.000950064	2.428941723
AB01FP3	37.26	57.86458641	AB01FP3	21.21	822700.7297	AB01FP3	22.15	397632.5909	0.000291045	4.137994463
AB01FP4			AB01FP4	21.1	868046.4392	AB01FP4	22.1	409597.7423	0	4.238531366

Appendix 10. The viral loads of normal skin

Sample	Cq	Calculated Concentration: copy number pe reaction (2 microliter)	Sample	Cq	Calculated Concentration: copy number pe reaction (2 microliter)	Sample	Cq	Calculated Concentration: copy number pe reaction (2 microliter)	Copy number per cell	
pCmPV-E1			pChHV5-Dpol			pGAPDH			pCmPV-E1	pChHV5-Dpol
CB83N			CB83N			CB83N	31.88	152.7934946	0	0
CB99N			CB99N	41.2	3.753341242	CB99N	26.21	4984.502474	0	0.001506004
CB105N			CB105N	44.98	0.62496252	CB105N	30.39	381.9397037	0	0.003272572
CB101N	35.11	7.092682819	CB101N			CB101N	31.07	251.6028899	0.056379979	0
CB102N	36.21	3.394476841	CB102N			CB102N	25.15	9585.920394	0.000708221	0
CB103N			CB103N			CB103N	27.41	2382.609796	0	0
CB104N			CB104N			CB104N	29.4	6637	0	0
CB106N			CB106N	36.99	14.03	CB106N	26.23	20610	0	0.001361475
CB107N			CB107N	36.02	6.204874251	CB107N	28.7	152.7934946	0	0.081219089
CB108N	32.9	247	CB108N	36.37	5.899	CB108N	27.34	10340	0.047775629	0.001141006
CB109N			CB109N			CB109N	29.67	689.5521974	0	0
CB110N	36.86	0.765761902	CB110N			CB110N	31.96	168.9975309	0.009062403	0
CB111N	37.21	11.29	CB111N	28.85	7756	CB111N	24.76	200700	0.000112506	0.077289487
BW01N			BW01N			BW01N	25.3	15850	0	0
BW99N	26	5545	BW99N			BW99N	20.4	43620	0.254241174	0
BW107N	36.19	1.198393685	BW107N			BW107N	28.03	1894.727398	0.001264977	0
BW108N	37.78	3.424	BW108N	39.02	113	BW108N	26.41	5107.794054	0.000162883	0.002028772
BW116N	34.25	4.383418913	BW116N			BW116N	26.8	4025.02978	0.00217808	0
BW117N	34.97	2.712908656	BW117N			BW117N	26.36	5270.715072	0.001029427	0
BW118N	38.1	0.336531884	BW118N			BW118N	27.9	2045.759584	0.000329004	0

BW119N	23	98000	BW119N			BW119N	26.01	26010	7.535563245	0
BW120N	32.13	29.30378719	BW120N			BW120N	30.06	575.9478123	0.101758481	0
BW121N	36.61	1.471263942	BW121N			BW121N	25.71	8326.584963	0.00035339	0
BW122N	25.73	6140	BW122N			BW122N	25.82	27270	0.450311698	0
BW124N			BW124N			BW124N	26.49	5151.826762	0	0
BW125N	31.42	46.79858551	BW125N			BW125N	26.72	4460.513256	0.020983498	0
BW126N	29.74	143.9676112	BW126N			BW126N	26.57	4905.243601	0.058699475	0
BW145N			BW145N			BW145N	30.1	559.6557106	0	0
GS52N	26.44	5089	GS52N			GS52N	21.28	115100	0.088427454	0
GS72N			GS72N			GS72N	27.67	3375.547347	0	0
GS73N			GS73N			GS73N	28.38	2107.845702	0	0
GS74N			GS74N	39.64	3.417531614	GS74N	28.1	2542.88885	0	0.002687913
MB01N			MB01N	35.56	25.52747528	MB01N	23.64	47467.25854	0	0.001075582
MB03N			MB03N	37.35	8.356	MB03N	29.14	10440	0	0.001600766
MB05N			MB05N			MB05N	28.22	2341.659378	0	0
MB06N			MB06N			MB06N	30.2	638.0339297	0	0

Appendix 11. The PCR results of the assymptimatic turtles

CmPV						ChHV5						Regions
Well	Sample	Cq	Efficiency	R^2	Result	Well	Sample	Cq	Efficiency	R^2	Result	
A1	1	32.84292	0.853357	0.99979		A1	1	-1	-1		Excluded	Cockle Bay
A2	2	37.38849	0.87802	0.99976		A2	2	-1	-1		Excluded	
A3	3	-1	-1		Excluded	A3	3	36.01417	0.632867	0.99686		
A4	4	29.01891	0.926999	0.99972		A4	4	34.9292	1.047588	0.99667		
A5	5	34.17774	0.970568	0.99885		A5	5	32.91783	1.188209	0.99806		
A6	6	33.07972	0.94694	0.99956		A6	6	29.94891	1.243854	0.99902		
B1	7	36.36904	0.839358	0.99989		B1	7	38.42288	0.387329	0.99004		
B2	8	32.82189	0.890966	0.99963		B2	8	-1	-1		Excluded	
B3	9	27.45897	0.858806	0.9999		B3	9	33.70222	0.774154	0.99392		
B4	10	34.1233	0.847383	0.99997		B4	10	37.44035	0.577215	0.99183		
B5	11	32.69771	0.865835	0.9998		B5	11	-1	-1		Excluded	
B6	12	32.72128	0.846145	0.99985		B6	12	-1	-1		Excluded	
C1	13	28.98123	0.879328	0.99983		C1	13	32.67983	1.098844	0.99564		
C2	14	24.5767	0.961327	0.99869		C2	14	38.80955	0.596639	0.98509		
C3	15	32.16668	0.930079	0.9988		C3	15	-1	-1		Excluded	
C4	16	31.29762	0.871909	0.99976		C4	16	33.43652	0.86454	0.99929		
C5	17	30.28316	0.913451	0.9993		C5	17	34.53646	1.06491	0.99773		
C6	18	37.37569	0.851615	0.99992		C6	18	33.23957	1.160148	0.99731		
D1	19	32.75267	0.941721	0.99897		D1	19	-1	-1		Excluded	
D2	20	27.27059	0.903667	0.99949		D2	20	38.60923	0.642376	0.98285		
D3	21	23.98634	0.965269	0.99873		D3	21	-1	-1		Excluded	
D4	23	32.31021	0.772485	0.99998		D4	23	33.82484	1.030884	0.99991		Bowen
D5	24	37.33206	0.886122	0.99993		D5	24	38.4633	0.613544	0.99203		

D6	25	37.10369	0.845499	0.99977		D6	25	36.66237	1.076112	0.99767		
E1	26	33.1031	0.946272	0.99889		E1	26	-1	-1		Excluded	
E2	27	-1	-1		Excluded	E2	27	-1	-1		Excluded	
E3	28	36.19606	0.902145	0.99989		E3	28	33.04266	1.098396	0.99867		
E4	29	-1	-1		Excluded	E4	29	33.12874	1.317859	0.99882		
E5	30	37.23884	0.785558	0.99951		E5	30	-1	-1		Excluded	
E6	31	36.153	0.92109	0.99956		E6	31	-1	-1		Excluded	
F1	32	-1	-1		Excluded	F1	32	-1	-1		Excluded	
F2	33	37.22896	0.867152	0.99994		F2	33	34.88464	0.928351	0.99845		
F3	34	37.1685	0.95819	0.99989		F3	34	37.68841	0.544927	0.99393		
F4	35	-1	-1		Excluded	F4	35	-1	-1		Excluded	
F5	36	-1	-1		Excluded	F5	36	-1	-1		Excluded	
F6	37	-1	-1		Excluded	F6	37	-1	-1		Excluded	
G1	38	36.23852	0.893938	0.99993		G1	38	-1	-1		Excluded	
G2	39	-1	-1		Excluded	G2	39	-1	-1		Excluded	
G3	41	-1	-1		Excluded	G3	41	38.11676	0.453549	0.98552		Gladstone
G4	42	-1	-1		Excluded	G4	42	36.49755	0.842868	0.98875		
G5	43	-1	-1		Excluded	G5	43	32.06167	1.104516	0.99642		
G6	44	-1	-1		Excluded	G6	44	37.64666	0.545385	0.99596		
H1	45	37.24093	0.988637	0.99948		H1	45	36.06076	0.787669	0.99256		
H2	46	-1	-1		Excluded	H2	46	-1	-1		Excluded	
H3	47	35.21976	0.880078	0.99978		H3	47	35.49237	1.129868	0.9955		
H4	48	33.01846	0.904992	0.99954		H4	48	-1	-1		Excluded	
H5	49	38.4031	0.918895	0.99972		H5	49	36.08271	0.891514	0.99449		

Appendix 12. The written confirmation of consent to the inclusion of published paper in the thesis

Thesis Title and Name of Candidate	SEA TURTLES DISEASE RISK ANALYSIS, ESTABLISHING PRIMARY CELL CULTURE AND QPCR FOR VIRAL SCREENING AND DISCOVERY OF THE FIRST AUSTRALIAN GREEN TURTLE (*Chelonia mydas*) PAPILLOMAVIRUS presented by **NARGES MASHKOUR**		
	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis
Chapter No. 4	Mashkour, N., Maclaine, A., Burgess, G. W., & Ariel, E. (2018). Discovery of an Australian *Chelonia mydas* papillomavirus via green turtle primary cell culture and qPCR. Journal of virological methods, 258, 13-23. https://doi.org/10.1016/j.jviromet.2018.04.004	E. Ariel and N. Mashkour conceived the study and drafted the ethics and permits to conduct the study. The cell culture experiment was done by NM. G. Burgess provided scientific advices in the molecular analysis section. The PCR assays were done by NM. and A. Maclaine. The publication was drafted by NM. and reviewed by all co-authors.	Name: Alicia Maclaine Signature: Name: Graham Burgess Signature: Name: Ellen Ariel Signature: